



3D-printed insect models offer a feasible method for mating studies of chrysomelid beetles

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Abstract

A variety of models have been used in mating bioassays of insects to assess the contribution of chemical and visual signals to mate location and mate selection. Although the use of such ‘dummies’ has had varying degrees of success, some insect species refuse to accept simplistic models. In the present study, we developed a 3D-printed model to explore whether more realistic models will be more successful than simplistic models in mating assays of difficult to manipulate species such as the flea beetle *Altica fragariae*. We ran five experiments to test (1) whether males could discriminate between males and females solely based on differences in cuticular hydrocarbons (CHCs), (2) whether males use shape or (3) color to choose mates, and (4) whether males can discriminate between 3D-printed models and freshly killed beetles either with or (5) without legs and antennae. The results of these experiments confirmed that male *A. fragariae* preferred models coated with CHCs of females over that of male CHCs, providing strong support for the role of CHCs in mate choice in *Altica*. We also showed that males use both shape and color in mate selection, and that males are capable of discriminating between the models and real beetle specimens. Together, the results indicate that 3D-printed models can provide a feasible and cost-effective method for mating studies of insects.

Keywords *Altica* · Chrysomelidae · 3D-printed model · Intraspecific recognition · Mating choice · Behavioral assay

Introduction

Chemical cues play an important role in mate recognition in insects (Johansson and Jones 2007; Smadja and Butlin 2009). One of the major chemical signals involved in inter-specific and intraspecific recognition are cuticular hydrocarbons (CHCs) (Singer 1998; Blomquist and Bagnères 2010). The CHCs of many insects are complex and involve saturated, unsaturated and branched chains with lengths between 21 and 50 carbons; they are usually important at short ranges and generally act as contact pheromones (Blomquist and Bagnères 2010). Within species, CHCs have documented functions ranging from discriminating the reproductive status of individuals to sex discrimination (Steiger et al. 2009; Schlechter-Helas et al. 2012). Determining the relative contributions of CHCs and other cues such as visual and tactile cues requires behavioral assays that directly manipulate CHCs and other cues. These experiments can be challenging because, for example, manipulation of CHCs may be impossible if chemicals continue to be excreted during a mating bioassay. Although some techniques, such as gas chromatography-electroantennographic detection and single

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sensillum recordings have been used to test the bioactivity of CHCs (Blomquist and Bagnères 2010; Gonzalez et al. 2014; Pask et al. 2017), bioassays are decisive and necessary for definitively identifying mating cues in insects.

In addition to live insects, behavioral assays have used ‘dummies’ or artificial models to assess the cues involved in mating decisions. In general, simple models have been frequently and successfully used in mating bioassays of insects, for example, glass rods and beads (e.g., Geiselhardt et al. 2012; Fujiwara-Tsujii et al. 2013; Shimomura et al. 2016; Isa et al. 2019), clay dummies (Rafter and Walter 2013), and even plastic centrifuge tubes (Fukaya et al. 1996; Zhang et al. 2003) and spatulas (Sugeno et al. 2006) have yielded information about mate discrimination. However, these simplistic models do not always work well for some insect species. For instance, male *Scirtothrips aurantii* (Faure) thrips would approach, contact and mount clay models treated with female cuticular extracts, but males did not copulate with the model (Rafter and Walter 2013). Similarly, male tea weevils, *Myloccerinus aurolineatus* (Voss), showed behavioral responses, but refused to copulate with glass rod models coated with female CHCs (Sun et al. 2017). Together, these results suggest that in addition to CHCs, the shape, color, texture and other physical characteristics of mates such as wing fanning or stridulation are also involved in mating decisions.

Since artificial models are too simplistic for insects that require a variety of mating cues, some mating studies have successfully employed freshly killed insects as surrogates (Schiestl and Ayasse 2000; Peterson et al. 2007; Tanigaki et al. 2007; Lacey et al. 2008; Buellesbach et al. 2013; Zhang et al. 2014; Xue et al. 2016a, b, 2018). In these systems, the CHCs can be removed from the surface of the dead insect and then reapplied for mating assays. For example, in two sympatric *Altica* flea beetles, *Altica fragariae* Nakane and *A. viridicyanea* (Baly), the mating preference of males reversed when they were given a choice between conspecific and heterospecific dead females with their CHCs exchanged, suggesting a key role of CHCs in behavioral isolation between close relatives (Xue et al. 2016a). Similar methods have been used to identify the role of CHCs in interspecific recognition in other species, for example, *Pidonia* longicorn beetles (Tanigaki et al. 2007), *Nasonia* jewel wasps (Buellesbach et al. 2013) and *Pyrhalta* leaf beetles (Zhang et al. 2014). There are, however, caveats to this approach. Specifically, both the effectiveness of removing all of the CHCs from the cuticle and the excretion of chemicals in freshly killed insects may obscure the mating preference of males during mating trials. This may have occurred in a study of intraspecific mate recognition cues in *Altica*, which has been used as a model for studies of mating behavior, host plant specialization, and ecological speciation (Xue et al. 2011, 2014, 2016a). In previous studies, we found that males could

discriminate between males and females as well as between sexually mature and immature females in mating bioassays with alive or freshly killed specimens (Xue et al. 2016b). Moreover, these groups of beetles had significantly different CHC profiles. Although the strength of preference was altered when the CHCs were exchanged between potential mating partners, the predicted reversal of mating choices were not observed. It is possible that the removal and reapplication of CHCs to freshly killed insects may have masked the subtle quantitative differences between individuals and obscured the mating choice of males. Alternatively, other cues may also be involved in mate discrimination other than CHCs (Xue et al. 2016b). Thus, to disentangle the effects of chemical and other cues on mate recognition, what is needed are more realistic models that can be readily manipulated.

