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Chemical and Mechanical Signals Trigger Courtship in the Wild Large-Headed Resin Bee *Heriades truncorum*

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Abstract Hymenopteran females are mostly highly selective, choosing their mating partner according to male quality signals, and may also signal their fertility through chemical advertisement. Male insects, in turn, often display a complex set of courtship behaviors to allure females to mate. In this study we explored the mechanisms triggering courtship behaviors in the oligolectic bee *Heriades truncorum*. In this species, males perform wing fanning followed by a sideways rocking motion before copulation. We tested the role of chemical cues in partner recognition by exposing males to dead females washed from cuticular extracts (i.e., sex pheromones) that were posteriorly coated, or not, with an extract of unmated females. Additionally, we experimentally reduced male wing length to test whether wing fanning was responsible for triggering sideways rocking motions. We found males to interact more often with females coated by extracts from unmated females, and to display wing fanning in the presence of these pheromones. Male wing fanning was important to perform sideways rocking motions, which are an essential behavior for successful mating and possibly an element used by females to evaluate male quality. Our study shows that both chemical and mechanical signals from female and male bees, respectively, are important elements in the mating system of *H. truncorum*.

Keywords Mating behavior · Precopulatory display · Sex pheromones · Sexual signal · Sideways rocking motion · Wing fanning

Introduction

Mating partner selection in insects usually combines chemical and mechanical cues. Sex pheromones produced either by males or females, have the function of bringing both sexes together for mating (Wyatt 2014). After the two sexes come together, further compounds acting at a close range stimulate so-called courtship behavior and copulation during which fitness information is exchanged and mating partners are selected (Paulmier et al. 1999; Ayasse et al. 2001). Sex pheromones (e.g., cuticular lipids) seem to work as an important source of information for potential mating partners (Ayasse et al. 2001). For instance, female mason bees (Megachilidae) use male volatiles to assess male quality (Conrad et al. 2010;

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Boff et al. 2022), while males of different bee species can distinguish unmated from mated females by their pheromones (Ayasse et al. 1999, 2001; Seidelmann and Rolke 2019). Therefore, a loss of "attractiveness" in females may lead to a reduction in inter-sex interactions or even to a complete lack of sexual interaction (Ayasse et al. 1999; Schiestl et al. 2000). Yet, unmated females may trigger a wide set of male behavioral responses before copulation, such as approaching and touching of females, thoracic vibration (Conrad et al. 2010), sideways rocking motions (Russell et al. 2018) and wing fanning (Weiss et al. 2015).

Wing fanning in the courtship process of insects can have several roles (Villagra et al. 2011; Benelli et al. 2012; Weiss et al. 2015). It can be used by wasps to attract a mating partner (Obara 1978), while providing information on male quality (Bredlau et al. 2013). In an experiment with the wasp *Psytallia concolor* (Braconidae) in which wing fanning was hindered by experimental reduction of the wings, mating was unsuccessful (Benelli et al. 2012). Male wing fanning was also reported in the courtship process of the solitary bee *Osmia bicornis* (Hymenoptera: Megachilidae) (Conrad and Ayasse 2015) and *Anthrenoides micans* (Hymenoptera: Andrenidae) (Dutra et al. 2021). Furthermore, the longer a male solitary bee stays on top of a female and lift females' abdomen, as reported to *Anthidium porterae* (Villalobos and Shelly 1991), and *Centris burgdorfi* (Sabino et al. 2017), respectively, the higher are males' chance to mate. Recently, a complex set of mating behaviors was described for *Heriades truncorum* (Hymenoptera: Megachilidae: Osmiini) (Boff 2022).

Heriades truncorum is a small solitary bee species whose male displays precopulatory behaviors to allure females to copulate, after touching the female's body with its antennae (Boff 2022). Copulation in this species happens on a substrate. While mounting over the females, males fan the wings, vibrate the thoracic muscles, and then display sideways rocking motions followed by lifting the female's abdomen (see figure of the behavioral steps in Boff 2022). These behaviors anticipate a copulation attempt (Boff 2022). Females of *H. truncorum* often react to male mating attempts (84% of the times) by bending the body and biting the male's abdomen. In response, males release the female or remain trying to copulate. Interestingly, this biting behavior does not suppress mating, which suggests that it can be a mechanism of male quality evaluation (Boff 2022). Behavioral responses, including wing fanning, seem to be important in the mating system of *H. truncorum*. However, previous studies on this species did not investigate the role of female pheromones on male attraction nor the potential consequences of a lack of male wing fanning in courtship.

