Animal Behaviour 111 (2016) 217–224

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Contact cuticular hydrocarbons act as a mating cue to discriminate intraspecific variation in *Altica* flea beetles

Huai-Jun Xue [a], Bin Zhang [a, b], Kari A. Segraves [c], Jia-Ning Wei [d], Rui-E. Nie [a], Ke-Qing Song [a, b], Jie Liu [a], Wen-Zhu Li [a], Xing-Ke Yang [a, *]

[a] Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
[b] University of Chinese Academy of Sciences, Beijing, China
[c] Department of Biology, Syracuse University, Syracuse, NY, U.S.A.
[d] State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

ARTICLE INFO

Article history:
Received 6 May 2015
Initial acceptance 5 June 2015
Final acceptance 6 October 2015
Available online
MS. number: 15-00375R

Keywords:
Chrysomelidae
cuticular hydrocarbon
intraspecific variation
mate choice
mating test
sex
sexual selection

Contact cuticular hydrocarbons (CHCs) are one of the major cues that allow many insects to identify interspecific and intraspecific variation between individuals, and often have mutually nonexclusive functions that can provide multiple types of signals. A previous study showed that two sympatric, closely related *Altica* beetles achieve behavioural isolation via species-specific CHC profiles. Here, we explored whether these CHCs also play a role in recognition of intraspecific variation. Specifically, we tested the hypothesis that differences in CHCs are a critical mating cue that allows males to discriminate the sex and age (sexual maturity) of females. We used CHC profile analysis and behavioural assays to examine mating cues in three closely related flea beetles, *Altica cirsicola*, *Altica fragariae* and *Altica viridicyanea* (Insecta: Coleoptera: Chrysomelidae). The results showed that (1) CHC profiles are sex and age specific, (2) male beetles can distinguish males from females and can also distinguish sexually mature females from immature ones and (3) CHCs are only one component of mate discrimination as additional cues also appear to be involved.

Although mate choice occurs in both sexes, much emphasis has been placed on female mate choice (Andersson & Simmons, 2006; Ben-Ari, 2000; Carazo, Sanchez, Font, & Desfilis, 2004). In contrast, male mate choice has been relatively neglected (Bonduriansky, 2001), primarily because the lower costs of reproduction for males drives them to be less discriminating than females (Andersson, 1994). Yet, when the benefits outweigh the costs of being choosy, male mate choice is also expected to evolve (Andersson, 1994; Bonduriansky, 2001; Pitnick, Spicer, & Markow, 1995). The costs that males pay while involved in sexual behaviour are diverse, and can be divided into direct energetic costs and trade-off costs (Scharf & Martin, 2013; Scharf, Peter, & Martin, 2013). In some instances, the production of costly sperm (e.g. Pitnick et al., 1995) or variation in female quality (e.g. Tuni & Berger-Tal, 2012) can drive the evolution of choosy males. Consequently, males should have traits that allow them to accurately assess mate quality such that they can invest appropriately.

A critical feature of assessing mate quality, and one of the major tasks of males, is male-female identification. Recognizing an opposite-sex conspecific is a prerequisite for an individual in any sexually reproducing species (Schlechter-Helas, Schmitt, & Peschke, 2012), yet male-male sexual behaviour in insects is prevalent (Bagemihl, 1999; Bailey & French, 2012; Bailey & Zuk, 2009; Burgevin, Friberg, & Maklakov, 2013; Dukas, 2010; Scharf & Martin, 2013). Although there are numerous explanations for same-sex sexual behaviour in insects (Bailey & Zuk, 2009; Scharf & Martin, 2013), this behaviour is typically considered an evolutionary dead end, and in most cases can be explained as mistaken identification by the mounting male (Scharf & Martin, 2013). In addition to sex recognition, males of many species might also benefit from assessing female quality in terms of sexual maturity and whether the female is virgin or mated (e.g. Aranaud & Haubruge, 1999). For example, in polygynandrous species, immature females tend to have underdeveloped oocytes (Carazo et al., 2004); thus, sexual maturity of females can influence male

* Correspondence: X.K. Yang, Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, No. 1 Beichen West Road, Chaoyang District, Beijing 100101, China.
E-mail address: yangxk@ioz.ac.cn (X. -K. Yang).

http://dx.doi.org/10.1016/j.anbehav.2015.10.025
0003-3472/

mating investment (Tuni & Berger-Tal, 2012), and there is now increasing evidence that male fitness can depend on such discrimination ability (Thomas, 2011).

