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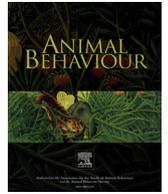
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## Experimental evidence for female mate choice in a noctuid moth

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Sexual signal evolution is shaped by whether only one or both sexes execute mate choice. When mate choice by both sexes is considered, the same signalling modality is generally inferred for males and females. In the noctuid moth *Chloridea (Heliothis) virescens* (Lepidoptera: Noctuidae), both sexes may be signallers and responders, as both emit a sex-specific pheromone. Male mate choice is based on the female sex pheromone, perceived via the antennae, and has been well documented. However, whether females choose partners and whether their choice is based on the male pheromone are unknown. Since female mate choice is expected when males vary in quality, we reared males on two different larval diets that affected their size, which correlated positively with their fitness. When given a choice, more females mated with larger than with smaller males, and these females produced more eggs and larvae. Female choice was not affected by the absolute amount or composition of the male pheromone. Moreover, we found that antennaless females mated as readily as intact females, indicating that antennal sensory input is not required for females to mate. To determine whether females make an active choice, we studied courtship behaviour in detail and observed that females determined the outcome of courtship by moving away from the male (avoidance) or by facilitating copulation with an abdominal bend (acceptance). Additionally, we discovered that tactile sensory stimuli may be involved during courtship. Because tactile interactions may mediate contact-based (chemical) communication, we also investigated putative pheromone components on moth legs, but found no differences between the sexes. Together, our study is the first comprehensive investigation of female mate choice in a heliothine moth.

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The evolution of sexual signals is affected by whether only one sex signals or both sexes are concurrently signallers and responders. If one sex signals, the selection pressure on sexual signals may come from both the choosing sex (intersexual selection) and the competing sex (intrasexual selection). Mate choice from both sexes can be expected when males and females both invest in mating and show similar variance in reproductive success (Trivers, 1972), and when both vary in quality (Parker, 1983; Johnstone, Reynolds, & Deutsch, 1996; Bergstrom & Real, 2000). When mate choice is identified in one sex, research often neglects to explore mate choice in the other sex, resulting in a one-sex biased perspective on mate choice (reviewed in Cotton, Small, & Pomiankowski, 2006). In various insect taxa, including Lepidoptera, male mate choice is well established, especially in relation to nuptial gifts (see Bonduriansky, 2001). However, the role of female mate choice in moths is understudied.

Females should be choosy when males invest more in reproduction than simply producing sperm (Parker, 1983; Johnstone et al., 1996). When there is variation in male quality, females are expected to select a specific mating partner rather than mating randomly (Parker, 1983; Owens & Thompson, 1994; Johnstone et al., 1996). Selecting high-quality mating partners allows the female to increase her individual fitness (Parker, 1983). Females can increase their fitness by producing either more offspring (traditionally referred to as ‘direct’ benefits) or offspring with higher fitness (referred to as ‘indirect’ benefits; Kokko, Brooks, Jennions, & Morley, 2003). To discriminate among males, females may assess males during courtship based on sexual signals. These are phenotypic traits that reflect an individual’s mate value (Edward, 2015). Males of high quality, and thus of high mate value, are expected to be more attractive to females.

To attract the opposite sex, males and females may use the same or different types of signals. We consider two signals the ‘same’ when they share a sensory modality and when both signals are produced through the same biosynthetic pathway. Contrastingly, we consider signals to be different when they operate via different

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sensory modalities (e.g. cricket males sing to attract females from a distance, while females choose partners based on a contact pheromone, Thomas & Simmons, 2009) and/or when signals are produced through different biosynthetic pathways. For example, females of the German cockroach, *Blattella germanica*, synthesize a volatile pheromone in their pygidial gland and contact-based cuticular lipid pheromones in abdominal oenocytes, while being attracted to male volatile pheromone produced in specialized tergal glands (Gemeno & Schal, 2004). Also in *Drosophila melanogaster*, male and female signals are derived through different biosynthetic pathways (Chertemps et al., 2007). In arctiid moths, female pheromones are produced in pheromone glands or in oenocytes and transported to pheromone glands (Jurenka, Blomquist, Schal, & Tittiger, 2017), while males produce a pheromone derived from plant secondary compounds (Henneken, Goodger, Jones, & Elgar, 2017; Nishida, 2002). Mate choice by both sexes on the same signal has been found in birds (Kraaijeveld, Kraaijeveld-Smit, & Komdeur, 2007), for example on plumage coloration in bluethroats, *Luscinia svecica* (Amundsen, Forsgren, & Hansen, 1997) and the European starling, *Sturnus vulgaris* (Komdeur, Oorebeek, van Overveld, & Cuthill, 2005), foot colour in the blue-footed booby, *Sula nebouxii* (Torres & Velando, 2005) and crest size in auklets, *Aethia cristatella* (Jones & Hunter, 1993). Also in invertebrate species, both sexes may use the same or different signals to evaluate partners. For example, in *Drosophila serrata*, both sexes use cuticular hydrocarbons (CHCs) to discriminate among mates, although males have a different preference than females, which results in contrasting selection pressures in each sex (Chenoweth & Blows, 2003, 2005). Similarly, in salticid spiders, both sexes exert mate choice on the same trait, body size, although mated females prefer small males, while males prefer large females (Cross, Jackson, & Pollard, 2007; Cross & Jackson, 2009).

Moths are well known for their chemical sexual signals, and a large body of research is focused on females attracting males from a distance by emitting species-specific sex pheromones (Baker, 1989; Harari & Steinitz, 2013; Löfstedt, 1993; Roelofs & Cardé, 1974). As in most insect species, females are thus considered the signalers and males the responders (reviewed in Bonduriansky, 2001). However, male moth pheromones have also been described (Birch, Poppy, & Baker, 1990; Conner & Iyengar, 2016; Hillier & Vickers, 2004; Lassance, Bogdanowicz, Wanner, Löfstedt, & Harrison, 2011) and most of the identified male and female pheromones consist of biosynthetically related compounds (i.e. both pheromones are partially derived from the same precursors and the same enzymes may catalyse steps in the pheromone production of both sexes; Symonds & Elgar, 2008; Jurenka et al., 2017). Of note however, are tiger moths (Arctiidae), where females emit either typical moth pheromones (aliphatic aldehydes, acetates, alcohols) or hydrocarbons and hydrocarbon derivatives from a pheromone gland associated with the ovipositor (Ando, Inomata, & Yamamoto, 2004), whereas male pheromones are often derivatives of plant secondary compounds and are emitted from specialized abdominal eversible glands (Iyengar & Conner, 2016; Weller, Jacobson, & Conner, 1999). Since pheromonal signals are emitted by male moths, and might convey information about male quality, females are expected to engage in mate choice based on these signals.

In the noctuid moth *Chloridea (Heliiothis) virescens*, both males and females may benefit from choosing a mating partner, because they face similar conditions for mating (Raulston, Snow, Graham, & Lingren, 1975), and both invest in each mating and increase their reproductive output by multiple matings (Gao, van Wijk, Clement, Egas, & Groot, 2020). In particular, both sexes mate multiply, but only once a night (Raulston et al., 1975). Also, males invest in mating by producing a spermatophore that weighs up to 5% of their body mass (Blanco, Rojas, Groot, Morales-Ramos, & Abel, 2009; Lamunyon,

2000). Males emit pheromone from elaborate structures, so-called hairpencils, near a female (Birch et al., 1990; Grant, 1970; Hendricks & Shaver, 1975; Hillier & Vickers, 2004; Teal, McLaughlin, & Tumlinson, 1981; Teal & Tumlinson, 1989). This pheromone consists of a blend of fatty-acid-derived components, with the major compound 16:OAc, and smaller amounts of 14:OH, Z7-16:OAc, Z9-16:OAc, Z11-16:OAc, 16:OH and Z11-16:OH (Hillier & Vickers, 2004; Teal & Tumlinson, 1989). So far, the male pheromone has been shown to play a role in species recognition (Hillier & Vickers, 2004, 2011; Lassance & Löfstedt, 2009) and in male–male competition (Hosseini et al., 2016). Also, it has been suggested that females discriminate males based on these volatiles (Baker & Cardé, 1979; Birch et al., 1990). However, empirical evidence for active female mate choice is lacking. In this study, we aimed to determine (1) whether there is female mate choice, (2) whether mate choice benefits females and (3) whether female mate acceptance is mediated through the male hairpencil pheromone. We hypothesized that females benefit from being choosy if males vary in quality and if females are more likely to mate with higher quality males. Further, we hypothesized that males signal their quality through the hairpencil pheromone.