In this study, we used manipulative experiments to further understand recently described behavioral responses of *H. truncorum* (Boff 2022). We first evaluated whether chemical compounds present on unmated females invite males to display courtship behaviors. For this, we tested whether male courtship was triggered by the presence of sex pheromones present in the surface of recently emerged unmated females. As a second step, we checked whether courtship behaviors, which occur in an order of events, happen thanks to wing fanning. This hypothesis was tested by reducing male wing size, which prevents or reduces air flow during wing fanning, and then comparing the outcome in courtship displays between treated and full-wing males. We evaluated the entire courtship process, including the time males remained mounted on the back of females, the sideways rocking motions, the lifting of the female's abdomen and female responses to male mounting. We predicted individuals with shorter wings to perform different courtship displays than full-winged males, which would impact their mating success. Lastly, since we performed a manipulative experiment to reduce the size of male wings, a negative impact of this manipulation on male strength should be visible in a decrease in mating attempts by treated males compared to untreated ones.

Material and Methods

The study was performed with a total of 58 freshly emerged males (emerged on that same day) and 68 females of *H. truncorum*, obtained from trap nests collected across Bavaria (Redlich et al. 2021) in 2019, and in the proximity of Bodensee, Biberach and Ulm, in Baden-Württemberg, Germany, in 2021.

The Role of Female Sex Pheromones on Male Courtship

We first aimed to understand whether males use olfactory cues (female sex pheromones) in mating partner recognition, i.e. the first antennal contact from males to females. A total of 39 freshly emerged females were washed together with the aid of a shaker in a 1.5 mL glass vial with a combination of hexane (hx) and dichloromethane (DCM) for two hours in a final volume of 600 µL (300 µL of each solvent). After washing the females ("dummy females" hereafter), the mix of solvents containing the extracted cuticular hydrocarbons from the 39 females were removed from the vial with the females and placed in a new vial, where the full volume was evaporated with a gentle nitrogen air stream to a final volume of 200 µL. Nine more dummy females were produced afterwards using the same procedure to have enough replicates (total number of dummies = 48) for the experiment.

To test if males were attracted to female sex pheromones, we ran experiments with the dummies in a dark room (dim red light) and room temperature. Three dry randomly chosen dummy females were placed in a Petri dish (5.5 cm diameter and 1.5 cm height) with three distinct zones marked in the external part of the Petridish (scheme in Fig S1). The first dummy was not exposed to any solvent (Washed), a second dummy was coated with 5 µl (appr. 1 female equivalent) of the extract of unmated females (Washed + CHC), and the third dummy received 5 µl of pure mix of 50% DCM and 50% hx to control for the solvent mixture (Washed + hx + DCM). The Petri dish remained for 30 s without the lid to let the small amount of solvents evaporate. Right after, an unmated male was placed in the middle of the females, and the Petridish was closed. The interaction of the male with the females was video recorded for five minutes. We counted the number of male–female interactions, i.e. the number of times that a male contacted a female with his antennae. Moreover, the time spent on courtship display (including antennation, mounting, wing fanning and sideways rocking motions, see Boff 2022)– towards dummies was annotated. This experiment was repeated 16 times (total of 80 min) with a total of 16 unmated males and 48 dummies. All bees placed in the arena were observed only once.

Electroantennography and Gas Chromatography for Identification of Sex Pheromone Candidates

To identify pheromones involved in male attraction, eight unmated females emerged on that same day were first frozen and then pooled together in a 1.5 ml glass vial, where they were immersed in 300 µL of pure pentane. The extraction of semiochemicals was performed for three minutes. A shaker was used to wash the complete surface of females. The resulting extract was concentrated with a gentle stream of nitrogen to a final volume of 100 µL.