Here, we take advantage of 3D-printing technology to develop a more realistic mating model for *Altica*. The development of 3D-printing techniques provides us with an opportunity to develop a more lifelike model for mating studies. In the present study, we used a flea beetle *Altica fragariae* (Coleoptera: Chrysomelidae) and 3D-printed models to test the feasibility of these models in behavioral mating assays of insects. We asked three questions. (1) Can males discriminate between the sexes based solely on differences in CHCs? (2) Do visual cues such as shape and color of the 3D model affect mate choice decisions? (3) Can males discriminate between real beetles and 3D-printed models?

Materials and methods

Beetles

Altica fragariae are small (3–4 mm), elongate-ovate chrysomelid leaf beetles. The body surface is black with a metallic blue reflection. Male *A. fragariae* are the choosier sex and is the primary partner controlling mate choice (Xue et al. 2018). Both sexes of *Altica* species mate several times with multiple partners over the course of their lifetime (Xue et al. 2014). Previous mating experiences do not affect mating preference of male; furthermore, although the CHC profiles are age-specific (Xue et al. 2016b), the CHC profiles of sexually mature males and females of *Altica* do not change after mating (HJX, unpublished). As a result, field-collected beetles are suitable for use in experiments testing whether males can discriminate between the sexes based on differences in CHCs. In the present study, we collected adults from Nankou (40.28° N, 116.02° E), Changping, Beijing and maintained them in the lab. Under our laboratory conditions (25 °C and with 16:8 h light:dark), newly emerged adult females of *A. fragariae* become sexually active after 5–7 days of maturation (Xue et al. 2007). Since the sexual maturity status of the field-collected beetles was unknown,

to ensure that the beetles were fully mature, we waited at least 7 days after collection before using them for mating bioassays.

3D-printed model

To create a 3D model of *Altica* (Fig. 1), we dried frozen specimens of *A. viridicyanae* in a chemical fume hood at 25 °C for 2 days and then used clear nail polish to glue them to the tip of a micropipette. The specimens were scanned with an Xradia MicroXCT-400 (Xradio Inc., California, USA) with the following parameters: beam strength was set to 60 kV and 133 μ A; pixel size of images was 3.71 μ m; optical magnification was 4 \times ; and exposure time was 1 s. We obtained 992 sections of images that were then imported into the Amira 6.0.1 software (Thermo Fisher Scientific, Hillsboro, Oregon, USA) for 3D reconstruction. Amira 6.0.1 was used to smooth and simplify the reconstructed model and to produce a final OBJ format file. The 3D beetles were then printed using isocyanate and polybasic alcohols by the 3Dmold Service Company (Shenzhen, Guangdong) (<http://www.3dmold.com.cn/>). The antennae and legs were not included on the 3D models because these appendages were too thin for current 3D-printing methods. We obtained both black and white models for our tests.

Chemical detection

Prior to conducting mating assays, we checked the mating assay models for any potential chemical odors that might affect the results. To do this, we used gas chromatography–mass spectrometry (GC–MS) to test for CHCs in both the black and white 3D-printed beetles (3DPB) as well as black glass bead models (diameter of 3 mm). We examined five samples per group. In addition to these models, we also included three replicates each of three treatments: hexane only (HPLC/Spectro grade, Tedia, USA), male *A. fragariae*, and female *A. fragariae*. To obtain the cuticular extracts from the beetle specimens, we euthanized the experimental beetles by placing them in a –30 °C freezer for 20 min, and submerged them in 40- μ L hexane for 15 min. In the mating assay models, we first removed any potential dissociative chemicals on the surface by rinsing them twice with hexane (0.5 mL/model), and then the models were submerged in 40- μ L hexane for 15 min to obtain the extracts.

We placed the prepared extracts into vial inserts (Agilent Technologies Inc.; 250 μ L, glass with polymer feet) and then transferred them to chromatography vials (Agilent Technologies Inc., screw cap vials, 1.5 mL) for GC–MS analysis (HP 7890 series). To separate the CHCs, an HP5 column (30 m \times 0.32 mm internal diameter \times 0.25 μ m film thickness, Agilent Technologies, Inc.)