## METHODS

### Insects

*Chloridea virescens*, originating from North Carolina State University, Raleigh, NC, U.S.A., (YDK strain) and the Max Planck Institute for Chemical Ecology, Jena, Germany, was reared at the Institute for Biodiversity and Ecosystem and Dynamics (IBED), University of Amsterdam, Amsterdam, Netherlands, in an environmental chamber at 60% relative humidity and  $25 \pm 1^\circ\text{C}$  with a 14:10 h light:dark photoperiod (lights off at 1100 CET). Larvae were reared on artificial pinto bean diet (Burton, 1970) in individual plastic cups (37 ml, Solo, Lake Forest, IL, U.S.A.). Pupae were checked daily for adult emergence. Newly emerged adult males and females were kept separately and fed 10% sucrose solution provided through 1 cm cotton dental wick. For all experiments, we used 2–3-day-old virgin males and females. Interaction and mating experiments were conducted exclusively with nonsibling pairs. The environmental conditions for rearing and all experiments were identical.

### Identifying Male Quality

#### Two diet treatments to increase male pupal mass range

Since laboratory cultured insects can be expected to exhibit a limited range in mate quality, and mate choice only makes sense if variation in partners exists, we increased the range of body mass by manipulating the larval diet, as this is a prominent determinant of quality in insects. A pinto bean diet was used for all the treatments (Burton, 1970). The nutritional value was either kept standard according to the regular rearing protocol (henceforth referred to as standard diet; see e.g. Groot et al., 2014) or lowered to 25% in nutritional value compared to the standard (referred to as reduced diet). The reduced diet was produced by adding 25% of each ingredient to the regular amount of agar and water. We placed individual neonate larvae, originating from mass matings with four to five adults of each sex, in clear plastic cups filled with standard or reduced diet. The great majority of the developing larvae reached the pupal stage by day 24. Therefore, we measured the pupal mass of each individual on day 24, using a high-precision scale (Sartorius MC1 Analytical Balance AC210S, Sartorius, Göttingen, Germany), and determined its sex. We checked each pupa daily and noted its date of eclosion, after which we fed the adult moths 10% sucrose solution. To determine whether male pupal mass differed significantly between the two diets, we first checked visually and with a

Shapiro–Wilk test whether the data were normally distributed after which we performed a Welch two-sample *t* test.

#### *Effect of larval nutrition on reproductive performance*

To assess the effect of larval diet and pupal mass on reproductive output, we measured fecundity and fertility in a separate experiment using single pair matings. Males and females from the standard and reduced diets were tested in a  $2 \times 2$  factorial design. A male and female pair was placed into a clear plastic cup (473 ml, Solo) covered with gauze, placed into the environmental chamber and checked for mating every 30 min, as matings in *C. virescens* last more than 3 h (Hosseini et al., 2016). When a mating pair was found, the cup was set aside and after they separated the male and female were placed in separate cups with 10% sucrose solution.

To measure individual lifetime reproductive output, the gauze with eggs was collected every 24 h until the female died. The gauzes with eggs from each female were kept in the original cup in the same environmental chamber for another 48 h to distinguish fertilized (dark-coloured) from unfertilized (green-whitish) eggs. All eggs and larvae were counted using a stereomicroscope. We quantified fertility (female lifetime reproductive success from a single mating) based on the sum of all fertilized eggs and larvae. We determined fecundity as the sum of all fertilized eggs, unfertilized eggs and larvae. We used the nonparametric Spearman's rank correlation coefficient for the relationship between male pupal mass and fecundity and fertility, respectively, as the data were not normally distributed. In the same way, we determined the correlation between individual male hairpencil pheromone compounds, the total amount of pheromone and the normalized compounds as ratios to the major compound 16:OAc to male pupal mass and fertility and fecundity, respectively. As we detected small quantities of 16:Ald in hairpencil extracts (see below), and it is known to be bioactive (Choi et al., 2016), we included this compound in our analyses.

#### *Female Choice for Different Quality Males*

To assess female choice, we conducted two-choice assays in BugDorm cages ( $30 \times 30 \times 30$  cm; [www.bugdorm.com](http://www.bugdorm.com)), in which we placed one female from the standard diet and two males. To identify males, we clipped one wing tip of each one either left or right in a randomized fashion. The experiment started about 10 min before the onset of the scotophase (dark period) by placing all moths in their respective cages with 10% sucrose solution. To check for newly formed mating pairs, we observed all cages at least once per hour. Other activities, such as female calling behaviour and male courtship, were also noted. To test whether male pupal mass differed between chosen and nonchosen males, we first determined whether wing clipping affected choice, using a two-tailed binomial test, after which we determined whether the mean mass of the chosen males was significantly higher than that of the unchosen males per cage, using a two-tailed *t* test.

To determine whether female mate choice was related to male pheromone, we extracted the hairpencils from all chosen and unchosen males used in the mating cages. To make sure that mating status would not affect the male pheromone composition, as soon as a mating pair was formed in the experimental cage, we mated the unchosen male with a donor virgin female in a separate cup. In the following photophase, all individuals were separated into their rearing cups and were kept at identical conditions in the environmental chamber. In the following scotophase (i.e. about 24 h after mating), we extracted the hairpencils of both males, following the protocol of Hosseini et al. (2016). The pheromone samples were prepared and analysed by gas chromatography with a synthetic multiple-component blend as a reference, following the procedure described in Groot, Claßen, Staudacher, Schal, and Heckel (2010).

Data analysis was conducted in R, version 3.6.3 (R Core Team, 2020). As the choice outcome represented a relative choice between the two males in a cage, we used the difference in mass and the difference in pheromone composition between these males as the explanatory variables to predict the response variable (female choice). To resolve the correlation between the amounts of pheromone compounds, we first normalized the amount of each compound (i.e. 16:Ald, 14:OH, Z7-16:OAc, Z9-16:OAc, Z11-16:OAc, 16:OH and Z11-16:OH) to the amount of the major male pheromone component 16:OAc. Subsequently, we calculated the difference in absolute amounts and normalized amounts between the two males from each cage. The difference in the total amount of pheromone that each male produced was added as a separate explanatory variable. To model the response variable choice, we randomly selected one male per cage that was either chosen or not chosen by the female. This procedure ensured that the sample size was identical to the number of choices made. Using this subset of males, we modelled how male mating probability can be explained by the difference in pupal mass and the differences in normalized amounts of hairpencil pheromone compounds, relative to the other male in the cage, in a main effects general linear model with a binomial error distribution.