Chemical analyses coupled with electroantennography were performed on an Agilent 7820 A Series gas chromatograph (Agilent Technologies, Germany) and an EAD Setup (Syntec Hilversum, Netherlands) equipped with a non-polar DB-5 MS capillary column (30 m × 0.25 mm inner diameter, J&W) and a flame ionization detector (GC-FID) with hydrogen as the carrier gas (constant flow, 2.0 ml/min). For each GC-EAD run, one antenna of *H. truncorum* was cut off at its basis and at the tip of the excised antenna and mounted between two glass-capillaries filled with insect Ringer solution (0.42 g KCl, 5 g NaCl, 0.125 g CaCl in 1 l demineralized water). The recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. One microliter of each sample was injected splitless into the gas chromatograph (injector temperature: 300 °C), operating at 60 °C for 1 min, after which the split valve was opened, and the temperature was increased continuously by 5 °C/min to a final temperature of 300 °C. The effluent was split (Gerstel outlet splitter, split ratio FID:EAD 1:1), and the outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the antenna to prevent dehydration. The outlet was heated (310 °C) to avoid condensation of the effluent in the cooler airflow. EAD and FID signals were recorded simultaneously on a PC running a GC-EAD program (Syntech, Hilversum, Netherlands). We considered a substance to be EAD-active when it proved to be active via antennae responses of different males.

All chemical analyses were performed on a gas chromatograph (Agilent 7890A, Agilent Technologies, Waldbronn, Germany) with a DB-5 MS capillary column (30 m × 0.25 mm inner diameter, J&W) and a flame ionization detector (FID). For the identification of the GC-EAD active semiochemical compounds in female extracts, indication of sex pheromone candidates, we used gas chromatography/mass spectrometry (Agilent 7890B GC plus 5977A MSD, Agilent Technologies, Santa Clara, CA, USA; method as described above for GC, carrier gas: helium) and compared mass spectra using references from the NIST11 library and GC retention times with those of authentic reference samples. Furthermore, we used reference mixtures containing synthetic alkanes and alkenes and compared compounds with the ones from our female extracts. Double bond positions of Alkens were identified by co-injection of samples with GC-EAD active compounds with reference mixtures of different alkene isomers. For quantitative analyses in each of the samples, 10 µL of an internal standard (tridecane (99%, Sigma, Germany), stock solution: 100 µg/mL in n-pentane) was added. GC-EADs and GC runs were performed at the Institute of Evolutionary Ecology and Conservation Genomics, Ulm University.

Wing Fanning as Functional Trait During Mating

Freshly emerged males ($n = 30$) and females ($n = 10$) of *H. truncorum* were placed in individual Petri-dishes (2.5 cm Ø, 1.5 cm high, with ad libitum sucrose solution in a feeder) and studied from 15th July to 15th August 2020 after emergence in lab environment (Department of Animal Ecology and Tropical Biology, University of Würzburg). The intertegular distance of the individuals was sized with the software Las Ez version 3.4.0 attached to a stereomicroscope (Leica EZ4D), and this information was used as variable of interest in the models (see below).

To assess the importance of *H. truncorum* males' wings in the mating process, we experimentally reduced the size of both fore and hind wings of males, creating three groups (Fig. S2): Full-winged males (untouched wings = FW), a second group with the tip of the wings removed (tip of the wing off = TW) and a third group with vestigial wings (basal part of the wing maintained = VW). One male from each treatment was placed in the arena together with an unmated female. To attenuate the effect of chemical and genetic signals on partner preferences, we only used males sampled from the same locality in each mating arena (Vereecken et al. 2007; Conrad et al. 2018) with a female from a different sampling site. At the end of the experiment, fore and hind wings of four individuals per treatment were cut off with micro-dissecting scissors and mounted on microscopic glass slides (76×26 mm, VWR International, Radnor, USA). We used an Axiocam 105 color microscope camera (Zeiss, Germany) mounted on a Stemi 508 stereo microscope (Zeiss, Germany) to photograph both wings of each individual. Wing size was measured based on the maximum distance from the closest wing vein at the insertion of the wing in the tegula to the tip end of the remaining wing by means of the analytical software ZEN 3.2 (blue edition, Zeiss). FW males had fore wing length average = 4.26 ± 0.09 mm, and hind wing length average = 3.17 ± 0.17 mm; TW males had fore wing length average = 2.93 ± 0.29 mm, hind wing length average = 2.3 ± 0.23 mm; VW males had fore wing length average = 1.46 ± 0.43 mm, hind wing length average = 1.21 ± 0.34 mm. We recorded 10 videos, ten minutes each, of individuals' interactions in Petri dishes. Since neither treatment (wing length shortening) nor the size of individuals reduced the ability of males to perform a mating attempt (see Results), we discarded an effect of manipulation on male strength and performed further observations of the same video recordings on the following additional behaviors: 1) the time a given male remained mounted on the back of the female, 2) the number of sideways rocking motions, 3) whether males lifted up the female's abdomen (yes/no), 4) the number of copulations, and 5) female responses to male mounting (biting attempts). The sideways rocking motions were estimated by counting the number of times that males bent the body laterally while mounted on females, measured with the video in slow-motion.