Given that male fitness can be tied to their ability to choose sexually mature females, males should be able to detect honest signals that indicate a female's quality or receptivity. Surprisingly, the cues that males use to assess potential mates are poorly known (Tuni & Berger-Tal, 2012). Although there are a variety of cues used to assess potential mates (Bonduriansky, 2001), such as olfactory, visual, acoustical, tactile and behavioural cues (Geiselhardt, Otte, & Hilker, 2009; Greenspan & Ferveur, 2000; Pureswaran & Poland, 2009; Swierk, Myers, & Langkilde, 2013; Tibbetts, 2002), chemical signals are generally regarded as the most ancient and widespread form of communication (Johansson & Jones, 2007). Contact cuticular hydrocarbons (CHCs) are one of the major chemical cues involved in insect species recognition (Peterson et al., 2007; Zhang et al., 2014), sex discrimination (Kather & Martin, 2012; Porco, Deharveng, & Gers, 2004; Singer, 1998), reproductive status determination (Dietemann, Peeters, Liebig, Thivet, & Hölldobler, 2003; Scott, Madjid, & Orians, 2008; Steiger, Whitlow, Peschke, & Müller, 2009; Tuni & Berger-Tal, 2012) and even colony or nestmate identification (Howard & Blomquist, 2005; Krasnec & Breed, 2013; Ozaki et al., 2005; Ruther, Sieben, & Schricker, 2002; Wagner, Tissot, Cuevas, & Gordon, 2000). These hydrocarbons are extremely complex usually involving components of chain lengths between 21 and 50 carbons (Blomquist & Bagnères, 2010).

The flea beetles *Altica cirsicola*, *Altica fragariae* and *Altica viridicyanea* (Insecta: Coleoptera: Chrysomelidae) are sympatric, closely related species that use distinct host plants (Xue, Li, Nie, & Yang, 2011). As in many other beetles, both sexes of *Altica* species mate several times with multiple partners during their lifetime (Xue, Li, & Yang , 2014), and the sexually active period may last more than 3 months (Xue, Wang, Li, Zhang, & Yang, 2007). Continuous oviposition leads to overlapping generations within populations (Xue, Egas, & Yang, 2007; Xue, Wang, et al., 2007; Xue & Yang, 2007); thus, males simultaneously encounter both mature and immature females. Because *Altica* beetles often cluster together in the field, males frequently encounter both females and males as well as other *Altica* beetle species. As a result, we might expect male *Altica* to use specific cues to find and recognize suitable mates. Indeed, previous work has shown that strong behavioural isolation between two closely related flea beetle species, *A. fragariae* and *A. viridicyanea*, has evolved (Xue et al., 2014). Further analysis revealed that the CHC profiles are species-specific and that the CHCs were used by males as contact sex pheromones to distinguish conspecific from heterospecific females (Xue et al., 2015). Given the importance of CHCs in species recognition, they may also play a role in mate quality assessment. Hence, our main aim was to test the extent to which *Altica* beetles use CHCs to recognize intraspecific variation in sex and sexual maturity status.

As chemical cues often have mutually nonexclusive functions and can provide multiple signals (Dietemann et al., 2003; Johansson & Jones, 2007; Steiger et al., 2009), we speculated that CHCs may also act as a critical mating cue to recognize intraspecific variation in partner quality in *Altica* species. We thus combined chemical analysis of CHC profiles with a suite of behavioural experiments to provide a rigorous test of this hypothesis in *A. cirsicola*, *A. fragariae* and *A. viridicyanea*. First, we determined whether there was significant variation in CHC profiles between mature and immature males and females for each species. Second, we tested whether males were able to discriminate sex (male versus female) and female sexual maturity (immature versus mature) using choice assays. Finally, we investigated whether male choice was mediated via CHC cues.