#### *Female Antennal Perception for Mating?*

##### *Mating latency of intact and antennaless females*

Because moth antennae house pheromone receptors in both sexes (Almaas & Mustaparta, 1990; Krieger et al., 2002, 2004; Hillier, Kleineidam, & Vickers, 2006; Wang, Vasquez, Schal, Zwiebel, & Gould, 2011) and male mate choice is based on the female sex pheromone perceived with the antennae, we assessed the necessity of antennal perception for female mate choice. If male signals are perceived by females through their antennae, females lacking antennae should either refuse to mate or mate readily. To obtain antennaless individuals, we cut both antennae at their base using small spring-scissors (Vannas-Tübingen Spring Scissors, no. 15003-08, Fine Science Tools, [www.finescience.com](http://www.finescience.com)) 1–2 h before the onset of the scotophase. We determined the mating latency of intact and antennaless females in a no-choice assay as follows. We placed either an intact or an antennaless female with an intact male into a clear plastic cup (473 ml, Solo) with a mesh cover, and mounted each plastic cup in a hanging grid underneath which a camera (GoPro Hero silver, GoPro, San Mateo, CA, U.S.A.) was placed. The experiment started at the beginning of the scotophase. To measure latency, we recorded a time lapse photo series with 1 picture/min with a camera. We then checked the digital pictures for newly formed mating pairs and calculated the time (min) until mating. To determine whether antennaless females mated significantly sooner or later than intact females, we performed a survival analysis with a log-rank test, using the R packages survival (Therneau & Lumley, 2015) and survminer (Kassambara, Kosinski, Biecek, & Fabian, 2019).

##### *Mating success of antennaless and intact individuals*

To further explore whether female mate choice is based on the male pheromone, we checked mating success of females with and without antennae in a no-choice assay with intact and antennaless individuals, using a full-factorial design. In total, we tested 83 pairs, 21 intact (I) pairs (IF  $\times$  IM), 18 pairs of antennaless (A) females with intact males (AF  $\times$  IM), 26 pairs of intact females with antennaless males (IF  $\times$  AM) and 18 antennaless pairs (AF  $\times$  AM). Each pair was placed into a clear plastic cup (473 ml, Solo) with 10% sugar water and covered with a gauze lid 30 min before the onset of the scotophase. All cups were placed randomly on shelves in the environmental chamber and checked every 30 min for matings.

Differences in mating success between the four possible pairings were determined by a two-tailed Fisher's exact test.

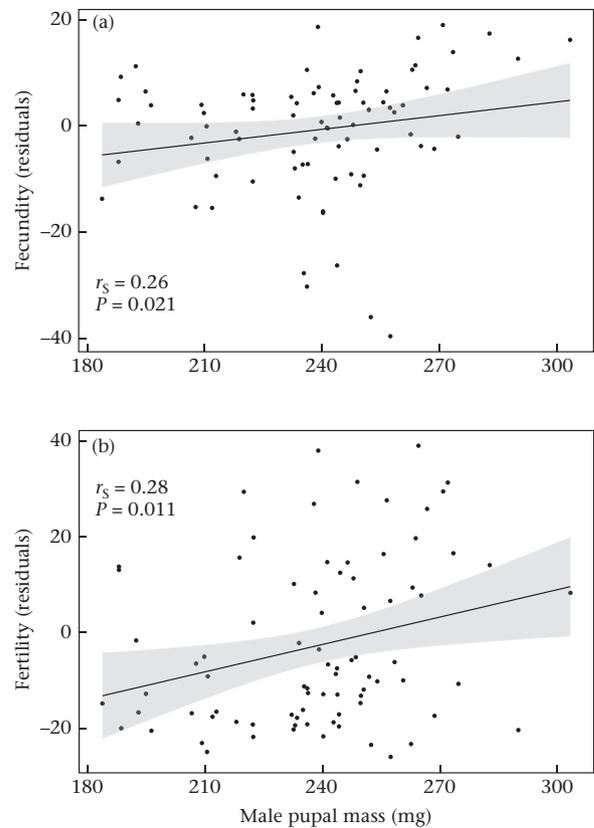
#### Analysis of Close-range Courtship Behaviour

To identify female pre-mating acceptance and rejection behaviours that amount to mate choice, we analysed close-range courtship in more detail. We recorded and analysed a total of 12 courtship clips of moth pairs that mated within 15 min, using video clips with 120 frames/s (fps) with a modified infrared-light-sensitive action camera (GoPro Hero4 black) on an infrared light panel as background. For the recording, we placed one 2–3-day-old, virgin female with cotton soaked with 10% sugar water into a cylindrical acrylic vial (10.9 cm height, 5 cm diameter with removable mesh lids on both sides) ca 30 cm in front of the infrared light panel in a horizontal position before the onset of the scotophase. We checked the females every 30 min for calling, which usually occurred 3–4 h into the scotophase. Once a female was calling, we added a male to the vial and started the camera. The pair was filmed until mating or for 15 min. The following day, we dissected all females to confirm mating by the presence of a spermatophore.

We inspected all video clips frame-by-frame and listed all behaviours for both sexes (see Table 1). Each behaviour was further assigned to one of the five categories: 'stationary', 'locomotion', 'maintenance', 'attempt' and 'none' (Table 1). To score the behaviours for each individual and to calculate transition frequencies from the ethogram, we used the behavioral observation research interactive software (BORIS; Friard & Gamba (2016); <https://boris.readthedocs.io/en/latest/#>). To identify female mate choice behaviours, we compared female behaviours between successful mating attempts, which we identified as a male approach resulting in mating, and failed mating attempts, which did not culminate in mating. Finally, we summarized the overall courtship process in a kinematic diagram.

#### Pheromone Compounds on Legs

Since our behavioural observations revealed that the moths show tactile interactions with their legs during close-range courtship, and moth tarsi contain putative pheromone compounds (Choi et al., 2016), we extracted male and female legs, using the same procedures used to extract pheromone glands (Groot et al., 2010). We removed and extracted all six legs from an individual in hexane for 30–60 min, after which the extract was transferred into a new glass insert and stored at -20 °C. All samples were processed within 2 weeks in a gas chromatograph for chemical analysis, as described in detail by Groot et al. (2010). As references, we used a synthetic



**Figure 1.** Relationship between *C. virescens* male pupal mass and both (a) fecundity and (b) fertility, corrected for female pupal mass (residuals). Solid black line: linear regression; grey area: 95% confidence interval. Spearman rank correlation coefficients are shown.  $N = 82$  for both (a) and (b).

multicomponent blend (see Groot et al., 2010) and a mix of alkane standards (C7–C30 alkane standard Sigma-Aldrich, 49451-U, Sigma-Aldrich, St Louis, MI, U.S.A.). Differences in the pheromone composition on the legs of males and females were compared using Welch two-sample *t* tests.

## RESULTS

### Male Pupal Mass is a Measure of Male Quality

We increased the range of male pupal mass by lowering the nutritional value of larval diet. Individuals reared on standard diet

**Table 1**  
Ethogram of behaviours observed in *C. virescens* close-range interactions

Behaviour	Definition	Category	Sex
Quiescent	No movement of legs or wings	Stationary	M + F
Wing fanning	High-speed movements with wings while resting	Stationary	M + F
Walking	Locomotion in any direction using the legs	Locomotion	M + F
Flying	Locomotion without contact to surface	Locomotion	M + F
Feeding	Everted proboscis with tip touching sugar water	Maintenance	M + F
Antennal grooming	Individual touches one of its own antennae with a leg	Maintenance	M + F
Eye grooming	Rubbing movement with foreleg over eye area	Maintenance	M + F
Calling	Everted ovipositor	none	F
Oviposition	Egg laying with brief bends of abdomen to the substrate	none	F
Hairpencil eversion (HP)	Everted brush-like organ from tip of the abdomen	Attempt	M
Abdominal curl (AC)	Bending of abdomen (in any direction)	Attempt	M + F
Grubbing (GB)	Grubbing motion with foreleg	Attempt	M + F
Mating	Copulation with abdomen staying attached	none	M + F

Stationary = behaviours shown while not moving around, locomotion = behaviours related to moving in space, maintenance = behaviours relevant for hygiene or body functions, attempt = behavioural elements of a (male) courtship attempt, other = any behaviours that do not fall into a specific category; M = male, F = female.