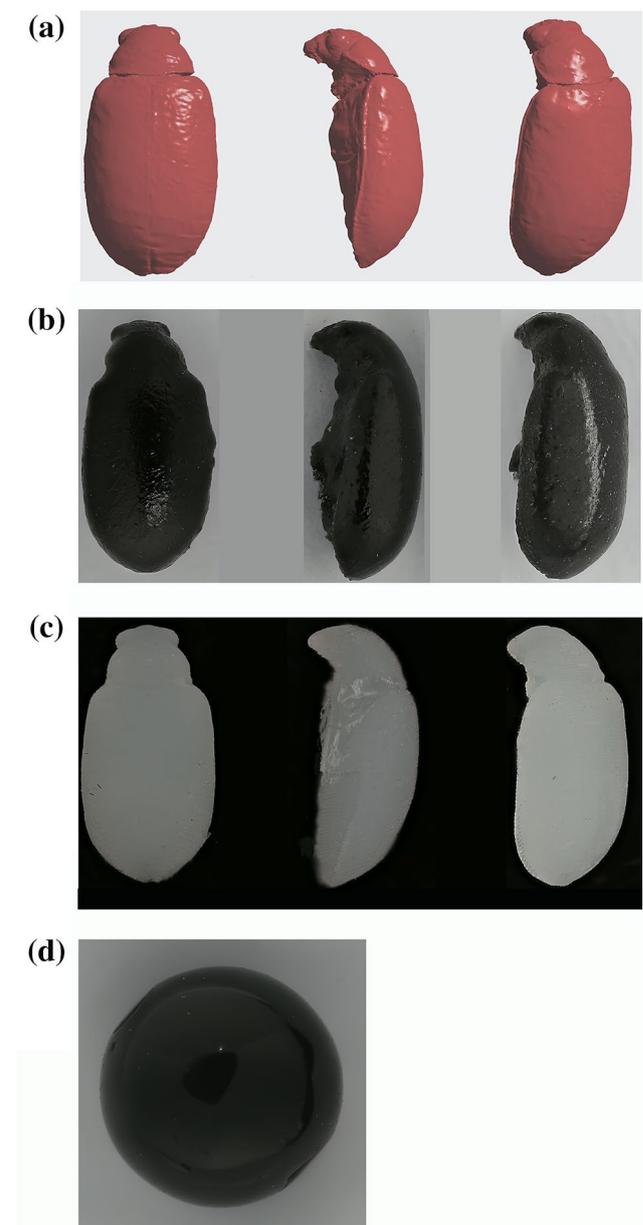


Fig. 1 Reconstructed 3D-printed model and printed samples of *A. fragariae*, and glass bead used in the present study. **a** Reconstructed 3D model; **b** black 3D-printed model; **c** white 3D-printed model; **d** black glass bead. For the 3D models, the columns from left to right are the dorsal view, lateral view, and 45° view

was used with helium as carrier gas (1.0 mL/min). An aliquot of 2 μ L per sample was injected in the splitless mode at 280 °C. The oven was programmed to hold at 40 °C for 1 min, then to increase by 8 °C per min to 300 °C, hold at 300 °C for 4 min, then increase 20 °C per min to 320 °C, and finally, to hold at 320 °C for 8 min. The mass spectrometer was in the electron impact mode set at 70 eV.

Mating bioassays

We used two-choice mate tests to examine the feasibility of the 3D-printed mating models. Two-choice tests have proven to be a reliable method for mate preference studies in *Altica* species (Xue et al. 2014, 2016a, b, 2018), and were used to conduct five sets of mating experiments in this study (Table 1). Mate choice experiments were carried out between 1200 and 1830 h because the sexual activity of *Altica* beetles is higher in the afternoon, although mating can also occur during the morning. These experiments were carried out in a temperature-controlled room held at 25–27 °C under natural light conditions. To prepare the models for the mating assay, they were coated with a CHC extract obtained from freshly killed beetles. About ten beetles were pooled and submerged in hexane (40 µL/each individual) for 15 min. A 40-µL aliquot (one beetle equivalent) of this hexane extract was transferred onto the surface of each model (3D-printed model, glass bead or dead beetle with the CHCs removed) by submerging them in the extract and allowing the solvent to evaporate in a chemical fume hood. Models were then glued to a small piece of triangular filter paper (length = 1 cm), and then glued to the wall of a glass Petri dish (9.0 × 1.2 cm) containing moistened filter paper (Xue et al. 2016a, b). The two mate options were placed approximately 1 cm apart. To prevent position bias during the mating assays, the order of the two mate options was alternated, with option A being on the right in the first assay and option B being on the right in the second assay, and so on. The side of dish with the mate options was oriented toward a window light source such that the mate options were equidistant from the light. At the beginning of the trial, the test male was placed in the center of the arena containing two choices placed 1 cm apart on the wall of the Petri dish. A successful mating was recorded when the beetle mounted the mate, waved his antennae quickly and exhibited aedeagus-protruding behavior for longer than 10 s. For each of the mating bioassays, due to beetle availability, 61–107 replicates were conducted. To increase the percentage of successful mating trials, mate choice was recorded over a

period of 5 h instead of 3 h as in previous studies of this species (Xue et al. 2016a, b, 2018). In between each mating assay, the petri dishes were washed with hexane and left to air dry, and the models and dead beetles were used in only a single trial.