Statistical Analyses

The Role of Female Sex Pheromones on Male Courtship

All analyses were performed in R version 4.2.0 (R Core Team 2018). To test if males were attracted to females via olfactory cues, we ran a Generalized Linear Model (GLM, MASS package, (Venables and Ripley 2002) with a negative binomial distribution, using the number of male approaches following antennation towards dummies as response variable, and dummy treatment as fixed factor with three levels (Washed, Washed + hx + DCM, Washed + CHC). Moreover, the time (in seconds) that males spent performing courtship displays was compared among dummy treatments. To do this, we ran a GLM with a negative binomial error distribution, using the time spent in courtship (mounted on the female) as response variable and dummy treatment as fixed factor. For this last analysis, we excluded those observations in which no courtship display was observed (77%).

Wing Fanning as Functional Trait During Mating

To test the importance of wing fanning in the mating behavior of the large-headed resin bee, we used Generalized linear mixed models (GLMMs, glmmTMB package, Brooks et al. 2017). First, we assessed whether the wing length treatment and the size of males and females affected the number of mating attempts by males. For this, we ran a GLMM with a poisson distribution, including the number of mating attempts as dependent variable, and the treatment, size of females and size of males as independent variables, and male identity as random effect. Then, we fitted a model with a Gaussian error distribution to analyze whether the time (in seconds and log transformed) that males remained mounted over the females was determined by wing length (fixed factor with three levels) and individual size (of both males and females). The second model, fitted with a Poisson distribution, included the number of times males performed the sideways rocking motion as response variable, and wing length as fixed factor with three levels. Then, we used a binomial error distribution to analyze if the male behavior of lifting the female's abdomen was explained by wing length. Since males performed multiple mating attempts, the identity of males was used as random factor in all models to account for pseudoreplication. Lastly, female response to male mounting (number of biting attempts) was tested in a model with a Poisson error distribution, with male wing length, number of mating attempts and male and female sizes as fixed factors.

The fit of the models was assessed with the package DHARMa (Hartig 2020).

Results

The Role of Female Sex Pheromones on Male Courtship

Our results confirmed that males interact differently with females depending on whether females have pheromones in their cuticle. A total of 136 male–female interactions (antennations) were recorded in 16 trials. On average, males interacted 4.31 (±3.2) times with dummies (females washed from pheromones) coated with extract of unmated females (Washed + CHC), 2.81 (±2.5) times with only washed dummies (Washed) and 1.37 (±2.01) times with dummies covered with a mixture of hexane and dichloromethane (Washed + hx + DCM). There was a significant effect of treatment in the number of antennations that males performed with dummies (GLM, $\chi^2 = 10.57$, df = 2, $p = 0.005$). The number of interactions was significantly lower with dummies exposed to solvent only, compared to dummies coated with extract of unmated females (Fig. 1). Males displayed no courtship behavior towards dummies exposed to solvent only (Washed + hx + DCM). Courtship towards dummies was recorded 11 times, six times towards dummies coated with surface extract of unmated females and five times towards washed dummies. However, courtship towards dummies coated with extract comprised 86% of the total courtship time (135 out of 157 s), including wing fanning. The number of interactions and the duration were not correlated (Pearson's r = 0.43 considering all observations, and Pearson's r = 0.19 considering interactions followed by mounting). However, males

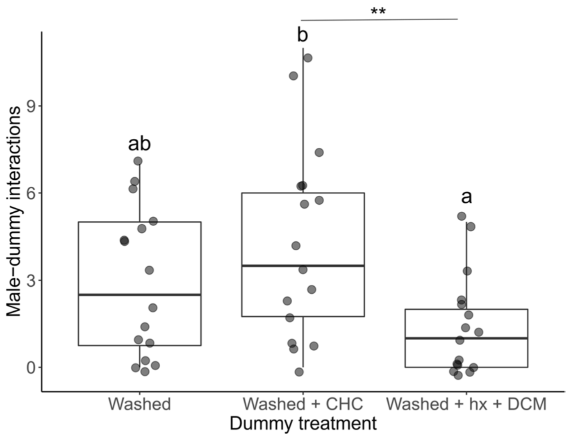

Fig. 1 The number of male-dummy interactions (i.e. number of antennations from males to dummy females) was significantly higher when comparing dummies exposed to CHC (Washed + CHC) and dummies exposed to solvent only (Washed + hx + DCM). Boxplots show mean and standard errors. Significance levels shown by letters: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

spent on average 22.5 (±24.8) seconds performing courtship towards Washed+CHC dummies, which was significantly higher than the 4.4 (±2.3) seconds that males spent with dummies that were not exposed to the extract (Washed) (GLM, $\chi^2=7.14$, df=1, $p=0.008$, Fig. S3).