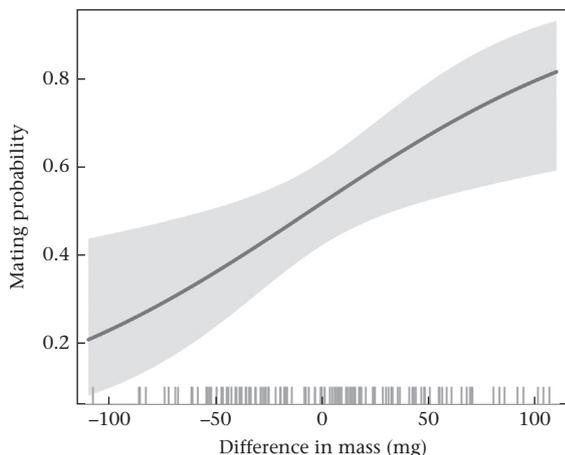
weighed  $254.4 \pm 2.4$  (SE) mg, whereas individuals reared on reduced diet weighed significantly less,  $217.9 \pm 2.3$  (SE) mg on average ( $t_{316} = 11.08$ ,  $P < 0.001$ ; Appendix Fig. A1).

Pupal mass was positively correlated with several measures of adult body size (Appendix Fig. A2a–d) and thus was a good predictor of adult body size (see Appendix for details). Pupal mass had the highest correlation coefficient with forewing length (Pearson  $r = 0.63$ ,  $P < 0.001$ ), followed by body length ( $r = 0.52$ ,  $P < 0.001$ ) and tarsus length ( $r = 0.51$ ,  $P < 0.001$ ). Tibia length showed the lowest, yet still statistically significant correlation with pupal mass ( $r = 0.33$ ,  $P < 0.001$ ).

We found a significant effect of male pupal mass on reproductive performance: females that mated with males of higher pupal mass showed significant positive Spearman correlation coefficients in both fecundity ( $r_s = 0.26$ ,  $N = 82$ ,  $P = 0.021$ ) and fertility ( $r_s = 0.28$ ,  $N = 82$ ,  $P = 0.011$ ), even when corrected for female pupal mass (Fig. 1a and b). Even though in this assay the total amount of hairpencil pheromone showed a weak, yet significant correlation with pupal mass ( $r_s = 0.26$ ,  $N = 82$ ,  $P = 0.007$ ), pheromone quantity was not significantly correlated with fecundity ( $r_s = -0.04$ ,  $N = 82$ ,  $P = 0.697$ ) or fertility ( $r_s = 0.08$ ,  $N = 82$ ,  $P = 0.596$ ; Appendix Fig. A3a, b). In addition, none of the individual compounds, normalized relative to the amount of the major pheromone component 16:OAc, was significantly correlated with either pupal mass, fecundity or fertility (all  $P > 0.05$ ; Appendix Fig. A3). Therefore, although the total pheromone content of the hairpencils might reflect male quality, the quantity of each pheromone compound alone does not appear to provide females with information about male size.

### Female Choice for Larger Males

In two-choice assays, we found that larger males had a higher mating probability than smaller males (Fig. 2), even though both males engaged in courtship. The mean pupal mass of successful males was on average 10.5 mg heavier (95% confidence interval, CI = 3.22–17.69) than the mean pupal mass of unsuccessful males (two-sample  $t$  test:  $t_{298} = 4.039$ ,  $P < 0.001$ ). Surprisingly, the logistic regression model with additive main effects revealed that only male pupal mass was relevant for the choice outcome



**Figure 2.** Mating probability in *C. virescens* in relation to male pupal mass, where the difference in mass is the relative difference between the two males in female choice experiments. The curve (solid line) shows the predicted values for mating probability in relation to the relative difference in male pupal mass, based on a full additive main-effects general linear model. Shaded area indicates the 95% confidence interval and the grey tick lines along the x-axis mark data points of a random subset, each tick line representing one male from every two-choice comparison.

( $\chi^2_1 = 8.35$ ,  $P = 0.004$ ). The total amount of hairpencil pheromone did not affect male mating probability (total sum of all compounds:  $\chi^2_1 = 0.27$ ,  $P = 0.600$ ), nor did any of the normalized hairpencil pheromone compounds (16:Ald/16:OAc:  $\chi^2_1 = 1.65$ ,  $P = 0.200$ ; 14:OH/16:OAc:  $\chi^2_1 = 1.76$ ,  $P = 0.184$ ; Z7-16:OAc/16:OAc:  $\chi^2_1 = 0.73$ ,  $P = 0.393$ ; Z9-16:OAc/16:OAc:  $\chi^2_1 = 0.263$ ,  $P = 0.608$ ; Z11-16:OAc/16:OAc:  $\chi^2_1 = 1.61$ ,  $P = 0.205$ ; 16:OH/16:OAc:  $\chi^2_1 = 1.61$ ,  $P = 0.204$ ; and Z11-16:OH/16:OAc:  $\chi^2_1 = 1.45$ ,  $P = 0.228$ ). These results indicate that females may choose males based on body size but not on hairpencil pheromone.

### Females do not Rely on Antennal Perception in Courtship

In comparing antennaless and intact females, we found that females without antennae mated within a similar time frame as females with intact antennae ( $\chi^2_1 = 0.6$ ,  $P = 0.44$ ; Fig. 3a). Intact pairs (IF  $\times$  IM) and pairs with antennaless females (AF  $\times$  IM) had similar high mating success (95.24% for intact pairs and 100% for pairs with intact males but antennaless females; Fig. 3b). In contrast, males without antennae failed to mate: in matings with antennaless males (IF  $\times$  AM), 2/26 pairs (7.69%) mated, and none of the mating pairs with two antennaless individuals (AF  $\times$  AM) mated. These differences in mating success were statistically significant (Fisher's exact test:  $P < 0.001$ ; Fig. 3b). Thus, females did not appear to rely on the perception of a male chemical signal for mate assessment, in contrast to the males which did not mate at all when lacking antennae.

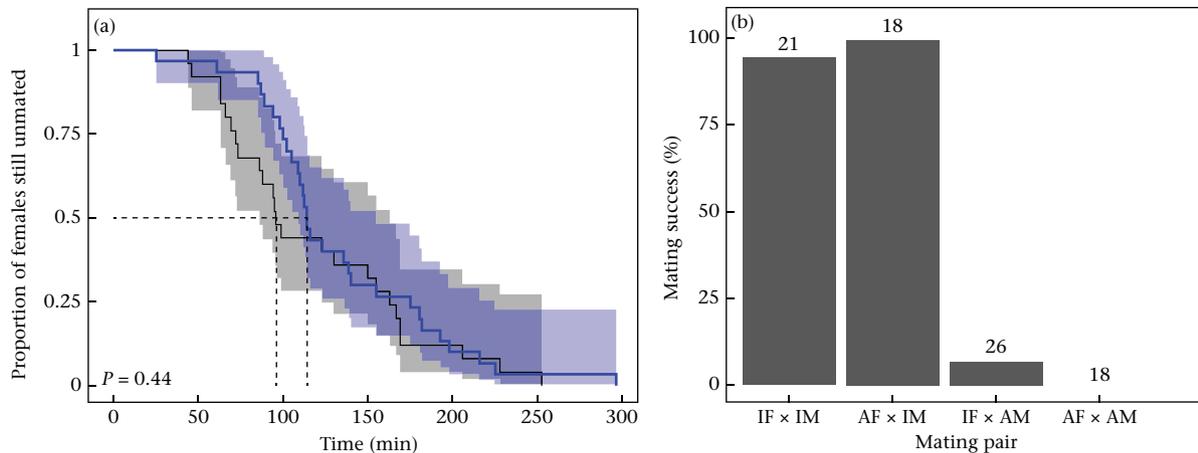
### Female Mate Choice Behaviours

#### Male courtship behaviours

By studying video-recorded close-range courtship behaviour in detail, we scored mating attempts when a male performed at least one of three mating attempt behaviours (Fig. 4). We observed that during a mating attempt, the male fully everted his hairpencils, followed by curling his abdomen towards the female. Simultaneously, the male moved one of his forelegs towards one female leg in a circular 'extend-and-bend' motion, a behaviour we termed 'grubbing'. To our knowledge, this behaviour has not been reported in insect courtship sequences before. Prior to successful mating, a male performed a combination of at least two behaviours, including hairpencil eversion and abdominal curl (Appendix Tables A1, A2).