In experiment I, we tested whether males were able to discriminate between the sexes based solely on CHC cues. A male was given a choice between a black 3DPB coated with female CHCs and a black 3DPB coated with male CHCs. Experiments II and III tested the role of visual cues in male mate choice decisions. We examined if the shape of the model altered mating behavior by comparing mate choice of males when given a black 3DPB coated with female CHCs and a round, black glass bead coated with female CHCs (Experiment II). We also compared whether the color of the model affected mating behavior by comparing mate choice of males presented with a black versus white 3DPB coated with female CHCs (Experiment III). In Experiments IV and V, we further tested the ability of males to discriminate between the 3DPB models from real beetles. Experiment IV compared mate choice of males presented with a black 3DPB coated with female CHCs and a dead female with the CHCs removed and then reapplied. Experiment V tested whether males could discriminate between a dead female with legs and antennae removed and a black 3DPB coated with CHCs from females. For all the experiments, we analyzed the mating preference data using Chi-square (χ^2) tests in SPSS 18.0 (IBM, Armonk, NY, USA).

Results

We first tested whether the models had any chemicals on the surface that might alter the outcome of mating trials. As compared to blank controls and *A. fragariae* samples, we were unable to detect any compounds associated with the study species on the 3D-printed or glass bead models under the given conditions (Fig. 2), indicating that these models are suitable for mating bioassays.