Chemical analyses identified a total of nine active compounds (Fig. 2). Two male's antennae detected the totality of compounds, while five male's antennae responded to eight out of nine compounds: One antenna did not respond (or was not detected) to the compound Z-(7) nonacosene and another to hentriacontane. From the nine compounds, five were alkans and four alkens with chain lengths varying between 23 and 31 carbon atoms. Alkens presented a double bond position at carbon seven (Table 1).

Table 1 Electrophysiologically active compounds present on the surface of recently emerged females of *Heriades truncorum* that released antennal responses of males

Chromatogram order	Class	Compound
1	Alken	(Z)-7-Tricosene
2	Alkan	Tricosane
3	Alken	(Z)-7-Pentacosene
4	Alkan	Pentacosane
5	Alken	(Z)-7 Heptacosene
6	Alkan	Heptacosane
7	Alken	(Z)-7 Nonacosene
8	Alkan	Nonacosane
9	Alkan	Hentriacontane

Wing Fanning as Functional Trait During Mating

The number of mating attempts (i.e. attempt to grab a female) was similar across males with different wing treatments ($n_{FW}=14$, $n_{TW}=13$, $n_{VW}=12$; $\chi^2=0.25$, df=2, $p=0.88$; Fig. S4a), and did not depend on the size of individuals ($\chi^2_{\text{Female size}}=0.03$, df=1, $p=0.854$; $\chi^2_{\text{Male size}}=0.22$, df=1, $p=0.64$; Fig. S4b and c). On average, full-winged males (FW) remained for a longer time (20.76±13.83 s) on the back of the female, but no statistical difference was found when compared to males with shortened wings (TW=14.57±12.94 s and VW=15.12±13.82 s; GLMM, $\chi^2=0.10$, df=2, $p=0.953$; Fig. S5a). The size of females and males did not affect the time males remained mounting the females (GLMM, $\chi^2_{Female}=1.00$, df=1, $p=0.318$, $\chi^2_{Male}=1.29$, df=1, $p=0.257$; Fig. S5b and c). On the other hand, wing length had a significant effect on sideways rocking motions (GLMM, χ^2 = 8.44, df=2, $p=0.015$; Fig. 3). Males with the shortest wing length displayed on average significantly less sideways rocking motions (VW=0.8±1.31) than males with longer wings (FW=3.81±4.41, TW=1.3±1.49; Fig. 3). Lifting females' abdomen was displayed more frequently by full-winged males, but differences among groups were not significant (GLMM, $\chi^2=3.62$, df=2, $p=0.163$). Nevertheless, the treatment reduced the number of times that males lifted the females' abdomen (FW=56%, TW=10% and VW=20%). Copulation attempts were observed only three times, and only by full-winged males. Although the low number of copulation events did not allow us to test

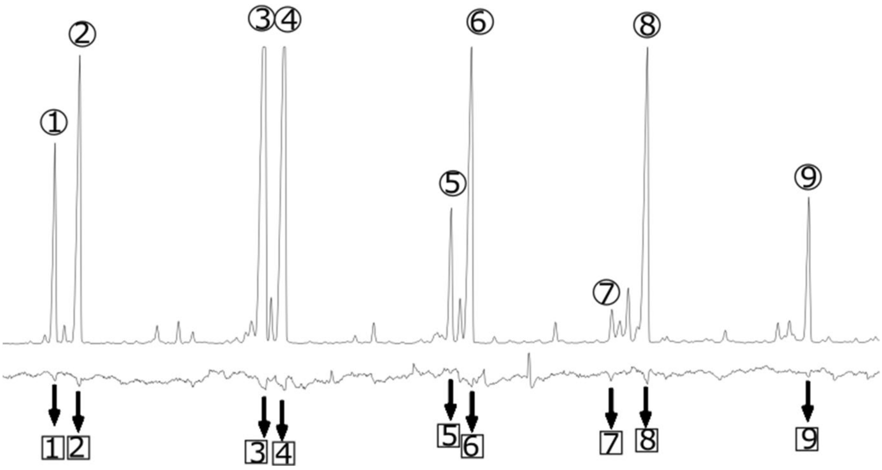

Fig. 2 Electroantennogram showing male antennal responses (squares) to unmated female sex pheromone candidates (circles)