#### Female courtship behaviours

By observing the female behaviours following a male mating attempt, we identified that the success of the attempt depended on the female's response (Fig. 4). The majority of unsuccessful male actions ( $N = 112$ ) were followed by female avoidance behaviours, such as walking away (31.3% of all attempts), wing fanning (27.8%), or flying away (18.3%). Less frequently, we observed that females curled their abdomen to the substrate (10.4%), started to call (6.1%), started to feed (2.6%), dropped to the floor ( $< 1\%$ ), showed grubbing-like behaviour ( $< 1\%$ ), started to groom the antennae ( $< 1\%$ ) or became quiescent ( $< 1\%$ ). In contrast, a male attempt was successful ( $N = 15$ ) when the female remained in one spot. In this stationary position, the female sometimes showed wing fanning and, most frequently, she curled her abdomen towards the male's genitalia (Fig. 4). This female acceptance behaviour was followed by a movement of the male hairpencils over the female's abdominal tip, after which the male clasped the female for copulation. Finally, the mating pair moved into a 180° position, facing away from each other. Thus, females determined the outcome of a male mating attempt in both avoidance and acceptance behaviour.



**Figure 3.** (a) Kaplan - Meyer survival curves, with the time course of mating as the proportion of unmated *C. virescens* females (black: intact females; blue: antennaless females). Shaded area around each curve represents the 95% confidence interval. Dashed lines: time point when 50% of the females mated. A log-rank survival model was fitted to assess whether the two lines differed. (b) Mating success of intact and antennaless males and females, tested in a full factorial design. IF x IM: two intact individuals; AF x IM: antennaless females with intact males; IF x AM: intact females with antennaless males; AF x AM: both individuals antennaless. Numbers above the bars indicate sample sizes.

### Pheromone Compounds on Legs

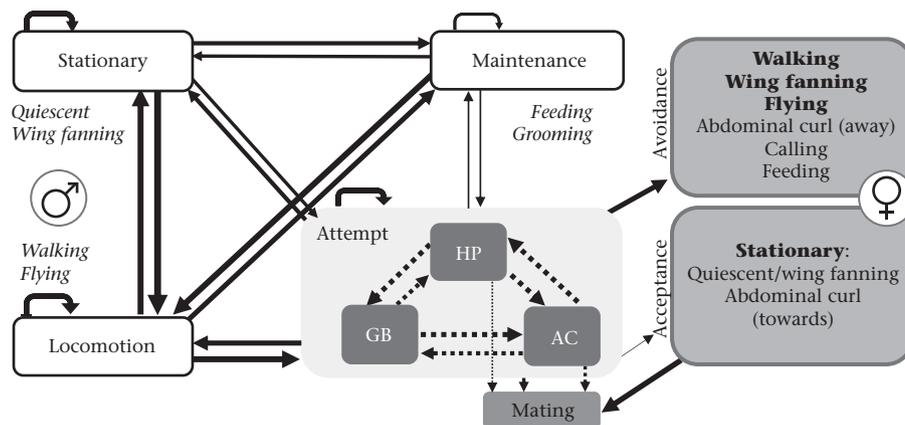
We detected four known pheromone compounds in extracts of male and female legs, including 16:Ald, 16:OAc, 16:OH and Z11-16:OAc. The absolute amounts did not differ between males and females (16:Ald:  $P = 0.965$ ; 16:OAc:  $P = 0.057$ ; Z11-16:OAc:  $P = 0.680$ ; the sum of all four compounds:  $P = 0.782$ ), except for more 16:OH in males ( $P = 0.001$ ; Fig. 5a). Accordingly, male legs contained higher relative amounts of 16:OH than female legs ( $P = 0.015$ ). Female legs contained greater relative amounts of 16:OAc ( $P = 0.049$ ; Fig. 5b). We found no difference between males and females in relative amounts of 16:Ald ( $P = 0.843$ ) and Z11-16:OAc ( $P = 0.935$ ). In addition to the described pheromone compounds, we consistently observed two longer-chain compounds in the leg samples, one corresponding in retention time to n-C25 hydrocarbon (C25) and another corresponding to n-C27 hydrocarbon (C27). While the amount of C25 was similar in males and females ( $P = 0.226$ ), C27 was significantly higher in female than in male leg extracts ( $P = 0.001$ ; Fig. 5c).

### DISCUSSION

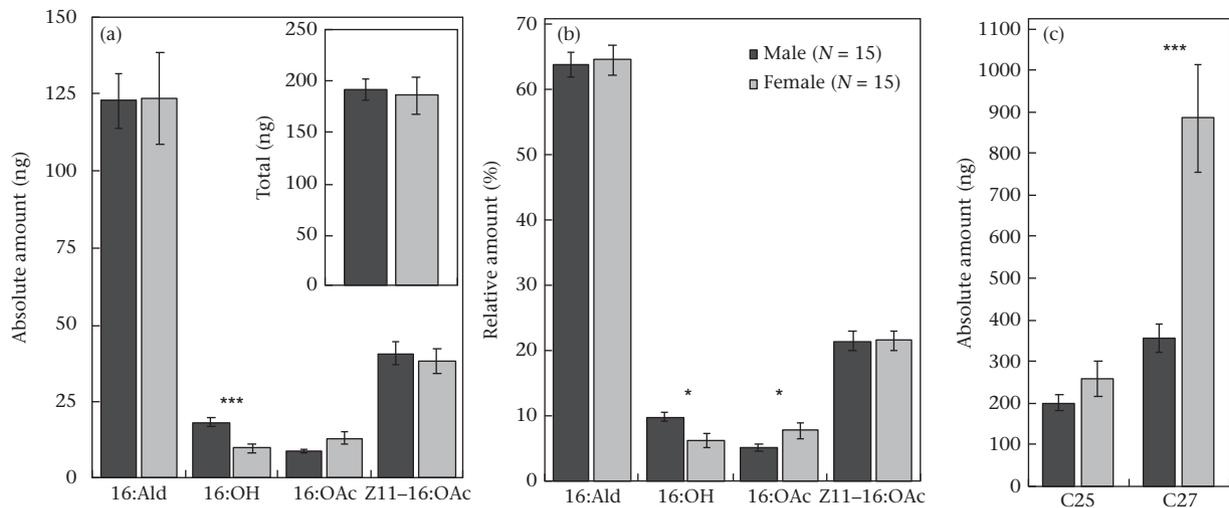
Our results demonstrate that *C. virescens* females prefer larger males, and that they gain benefits by mating with larger males. Surprisingly, however, female mate choice was not related to the male hairpencil pheromone, as evidenced by (1) the willingness of antennaless females to mate and (2) no correlation between the amount or composition of the male's hairpencil pheromone and his mating probability in two-choice assays, when corrected for the difference in male body mass. Thus, in contrast to our hypothesis, we did not find empirical support for female mate choice based on the male hairpencil pheromone. Detailed close-range mating observations using high-frame rate video recordings showed that females perform specific acceptance and rejection behaviours. These findings reveal that microscale female behaviour essentially determines the outcomes of mating attempts.