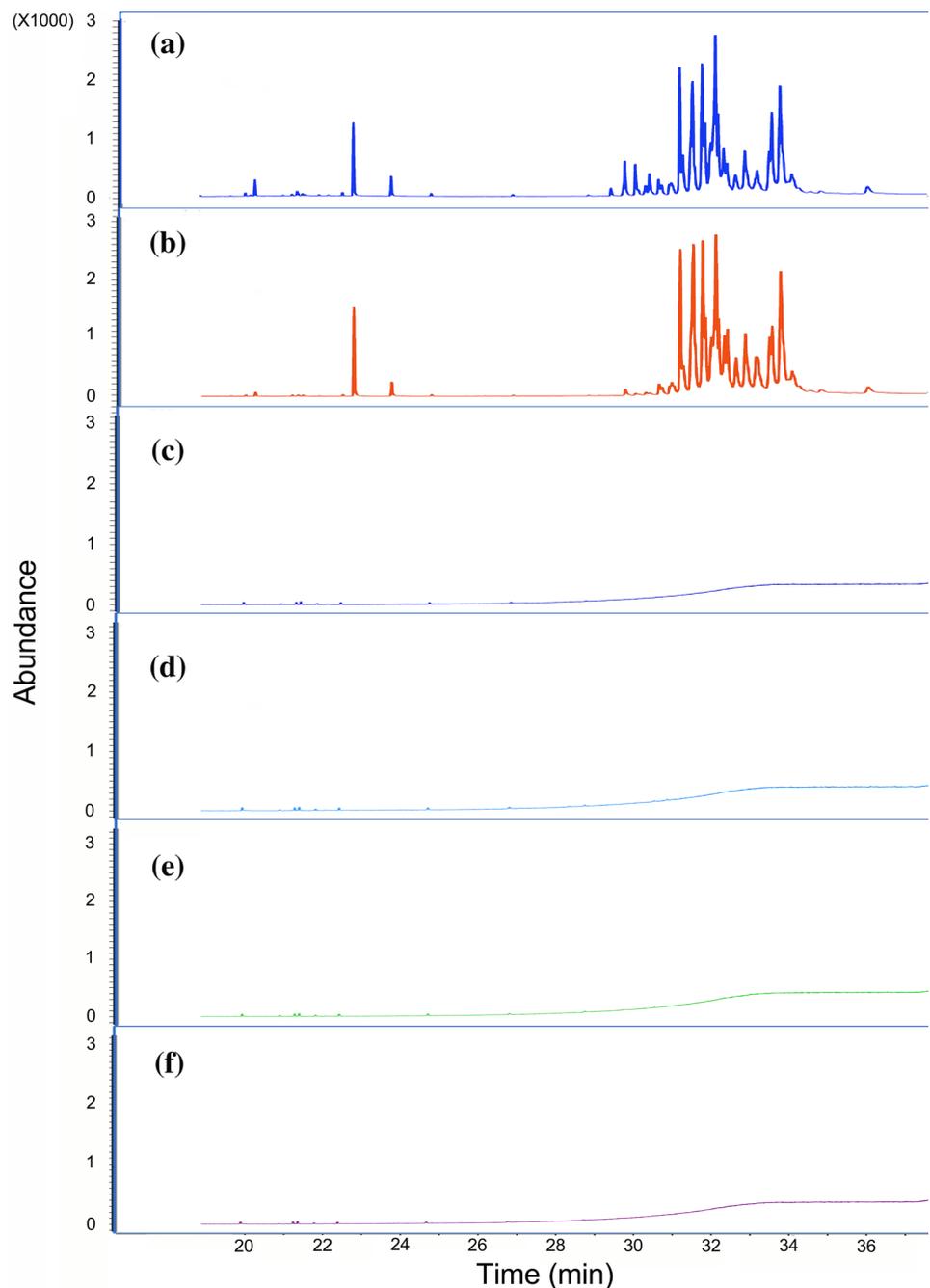
Table 1 Two-choice mating bioassays

Experiment	Question	Mating test design
I	Can males discriminate the sex of beetles solely by CHCs?	Black-3DPB + male CHCs vs. Black-3DPB + female CHCs
II	Does model shape contribute to mate choice?	Black-glass-bead + female CHCs vs. Black-3DPB + female CHCs
III	Does model color contribute to mate choice?	White-3DPB + female CHCs vs. Black-3DPB + female CHCs
IV	Can males discriminate 3DPB from real beetles?	#Dead female + female CHCs vs. Black-3DPB + female CHCs
V	Can males discriminate 3DPB from real beetles with antennae and legs removed?	#Dead female with antennae and legs removed + female CHCs vs. Black-3DPB + female CHCs

3DPB 3D-printed beetle

#Indicates tests in which the CHCs of dead females were removed and then female CHCs were reapplied

Fig. 2 Representative gas chromatogram profiles of cuticular hydrocarbons of *Altica fragariae*, hexane control, glass bead, and 3D-printed beetles. **a** Male *A. fragariae*; **b** female *A. fragariae*; **c** hexane control; **d** black glass bead; **e** black 3D-printed model; **f** white 3D-printed model



Behavioral assays testing the response of males to 3D models coated with male versus female CHCs showed that male *A. fragariae* significantly preferred female CHCs over that of males ($X^2 = 10.667$, $P = 0.001$; Fig. 3a). In terms of tests of the role of visual cues, males presented with black glass beads and black 3DPBs preferred the 3D-printed model over the round beads ($X^2 = 5.143$, $P = 0.023$; Fig. 3b). Similarly, males tested on black versus white 3DPBs all chose the black models (no statistics; Fig. 3c).

However, when males were offered dead females with the reapplied CHCs, males preferred dead females over black 3DPBs coated with female CHCs ($X^2 = 19.882$, $P < 0.001$; Fig. 3d). This pattern held even when we removed the legs and antennae of the dead females to make them more similar to the 3D-printed model ($X^2 = 7.348$, $P = 0.007$; Fig. 3e), although we did observe a significant reduction in the percent of males choosing to mate as compared to the observations in Experiment IV ($X^2 = 5.592$, $P = 0.018$).

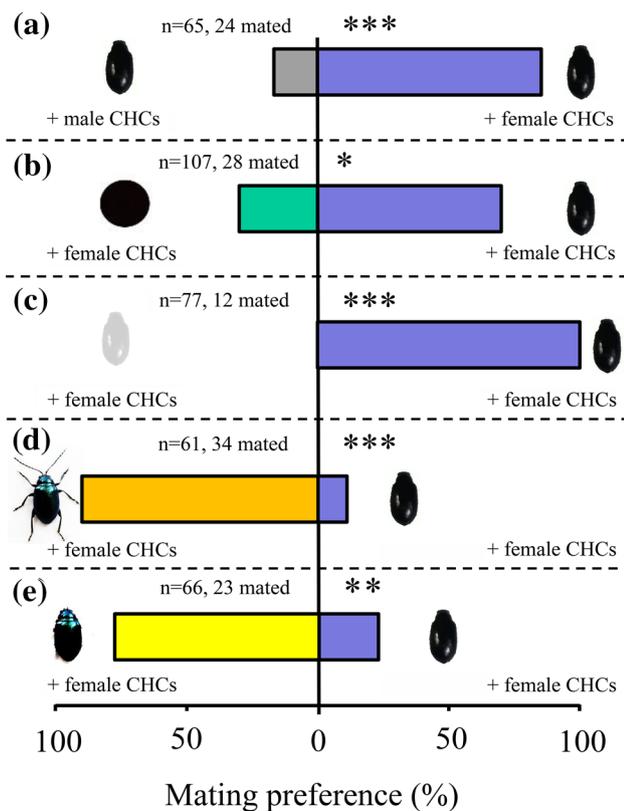


Fig. 3 Mating preference of male *Altica fragariae* comparing black 3D-printed beetles coated with female CHCs (right hand column) to alternative models (left hand column). **a** Black 3D-printed beetle coated with male CHCs versus black 3D-printed beetle coated with female CHCs; **b** black glass bead coated with female CHCs versus black 3D-printed beetle coated with female CHCs; **c** white 3D-printed beetle coated with female CHCs versus black 3D-printed beetle coated with female CHCs; **d** dead female with CHCs removed and reapplied versus black 3D-printed beetle coated with female CHCs; **e** dead female with antennae and legs removed and CHCs removed and reapplied versus black 3D-printed beetle coated with female CHCs. Asterisks indicate significant differences in mating preference (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). “Mated” refers to the number of males that mounted the mate, waved his antennae quickly and exhibited aedeagus-protruding behavior for longer than 10 s