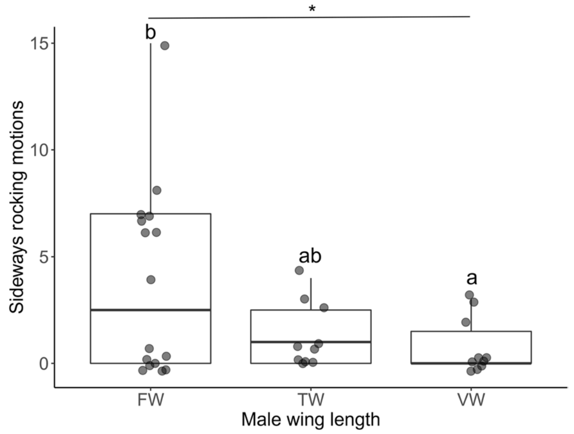

Fig. 3 Males with the shortest wings (VW) displayed significantly less sideways rocking motions than full-winged males (FW). FW = males with full-wing length, TW = males with the tip of the wing off, VW = vestigial wings. Boxplots show mean and standard errors. Significance levels shown by letters: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

for significant differences, the three males displayed the whole precopulatory behavior, i.e. sideways rocking motions and lifting up the female's abdomen.

Female's response to male mounting (biting attempts) was significantly affected by the number of mating attempts (GLMM, $\chi^2 = 18.63$, df = 1, $p < 0.001$; Fig. 4a). However, the number of female responses was independent of wing length (GLMM, $\chi^2 = 1.93$, df = 2, $p = 0.381$; Fig. 4b) and the size of the mating pairs (GLMM, $\chi^2_{Female} = 0.75$, df = 1, $p = 0.386$; $\chi^2_{Male} = 0.55$, df = 1, $p = 0.456$).

Discussion

Our results showed that chemical compounds present on unmated females are key to stimulate males to perform a courtship display and suggest that wing fanning is an important mechanism affecting sideways rocking motions prior to copulation.

The Role of Female Sex Pheromones on Male Courtship

Cuticular hydrocarbons are important components for attracting mating partners in some bee species (Ayasse et al. 1999, 2001), and this seems to be also the case for the oligolectic bee *H. truncorum*. Here, unmated males of *H. truncorum* interacted more often with dummies (odorless females washed with solvent) that were coated with extracts from a pool of dozens of unmated females than with those that received pure solvent without extracts of unmated females when deprived of visual cue. However, the number of interactions between males and dummies treated

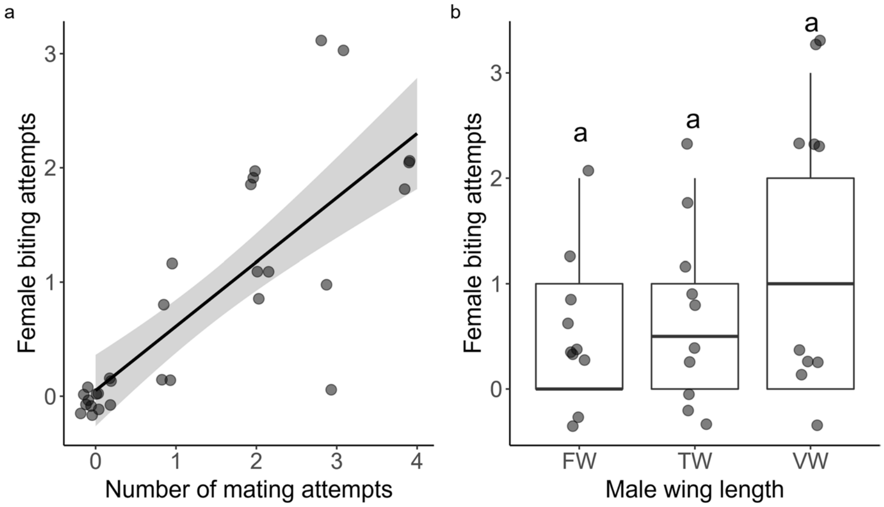

Fig. 4 a Female response to male mounting (biting attempts) increased significantly with male mating attempts. The gray shadow represents lower and upper limits for a 95% confidence interval. b Female response to male mounting did not change with male wing length. FW = males with full-wing length, TW = males with the tip of the wing removed, VW = vestigial wings. Boxplots show mean and standard errors. Significance levels shown by letters: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