#### Mate Choice is Beneficial to Females

Female mate choice is expected when males differ in quality. Because *C. virescens* females that mated with bigger males had



**Figure 4.** Kinematic diagram of *C. virescens* male transitions between behavioural elements grouped in categories in close-range interactions with a female. The thickness of the arrows indicates transition frequency, with thicker arrows indicating more frequent transitions. The grey box highlights the male copulation attempt with three key behaviours that lead to mating only when the female cooperates (AC: abdominal curl; GB: grubbing; HP: hairpencil eversion). Female responses, grouped as acceptance and avoidance, are listed to the right with most frequent behaviours in bold.



**Figure 5.** Chemical analysis of *C. virescens* legs extracts in (a) absolute and (b) relative amounts of male hairpencil pheromone compounds and (c) absolute amounts of cuticular hydrocarbon compounds. The hairpencil pheromone compounds have been described elsewhere (Choi et al., 2016; Hillier & Vickers, 2004; Teal & Tumlinson, 1989). The inset in (a) shows the total amounts of hairpencil pheromone compounds in males and females. Error bars show SE values. \* $P \leq 0.05$ ; \*\*\* $P \leq 0.001$ .

higher reproductive output, even when corrected for female pupal mass (Fig. 1), bigger males are higher quality partners. Correspondingly, in our two-choice tests we found female choice for larger males. In insects, large body size is a common indicator of high fertility (Bonduriansky, 2001; Honěk, 1993; Servedio & Lande, 2006), although there are exceptions (Klepetka & Gould, 1996). Females may benefit from larger partners by obtaining a larger spermatophore, more seminal secretions and/or more nuptial gifts (Bissoondath & Wiklund, 1996; Svård & Wiklund, 1989). In insects in general, larger spermatophores consistently increase female reproductive output (South & Lewis, 2011). In Lepidoptera, spermatophores contain not only fertilizing (eupyrene) but also non-fertilizing (apyrene) sperm (Cook & Wedell, 1999; Silberglied, Shepherd, & Dickinson, 1984). In *C. virescens*, females that receive a larger spermatophore store more eupyrene sperm and male pupal mass has been found to be positively associated with spermatophore size (Lamunyon, 2000). Perhaps apyrene sperm can be viewed as a nuptial gift. Zinc is another possible nuptial gift substance, since *C. virescens* males transfer this trace element in the ejaculate, which is later incorporated into the eggs (Engebretson & Mason, 1980). Male seminal secretions also contain other bioactive substances (e.g. juvenile hormone, Park, Shu, Ramaswamy, & Srinivasan, 1998; Pszczolkowski, Tucker, Srinivasan, & Ramaswamy, 2006) that may further contribute to female reproductive output and stimulate oviposition (Jin & Gong, 2001; reviewed in Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011).

#### Female Mate Choice is Unrelated to Pheromone

Contrary to our expectations, male hairpencil pheromone did not explain female choice in two-choice assays, as these volatiles have long been suggested to act as the signal that females may use to assess male quality in various moths (Baker & Cardé, 1979; Birch et al., 1990). Hillier and Vickers (2004) showed the relevance of male volatiles from hairpencils for species recognition, as they found higher mate acceptance in female *C. virescens* when conspecific hairpencil volatiles were present than when the hairpencils were treated with a heterospecific extract. However, in intraspecific interactions, we found no correlation between hairpencil pheromone and the reproductive output of the male's mate (Appendix Fig. A3), or between male pheromone and female choice. Since we previously found that the male pheromone

makes *C. virescens* females unattractive for at least 24 h post-mating (Hosseini et al., 2016), and thus that it acts as an antagonistic signal for approaching and competing males, the hairpencil pheromone may be an armament rather than an ornamental signal to females (Berglund, Bisazza, & Pilastro, 1996). As calling females attract multiple males simultaneously, intrasexual male competition could be an important mechanism in nature (Mitchell, Tumlinson, Copeland, Hines, & Brennan, 1974; van Wijk, Heath, Lievers, Schal, & Groot, 2017).

#### Females do not Require Antennae for Mate Choice

Antennal detection of a male signal does not seem to be essential for females to accept a mate, because antennaless females showed no difference in mate acceptance and latency to mating compared to intact females. We were surprised by this result, because Hillier et al. (2006) reported that the antennae of female *C. virescens* respond strongly to male hairpencil compounds in electrophysiological tests, and Hillier and Vickers (2004) found that female antennal detection of male hairpencil pheromone was an essential component of mate acceptance. We reason that antennal perception does not play the same role in females as it does in males. While antennaless males did not initiate any mating behaviour, antennaless females did mate. One could argue that antennectomy significantly hampers mobility of the females, as antennae are important organs for the sense of balance in moths (Sane et al., 2007). Without the possibility of flying, but also without any olfactory input from the antennae, antennaless females would not be able to avoid a courting male and might have simply accepted any male. However, if this were the case, we would have seen a difference in mating latency between antennaless and intact females, because the latter still possess their full capacity to assess a male, but we did not. Further, our result is consistent with studies in other noctuid moth species, where antennaless females were found to mate as readily as intact females (Ellis & Brimacombe, 1980; Hirai, 1977). In addition, female *Manduca sexta* and *Spodoptera littoralis* with knock-out mutations in the odorant coreceptor Orco mated at normal rates, while smell-impaired males did not mate at all (R. A. Fandino and F. A. Kourtroumpa, personal communication, 3 October 2018). Nevertheless, mate assessment by females could still involve chemical signals, as olfactory receptors have also been found on the abdomen (Krieger

et al., 2002, 2004) and on legs (Krieger et al., 2002) of several Lepidoptera species (reviewed in Jacquin-Joly & Merlin, 2004).

### Females Show Acceptance and Rejection Behaviours

Our finding that female choice is related to male size suggests that females perceive an associated signal. In our quest to find alternative signals that females may use, we conducted detailed observations on close-range courtship behaviours, and found that females can reject or accept courting males and that female cooperation is essential for mating. Such cooperation is not ubiquitous in Lepidoptera, as forced matings have been reported in several species (Hill, Wenner, & Wells, 1976; Pliske, 1975; Orr, 1999; Cannon, 2019). Female mate choice may happen through rejection of nonpreferred males (Gomes & Cardoso, 2018; Rosenthal, 2017). We also found this in *C. virescens* females, which rejected males by flying or moving away, or by curling the abdomen away from the courting male. Although small abdominal movements that disable copulation have been noted before in other moth species (Charlton & Cardé, 1990; Farrell & Andow, 2017; Gothilf & Shorey, 1976; Haynes & Birch, 1984; Phelan & Baker, 1990), these behaviours have not been recognized as components of female mate choice. *C. virescens* females also showed acceptance behaviour, which started by assuming a quiescent, stationary position, a behaviour that has been described in other Lepidoptera species as well (e.g. Birch, Lucas, & White, 1989; Curkovic, Brunner, & Landolt, 2006; Nieberding et al., 2008). Final acceptance occurred when a female curled her abdomen towards the male's abdomen, which was also described by Hillier and Vickers (2004).

### What Close-range Signal do Females Use?

Interestingly, in the close-range courtship analysis, we found distinct tactile interactions, whereby a male 'grubs' and interlaces his foreleg with the female before mating. We speculate that this behaviour facilitates physical alignment between males and females during courtship and/or this physical contact influences sexual communication. Tarsal contact during moth courtship has been reported before (e.g. Shorey, 1964; Charlton & Cardé, 1990; Conner, Eisner, Vander Meer, Guerrero, & Meinwald, 1981; Birch et al., 1989; Sanders & Lucuik, 1992; Curkovic et al., 2006), but we are not aware of any report of leg-to-leg interactions specifically. In analysing the chemical composition of male and female legs, we found pheromone-like compounds related to the male pheromone blend, and confirmed the presence of a female pheromone compound, 16:Ald (Choi et al., 2016). Since Choi et al. (2016) found significantly higher amounts of 16:Ald on male than female tarsi, this compound was hypothesized to act as a male sexual signal. In our study, however, we found that males and females contained similar amounts of this and other pheromone compounds on their legs. We therefore conclude that these compounds are not likely to be used in female mate choice.