Discussion

There is little doubt that CHCs play a key role in interspecific recognition among *Altica* species (Xue et al. 2016a, b, 2018); however, in bioassays focused on intraspecific mate discrimination, the patterns of male mate choice may be unreliable because of the use of dead females with manipulated CHCs. This may be particularly true in assays offering mating partners with exchanged CHCs. For example, against the predictions, males of *A. cirsiicola* showed no preference for female CHCs over male CHCs, whereas males of *A. fragariae* and *A. viridicyanea* preferred females when the CHCs of males and females were exchanged (Xue

et al. 2016b). These results suggest that additional cues are involved in intraspecific mate discrimination in at least *A. cirsiicola*. Interestingly, the results from Experiment I of the present study were consistent with a previous study that used living or freshly killed *A. fragariae* as potential mating partners (Xue et al. 2016b): males preferred females (Xue et al. 2016b) or 3DPB coated with CHCs of females (present study). This consistency between studies suggests that the approaches used in the present approach could be used to supplement mating experiments that manipulate CHCs. In addition to behavioral cues such as wing fanning and posturing, additional cues could be used by insects in mate choice such as olfactory, visual, acoustical and tactile cues (Brown 1999; Demary et al. 2000; Greenspan and Ferveur 2000; Benelli et al. 2014; Oike et al. 2017; Jones and Conner 2017; Johnson et al. 2019), Experiment I of the present study indicates that *A. fragariae* can discriminate males from females based solely on differences in CHCs because all the cues were identical with the exception of the CHCs.

Chemical cues are important for mate choice decisions in *Altica*; however, the results indicate that males are also using visual cues. Visual cues actually had a more important role than olfactory cues in host finding in the monophagous *Altica engstroemi* (Sahlberg) (Stenberg and Ericson 2008), but the function of visual cues in mating behavior has not been previously assessed in *Altica*. The results of Experiment II showed that the shape of the model strongly impacted mate choice, and Experiment III demonstrated that the color of the model was critical. If visual cues are being used as long-distance attractors, the shape and color of the model may affect a male’s initial orientation to potential mating partners. Upon direct contact, suitable mates are likely further assessed using the nonvolatile CHCs. Similar orientation responses have been shown in males of the white-spotted longicorn beetle, *Anoplophora malasiaca*, and *Cicindela* tiger beetles where the color of the model affected male responses (Fukaya et al. 2004; Jones and Conner 2017). Here, our results not only indicate that visual cues are playing a role in mate preference but they also show that models that more closely mimic female beetles can motivate mating behavior.

Although the 3DPBs triggered mating behaviors at a reasonable level, the results showed that these models still lacked details that would have made them commensurate with real females. For instance, the reduction in the percentage of males choosing mates in experiments involving only models versus those including dead females may indicate that tactile cues such as hardness or texture affect mate choice. Experiments IV and V showed that males overwhelmingly preferred dead females over the 3D models, further suggesting that tactile cues and/or other visual cues are involved in mate decisions. For example, we were unable to print antennae and legs on the 3D models

because these appendages were too thin in this small beetle. In Experiment V, we removed the legs and antennae of the dead females to test whether the removal of the visual cues impacted mate choice. Although males still preferred dead females even after the legs and antennae were removed, the percent of males choosing mates was significantly reduced as compared to that of Experiment IV (Fig. 3), suggesting that those visual cues were being used by the males. In *Altica*, then, a combination of cues is being used for mate discrimination, a result consistent with other studies showing that mating behaviors are the result of a synergism between visual, olfactory and tactile senses (Benelli et al. 2014).

Despite the fact that the results showed that males preferred to mate with dead females over our 3D models and that there was a lower percentage of males choosing mates in trials involving only 3D models, the pattern of selectivity in Experiment I supports the idea that 3D-printed models could be a feasible and cost-effective method to expand the array of options available to researchers conducting mating studies in chrysomelid beetles and potentially other insect groups. Further, the results observed here were consistent with previously published work in this system, suggesting that despite the caveats of using 3D models, this approach can yield reliable data. Developing even more realistic models in terms of shape, color, texture and other physical characteristics will allow future research to tease apart the role of multiple cues involved in insect mate discrimination.

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Author contributions HJX and YW conceived and designed the study; LZ, XYZ and SQG constructed the 3D model; YW and HJX conducted the GC–MS analysis and bioassays; HJX, KAS, YW and LZ drafted the paper. All the authors gave final approval for publication.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding authors on request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was carried out in full compliance of the laws of the country (China), and no specific permits were required for the experiments described above. We collected beetles in the field by hand and kept them in growth chamber till experiments. Some experimental beetles were euthanized by placing them in a –30 °C freezer for 20 min. At the end of the study, left experimental beetles were euthanized by freezing overnight at –30 °C.