with extracts of unmated females was similar to the number of interactions between males and dummies just washed with odorless solvent. Hence, even when the presence of unmated female extract was more attractive than the potential repellent effect of the solvent, the effect is not as strong as expected. First, it is possible that although we let the solvent evaporate, traces of the solvent alone could have repelled males. This could explain the lower number of interactions towards dummies only coated with solvent and the not significant difference between dummies coated with extract of unmated females (applied with the same solvent) and washed dummies. Second, even if we washed females for a two-hour period to produce odorless dummies, some dummies might have preserved traces of the active compounds, which could explain why some males interacted with them. Third, since our experiment was performed in the dark with dead females, we cannot discard that males of *H. truncorum* might have an innate response (e.g. grabbing behavior) to females' body morphology. We can exclude that these contacts may have occurred randomly, as males used to actively explore the arena.

Apart from the number of interactions, dummies that received extract from unmated females elicited 37.5% of the males to perform courtship, and the time that males spent interacting with these dummies with respect to dummies coated with solvent only was six times higher. Considering the higher number of interactions and the longer time that males spent performing courtship to dummies coated with extract of unmated females, our results support a pivotal role of pheromones on female-male sex communication in *H. truncorum*, as described for other hymenopteran species such as *Lasioglossum malachurum* (Ayasse et al. 1999), *Amegilla dawsoni* (Simmons et al. 2003) and *Stizus continuus* (Polidori et al. 2017).

Although semiochemicals of different volatility may be linked to different behavioral responses, previous research on a parasitic wasp linked a contact sex pheromone with male wing fanning (Kühbandner et al. 2012). Courtship in hymenopterans, and particularly wing fanning, seems to be driven by sex pheromones present on the surface of unmated females (Wcislo 1992). This suggests a conserved mechanism in the courtship display of male hymenopterans (Wcislo 1992; Ayasse et al. 1999; Kühbandner et al. 2012; Benelli et al. 2013; Polidori et al. 2017; Würf et al. 2020).

Here, we have determined that males of *H. truncorum* respond to nine different chemical compounds present on the surface of unmated females. Some of these compounds are common in recruiting behaviors, showing a conserved function across species. For example, Z-(7)-Tricosene is one of the sex pheromones in the moth *Coscinoptycha improbana* (Gibb et al. 2006). Females of house flies produce a tricosene with a double bond position at carbon 9th to attract males (Reed et al. 1994). The compound tricosane was found to negatively regulate mating activity in *Drosophila suzukii* (Snellings et al. 2018). n-Pentacosane is a female contact and sex pheromone in other insect species such as the beetle *Myllocerinus aurolineatus* (Sun et al. 2017) and *Andrena nigroaenea* (Schiestl et al. 2000). Many of the active compounds present in females of *H. truncorum* were also present in the surface of workers and queens of the species *Lasioglossum malachurum* (Ayasse et al. 1999; Steitz and Ayasse 2020) and *Megachile rotundata* (Paulmier et al. 1999), supporting the role of these semiochemicals in co-specific interactions. In the Cerambycidae *Anoplophora glabripennis*, males were also attracted to dummies in the presence of cuticular extracts, including Alkens such as tricosene, (Z)-7-pentacosene and (Z)- 7-heptacosene (Zhang et al. 2003). Hentriacontane was also indicated as candidate sex pheromone together with other Alkanes in *Araneus diadematus* (Fischer et al. 2021). In this study, we are limited to discuss the individual role of each compound in the courtship and mating behavior of *H. truncorum*, and it is even possible that the combination of semiochemicals (Zhang et al. 2003; Sun et al. 2017) and ratios (Snellings et al. 2018) work synergistically in partner attraction. Nevertheless, future studies could try to link the function of each compound to a specific mating behavior of males. Besides, these results should be taken with caution, since we did not perform bioassays with mated females. Understanding how the chemical profile of females changes to signal their mated state, and whether males interact with extract of mated females, would further confirm our findings.

Despite the clear mating attempts performed by males to dummies coated with extracts of unmated females, in 73% of the observations males released females and stopped interacting with them after 3.8 s on average. This might indicate that, in this mating system, a behavioral response of females (see below) can be necessary for effective copulation.