In addition to the above-mentioned pheromone compounds, cuticular hydrocarbons (CHCs) are known to mediate sexual communication (Blomquist & Bagnères, 2010; Ferveur, 2005). Since CHCs and butyrate esters have been identified on tarsi of *C. virescens* (Böröczky et al., 2008; Choi et al., 2016), we also compared the longer-chain compounds in our extracts and found differences between males and females. However, males and females had similar amounts of a C25 hydrocarbon and males contained lower amounts of a C27 hydrocarbon than females, suggesting that these hydrocarbons likely do not inform females about the quality of potential partners. Legs may also be used to detect sexual signals. For example, *Drosophila* species perceive contact-based chemical cues via their foreleg tarsi during courtship

(Fan et al., 2013; Ferveur, 2005; Manning, 1959). Chemoreceptors are present on tarsi of noctuid moths (Blaney & Simmonds, 1990), but whether these receptors are involved in detecting sexual signals has not been investigated.

Alternatively, females may use other signal modalities, such as visual or acoustic signals, to assess males. Acoustic signals have been found to play a role in close-range courtship in other moth species (Nakano et al., 2009). All noctuid moths have tympanal ears (Eggers, 1919; Ghiradella, 1971; Surlykke, 1984) that can detect sexual signals of conspecifics (Spangler, 1988; Nakano, Takanashi, & Surlykke, 2015). However, to our knowledge, no sound-producing organs have been described in male *C. virescens*. Recently, courtship ultrasounds have been discovered in noctuids, including in another heliothine moth, *Helicoverpa zea* (Nakano et al., 2009a, 2009b). This discovery highlights the potential to explore acoustic communication further.

### Limitations of this Study

We acknowledge two potential constraints of our study. First, all our chemical analyses were based on extracts of tissues (hairpencils, legs). It is possible that large and small males differ in emission of compounds that females use to choose mates. However, as antennaless females mated as readily as intact females, we find it more likely that females use other types of signals. Second, female preference for larger males may reflect more vigorous courtship by large males. Even though we did not observe a difference in courtship display between larger and smaller males, in the female choice experiments there were male–male interactions. However, in our close-range behavioural analysis, we clearly found female behaviours showing active female choice. Further studies are needed to determine the relative contribution of male–male competition and female choice in matings.

### Conclusion

In this study, we have demonstrated female mate choice in *C. virescens* and showed that females benefit from choosing bigger, higher quality males. In contrast to our expectations, male attractiveness was not associated with the biosynthetically related male hairpencil pheromone, but with another signal that potentially conveys information about male size. The nature of this signal has yet to be determined. The new empirical evidence we provided for female mate choice suggests that in *C. virescens*, both sexes execute mate choice, but their respective mate choices are based on different types of signals.

### Acknowledgments

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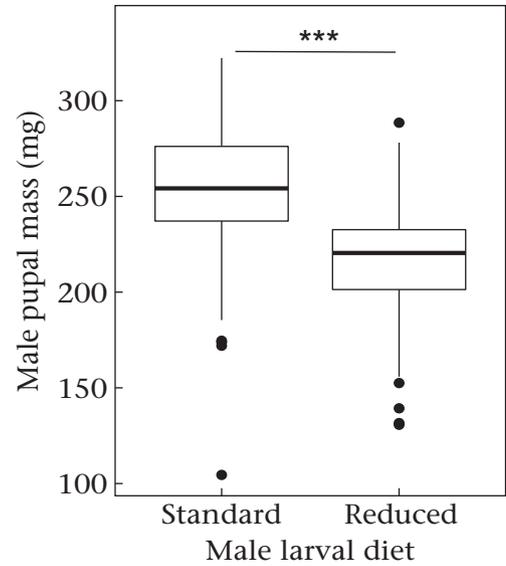
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**Appendix**

To test the reliability of pupal mass as a proxy for adult body size, we measured body length, leg length (tarsus and tibia length of the hindleg) and forewing length of 160 adult moths. After emergence, the moths were frozen and stored at  $-20\text{ }^{\circ}\text{C}$ . To measure the body parts, we ablated the left front wing and left hindleg of each individual with watchmaker’s tweezers and fixed the body parts onto A4 paper using transparent tape. The document was scanned together with a ruler using a copy machine (Canon imageRUNNER ADVANCE C3330i). The moth bodies were lined up on white A4 paper for optimal contrast next to their ID and photographed with a smart phone (Apple iPhone 8). The digital files were analysed using ImageJ (Rasband, 1997) and the body length, tarsus length, tibia length and forewing length of each individual were measured. We statistically analysed the correlations between pupal mass and adult body measures, using Pearson correlation coefficients for pupal mass with each response variable.



**Figure A1.** Box plots of *C. virescens* male pupal mass when reared on standard ( $N = 165$ ) or reduced ( $N = 153$ ) diet. The line within each box represents the median value and the lower and upper borders of each box show the first and third quartiles. Whiskers above and below the boxes mark the range of maximally 1.5 times the interquartile range. Outliers are indicated with circles. \*\*\*  $P \leq 0.001$ .

**Table A1**

Transition frequency matrix of *C. virescens* individual male behaviours in close-range courtship ( $N = 12$ )

	Q	WF	AG	EG	E	F	W	AC	GB	HP	M
Quiescent (Q)	NA	0.008	0.000	0.004	0.000	0.000	0.001	0.000	0.000	0.001	0.000
Wing fanning (WF)	0.006	NA	0.009	0.005	0.004	0.009	0.101	0.000	0.006	0.005	0.000
Antennal grooming (AG)	0.001	0.005	NA	0.000	0.001	0.001	0.004	0.000	0.000	0.001	0.000
Eye grooming (EG)	0.001	0.004	0.001	NA	0.000	0.001	0.005	0.000	0.000	0.000	0.000
Feeding (E)	0.000	0.003	0.000	0.000	NA	0.008	0.031	0.001	0.000	0.005	0.000
Flying (F)	0.000	0.018	0.000	0.000	0.003	NA	0.085	0.000	0.003	0.011	0.000
Walking (W)	0.003	0.066	0.004	0.004	0.029	0.078	NA	0.016	0.016	0.050	0.000
Abdominal curl (AC)	0.000	0.001	0.000	0.000	0.000	0.004	0.005	NA	0.031	0.069	0.013
Grubbing (GB)	0.000	0.001	0.000	0.000	0.001	0.006	0.011	0.039	NA	0.029	0.000
Hairpencil eversion (HP)	0.001	0.033	0.000	0.000	0.009	0.009	0.020	0.066	0.031	NA	0.003
Mating (M)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	NA