References

- Benelli G, Bonsignori G, Stefanini C, Raspi A (2014) First quantification of courtship behavior in a silver fly, *Leucopis palumbii* (Diptera: Chamaemyiidae): role of visual, olfactory and tactile cues. *J Insect Behav* 27:462–477. <https://doi.org/10.1007/s10905-014-9443-7>
- Blomquist GJ, Bagnères A-G (2010) *Insect hydrocarbons: biology, biochemistry, and chemical ecology*. Cambridge University Press, New York
- Brown WD (1999) Mate choice in tree crickets and their kin. *Ann Rev Entomol* 44:371–396. <https://doi.org/10.1146/annurev.ento.44.1.371>
- Buellesbach J, Gadau J, Beukeboom LW, Echinger F, Raychoudhury R, Werren JH, Schmitt T (2013) Cuticular hydrocarbon divergence in the jewel wasp *Nasonia*: evolutionary shifts in chemical communication channels? *J Evol Biol* 26:2467–2478. <https://doi.org/10.1111/jeb.12242>
- Demary K, Michaelidis CI, Lewis SM (2010) Firefly courtship: behavioral and morphological predictors of male mating success in *Photinus greeni*. *Ethology* 112:485–492. <https://doi.org/10.1111/j.1439-0310.2005.01176.x>
- Fujiwara-Tsujii N, Yasui H, Wakamura S (2013) Population differences in male responses to chemical mating cues in the white-spotted longicorn beetle, *Anoplophora malasiaca*. *Chemoecology* 23:113–120. <https://doi.org/10.1007/s00049-013-0126-1>
- Fukaya M, Yasuda T, Wakamura S, Honda H (1996) Reproductive biology of the yellow-spotted longicorn beetle, *Psacotha hilaris* (Pascoe) (Coleoptera: Cerambycidae). III. Identification of the contact sex pheromone on female body. *J Chem Ecol* 22:259–270. <https://doi.org/10.1007/BF02055097>
- Fukaya M, Akino T, Yasuda T, Yasui H, Wakamura S (2004) Visual and olfactory cues for mate orientation behaviour in male white-spotted longicorn beetle, *Anoplophora malasiaca*. *Entomol Exp Appl* 111:111–115. <https://doi.org/10.1111/j.0013-8703.2004.00147.x>
- Geiselhardt S, Otte T, Hilker M (2012) Looking for a similar partner: host plants shape mating preferences of herbivorous insects by altering their contact pheromones. *Ecol Lett* 15:971–977. <https://doi.org/10.1111/j.1461-0248.2012.01816.x>
- Gonzalez PV, Audino PAG, Masuh HM (2014) Electrophysiological and behavioural response of *Aedes albopictus* to n-heptacosane, an ovipositional pheromone of *Aedes aegypti*. *Entomol Exp Appl* 151:191–197. <https://doi.org/10.1111/eea.12184>
- Greenspan RJ, Ferveur JF (2000) Courtship in *Drosophila*. *Ann Rev Genet* 34:205–232. <https://doi.org/10.1146/annurev.genet.34.1.205>
- Isa M, Kumano N, Tatsuta H (2019) When a male perceives a female: the effect of waxy components on the body surface on decision-making in the invasive pest weevil. *R Soc Open Sci* 6:181542. <https://doi.org/10.1098/rsos.181542>
- Johansson BG, Jones TM (2007) The role of chemical communication in mate choice. *Biol Rev* 82:265–289. <https://doi.org/10.1111/j.1469-185x.2007.00009.x>
- Johnson TD, Hanson E, Yu A (2019) Adults of the cerambycid beetle *Megacyllene caryae* use both olfactory and visual information to locate mates. *Entomol Exp Appl* 167:500–506. <https://doi.org/10.1111/eea.12781>
- Jones TK, Conner WE (2017) Pre-mating reproductive isolation in tiger beetles (Carabidae: Cicindelinae): an examination of the role of visual and morphological feedback. *J Insect Behav* 31:672–688. <https://doi.org/10.1007/s10905-018-9704-y>
- Lacey ES, Ginzler MD, Millar JG, Hanks LM (2008) 7-Methylheptacosane is a major component of the contact sex pheromone of the cerambycid beetle *Neoclytus acuminatus acuminatus*. *Physiol*