Wing Fanning as Functional Trait During Mating

The number of copulations observed in the study was low ($n=3$), but they were only registered in full-winged males, supporting preliminary observations that wing fanning forego copulation in *H. truncorum* (Boff 2022). Our experiment showed that wing fanning is not a requirement for males to be able to mount females, although full-winged males could hold females for a longer time.

Additionally, we corroborated that wing fanning is needed to perform sideways rocking motions. Males that could not fan their wings due to shorter wing length significantly reduced the number of sideways rocking motions. Besides, although differences were not significant, full-winged males could lift the females' abdomen more frequently than bees with reduced wings. It is possible that a bigger sample size would reveal wing fanning as an important mechanism for males to hold on the back of females and to lift their abdomens. It is worth highlighting that males subjected to different wing length treatments engaged in a similar number of mating attempts, evidencing no negative effect of wing shortening on the will of males to mate.

Even though wing fanning may be a common behavior in several hymenopterans, including solitary bee species, its sexual meaning may differ across species. In *Diadasia rinconis* (Hymenoptera: Apidae) males perform this behavior at regular intervals after genital coupling began (Russell et al. 2018), whereas in *Aphidius ervi* (Hymenoptera: Braconidae) wing fanning is related to a courtship signal: The sound produced by wing fanning determine the female acceptance of the mating partner (Villagra et al. 2011). Here, although wing fanning had an impact on sideways rocking motions, we do not know if females obtained information on male quality from the air flow produced by male wing fanning (Benelli et al. 2013; Bredlau et al. 2013), such as some sort of chemical sexual signal (Obara 1978; Peters et al. 2017; Li et al. 2018).

Females of several hymenopterans, including *Osmia bicornis* (Conrad et al. 2010) and *O. cornuta* (Boff et al. 2022) like *H. truncorum*, display behavioral responses to evaluate the quality of potential mating partners. In mason bees, males vibrate their thoracic muscles, and females use this information to evaluate male quality, especially regarding the frequency of modulation (Conrad et al. 2010; Boff et al. 2022). *H. truncorum* females evaluate males by displaying a response to male mounting (Boff 2022). Females turn their body ventrally and bite the male's abdomen while the male attempts to copulate by vibrating the thorax and fanning the wings. Here, we confirm the observation that *H. truncorum* females evaluate male quality, as other species do (Ayasse et al. 2001; Conrad et al. 2017; Boff et al. 2022). Nearly 60% of the males that attempted to mate received bites. The number of biting attempts did not differ among males of different wing size, but vestigial-winged (VW) males received bites from females in 85% of their mating attempts, while this proportion dropped to 33% in full-winged conspecifics. In the parasitic wasp *Mormoniella vitripenis,* males of older age are less keen to fan the wings and therefore more often rejected by females (Barrass 1960). Despite the lack of significant differences among males of different wing length, it is interesting that females' responses increased with decreasing ability of males to fan the wings. As mentioned above, it is likely that a bigger sample size would be needed to clarify uncovered significant differences. On the other hand, although aspects of chemical communication (e.g., transference of pheromones) related with wing fanning in *H. truncorum* remain to be explored, the higher female evaluation towards VW males might indicate that wing fanning is used for male quality evaluation, as shown for other species (Barrass 1960; Benelli et al. 2013).

Courtship display and subsequent mating of *H. truncorum* relied on chemical advertisement present in the surface of unmated females. The absence of these compounds limited male courtship displays. Preventing males to perform wing fanning also limited their ability to perform follow-up courtship displays (sideways rocking motions). Our results suggest that the complete male courtship relies on attributes from both females and males. Additionally, it is possible that wing fanning is an element used by females to evaluate male quality. In conclusion, both sex pheromones and wing fanning are important components of the mating system of *H. truncorum*, which is therefore dependent on both chemical and mechanical signals.

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Author's Contribution SB designed and performed the experiments. CG organized and separated all studied individuals straight after their emergence. SB recorded videos of the bees and interpreted their behavior. SB performed electroantennography analyses and interpreted responses with MA. SB and MB performed chemical compound identification. SB and CG performed statistical analysis and SB drafted the manuscript. SB, CG and MA wrote the final version and agreed with the submission of the manuscript.

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Declarations

Conflicts of Interest Authors disclose all potential conflicts of interest.

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