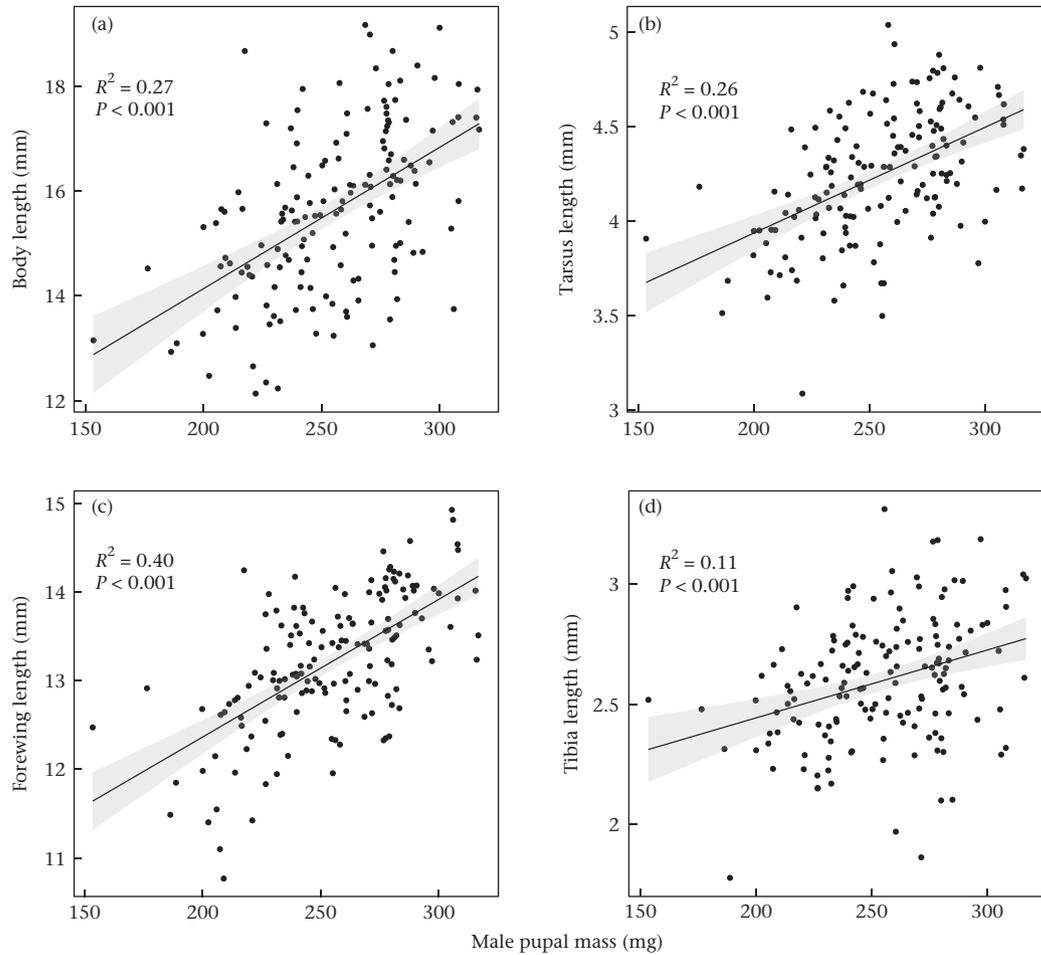
Numbers indicate the frequency of transitioning from one behaviour to another.

**Table A2**

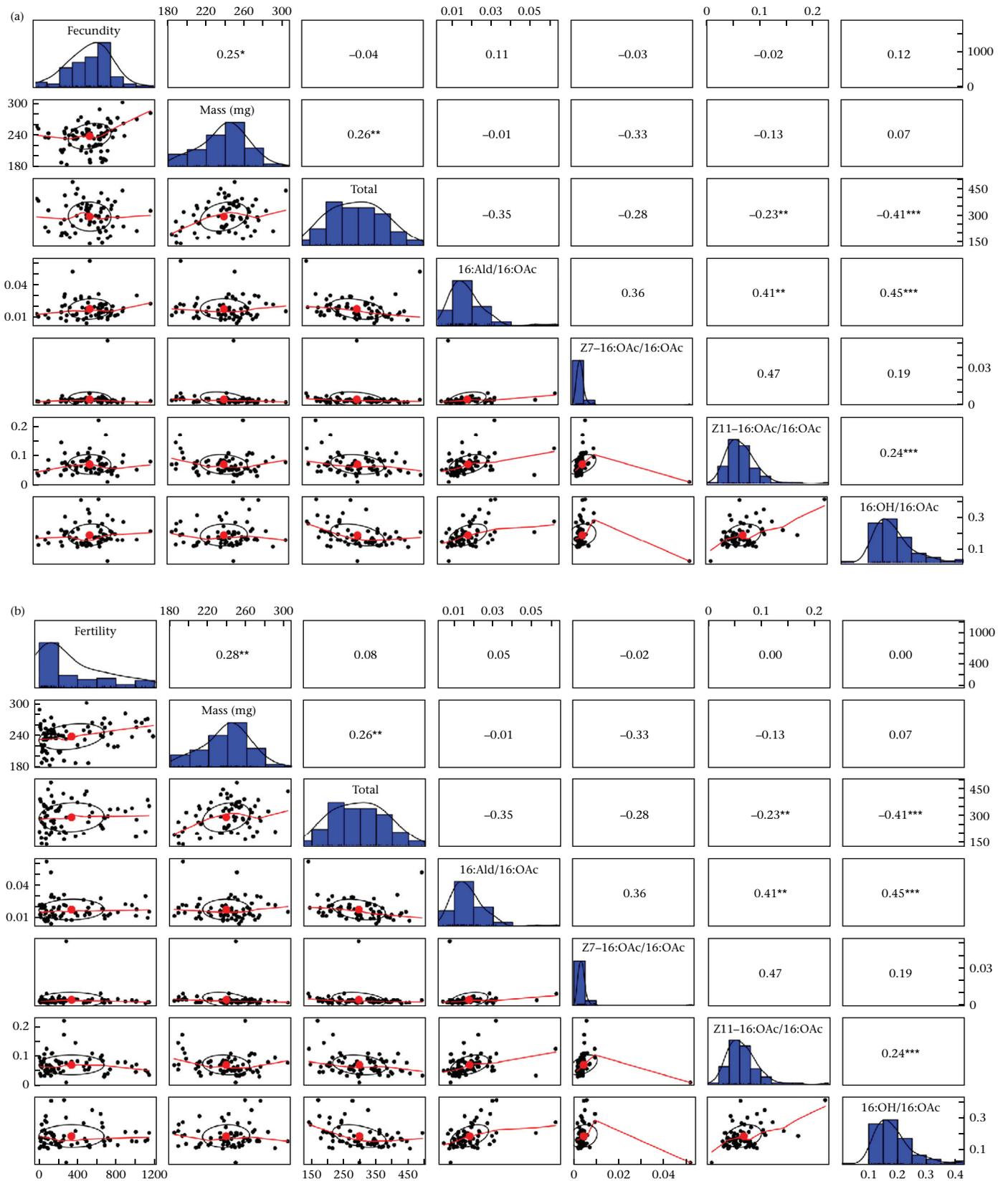
Transition frequency matrix of *C. virescens* male behaviour between categories and individual elements of a mating attempt

	Stationary	Maintenance	Locomotion	AC	GB	HP	Mating
Stationary	0.014	0.021	0.111	0.000	0.006	0.006	0.000
Maintenance	0.014	0.003	0.050	0.001	0.000	0.006	0.000
Locomotion	0.086	0.039	0.163	0.016	0.019	0.061	0.000
AC	0.001	0.000	0.009	NA	0.031	0.069	0.013
GB	0.001	0.001	0.018	0.039	NA	0.029	0.000
HP	0.034	0.009	0.029	0.066	0.031	NA	0.003
Mating	NA	NA	NA	NA	NA	NA	NA

Numbers indicate the frequency of transitioning from one behaviour to another. AC: abdominal curl; GB: grubbing; HP: hairpencil eversion.



**Figure A2.** Correlations and linear regressions of *C. virescens* pupal mass and (a) body length, (b) tarsus (hindleg) length, (c) forewing length and (d) tibia of the hindleg, based on the measurements of 155 males. Black solid lines: linear regressions; grey shaded area: 95% confidence interval. Correlation coefficients are indicated as  $R^2$  values with corresponding  $P$  values.



**Figure A3.** Scatter plot matrices, showing correlations between (a) fecundity and (b) fertility with *C. virescens* pupal mass and normalized amounts of hairpencil pheromone (ratios to the major compound 16:OAc). Spearman correlation coefficients are displayed above the diagonal. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ . The data are shown below the diagonal with a linear regression line in red and the correlation ellipse.