- Entomol 33:209–216. <https://doi.org/10.1111/j.1365-3032.2008.00624.x>
- Oike M, Kanayama S, Wakamura S (2017) Mate-searching behavior of the black chafer *Holotrichia kiotonensis* (Coleoptera: Scarabaeidae): identification of a sex pheromone, and male orientation behavior controlled by olfactory and visual cues. *Appl Entomol Zool* 52:519–526. <https://doi.org/10.1007/s13355-017-0504-1>
- Pask GM, Slone JD, Millar JG, Das P, Moreira JA, Zhou XF, Bello J, Berger SL, Bonasio R, Desplan C, Reinberg D, Liebig J, Zwiebel LJ, Ray A (2017) Specialized odorant receptors in social insects that detect cuticular hydrocarbon cues and candidate pheromones. *Nat Commun* 8:297. <https://doi.org/10.1038/s41467-017-00099-1>
- Peterson MA, Dobler S, Larson EL, Juárez D, Schlarbaum T, Monsen KJ, Francke W (2007) Profiles of cuticular hydrocarbons mediate male mate choice and sexual isolation between hybridising *Chrysochus* (Coleoptera: Chrysomelidae). *Chemoecology* 17:87–96. <https://doi.org/10.1007/s00049-007-0366-z>
- Rafter MA, Walter GH (2013) Mate recognition in the South African citrus thrips *Scirtothrips aurantii* (Faure) and cross-mating tests with populations from Australia and South Africa. *J Insect Behav* 26:780–795. <https://doi.org/10.1007/s10905-013-9391-7>
- Schiestl FP, Ayasse M (2000) Post-mating odor in females of the solitary bee, *Andrena nigroaenea* (Apoidea, Andrenidae), inhibits male mating behavior. *Behav Ecol Sociobiol* 48:303–307. <https://doi.org/10.1007/s002650000241>
- Schlechter-Helas J, Schmitt T, Peschke K (2012) Learning individual signatures: rove beetle males discriminate unreceptive females by cuticular hydrocarbon patterns. *Anim Behav* 84:369–376. <https://doi.org/10.1016/j.anbehav.2012.05.003>
- Shimomura K, Matsui S, Ohsawa K, Yajima S (2016) Saltational evolution of contact sex pheromone compounds of *Callosobruchus rhodesianus* (Pic). *Chemoecology* 26:15–23. <https://doi.org/10.1007/s00049-015-0204-7>
- Singer TL (1998) Roles of hydrocarbons in the recognition systems of insects. *Am Zool* 38:394–405. <https://doi.org/10.2307/4620153>
- Smadja C, Butlin RK (2009) On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity* 102:77–97. <https://doi.org/10.1038/hdy.2008.55>
- Steiger S, Whitlow S, Peschke K, Müller JK (2009) Surface chemicals inform about sex and breeding status in the biparental burying beetle *Nicrophorus vespilloides*. *Ethology* 115:178–185. <https://doi.org/10.1111/j.1439-0310.2008.01600.x>
- Stenberg JA, Ericson L (2008) Visual cues override olfactory cues in the host-finding process of the monophagous leaf beetle *Altica engstroemi*. *Entomol Exp Appl* 125:81–88. <https://doi.org/10.1111/j.1570-7458.2007.00597.x>
- Sugeno W, Hori M, Matsuda K (2006) Identification of the contact sex pheromone of *Gastrophysa atrocyanea* (Coleoptera: Chrysomelidae). *Appl Entomol Zool* 41:269–276. <https://doi.org/10.1303/aez.2006.269>
- Sun XL, Zhang XZ, Wu GY, Li XW, Liu FJ, Xin ZJ, Zhang J (2017) *n*-Pentacosane acts as both contact and volatile pheromone in the tea weevil, *Mylokerinus aurolineatus*. *J Chem Ecol* 43:557–562. <https://doi.org/10.1007/s10886-017-0857-5>
- Tanigaki T, Yamaoka R, Sota T (2007) The role of cuticular hydrocarbons in mating and conspecific recognition in the closely related longicorn beetles *Pidonia grallatrix* and *P. takechii*. *Zool Sci* 24:39–45. <https://doi.org/10.2108/zsj.24.39>
- Xue HJ, Egas M, Yang XK (2007) Development of a positive preference-performance relationship in an oligophagous beetle: adaptive learning. *Entomol Exp Appl* 125:119–124. <https://doi.org/10.1111/j.1570-7458.2007.00605.x>
- Xue HJ, Li WZ, Nie RE, Yang XK (2011) Recent speciation in three closely related sympatric specialists: inferences using multi-locus sequence, post-mating isolation and endosymbiont data. *PLoS ONE* 6:e27834. <https://doi.org/10.1371/journal.pone.0027834>
- Xue HJ, Li WZ, Yang XK (2014) Assortative mating between two sympatric closely-related specialists: inferred from molecular phylogenetic analysis and behavioral data. *Sci Rep* 4:5436. <https://doi.org/10.1038/srep05436>
- Xue HJ, Wei JN, Magalhães S, Zhang B, Song KQ, Liu J, Li WZ, Yang XK (2016a) Contact pheromones of 2 sympatric beetles are modified by the host plant and affect mate choice. *Behav Ecol* 27:895–902. <https://doi.org/10.1093/beheco/arv238>
- Xue HJ, Zhang B, Segraves KA, Wei JN, Nie RE, Song KQ, Liu J, Li WZ, Yang XK (2016b) Contact cuticular hydrocarbons act as a mating cue to discriminate intraspecific variation in *Altica* flea beetles. *Anim Behav* 111:217–224. <https://doi.org/10.1016/j.anbehav.2015.10.025>
- Xue HJ, Segraves KA, Wei J, Zhang B, Nie RE, Li WZ, Yang XK (2018) Chemically mediated sexual signals restrict hybrid speciation in a flea beetle. *Behav Ecol* 29:1462–1471. <https://doi.org/10.1093/beheco/ary105>
- Zhang AJ, Oliver JE, Chauhan K, Zhao BG, Xia LQ, Xu ZC (2003) Evidence for contact sex recognition pheromone of the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae). *Naturwissenschaften* 90:410–413. <https://doi.org/10.1007/s00114-003-0452-1>
- Zhang B, Xue HJ, Song KQ, Liu J, Li WZ, Nie RE, Yang XK (2014) Male mate recognition via cuticular hydrocarbons facilitates sexual isolation between sympatric leaf beetle sister species. *J Insect Physiol* 70:15–21. <https://doi.org/10.1016/j.jinsphys.2014.08.006>