METHODS

Study System

To create laboratory colonies of the three species, adult *A. cirsicola* were collected in Olympic Park (40.01°N, 116.38°E), Chaoyang, Beijing, and adults of *A. fragariae* and *A. viridicyanea* were collected in Nankou (40.28°N, 116.04°E), Changping, Beijing. Approximately 25 adults were collected for each species. The three species were maintained separately in growth chambers held at 16:8 h light:dark and 25 °C and fed their normal host plants (*A. cirsicola*: *Cirsium setosum* (Willd.) MB.; *A. fragariae*: *Duchesnea indica* (Andrews) Focke; *A. viridicyanea*: *Geranium nepalens* (Sweet)). They were allowed to mate and oviposit, creating a second generation fully reared in the laboratory. Because we needed to know the precise age and mating history of female beetles, we collected beetles of this second generation for use in the present study (ca. 1600 beetles per species were reared and ca. 1200 beetles per species were used in subsequent experiments). Under our laboratory conditions, newly emerged females of these species became sexually active and began to oviposit after 5–7 days (Xue, Egas, et al., 2007; Xue et al., 2015); hence, we defined sexually mature females as older than 10 days and immature females as less than 3 days old.

Chemical Analysis of Cuticular Hydrocarbon Extracts

Approximately 30 replicate cuticular extracts from four groups (immature and mature beetles of both sexes) were obtained per *Altica* species. Each beetle was dipped in 40 μl hexane for 30 min to obtain the cuticular extracts for gas chromatography/mass spectrometry (GC–MS) analysis. Prepared extract samples were transferred into a vial insert (Agilent Technologies Inc., Santa Clara, CA, U.S.A.; 250 μl glass with polymer feet), and then placed in chromatography vials (Agilent Technologies Inc., screw cap vials, 1.5 ml) for GC–MS analysis (HP 7890 series GC – HP 5975 MSD; GC–MS) with the MS Library NIST2005 (Agilent Technologies, Inc.). An HP5 column (30 m × 0.32 mm internal diameter × 0.25 μm film thickness, Agilent Technologies, Inc.) was used, with helium at 1.0 ml/min carrier gas. A 2 μl volume of sample was injected and the injector set to 280 °C. The oven was programmed as follows: 40 °C for 1 min, 8 °C/min from 40 to 300 °C, then 20 °C/min to 320 °C. The MS was in the electron impact mode (70 eV). Two microlitres of each extract was injected in the splitless mode. The *n*-alkane (C6–C40) standard was also injected to calculate retention indices (RI). Individual compounds were identified by integrative analysis of their mass spectra (Doolittle, Proveaux, Alborn, & Heath, 1995; Nelson, Sukkestad, & Zaylskie, 1972; Pomonis, Nelson, & Fatland, 1980) and RIs (Carlson, Bernier, & Sutton, 1998). The flame ionization detector (FID) exhibits greater precision than MS in chemical quantification (Dodds, McCoya, Reac, & Kennisha, 2005), so the relative quantification of CHCs was performed by GC-FID under the same conditions as described above. In parallel with the CHC extracts, a set of reference compounds (e.g. 2-methyl-octacosane, 7-methyl-nonacosane) were also run using GC–MS to confirm the identification of the compounds observed in the beetles.

The peaks with a mean relative proportion of more than 0.5% in at least in one group (mature female and male, immature female and male) within a given species were used for further analysis. Quantitative differences between the CHC profiles of the four groups from one species were statistically analysed using a MANOVA with sex and maturation status as main effects. Prior to multivariate statistics, the CHC data were centred log-ratio transformed as follows: $z_{ip} = \ln[A_{ip}/g(A_p)]$, where A_{ip} is the area of peak i for beetle p, $g(A_p)$ is the geometric mean of all peaks for beetle p

and z_{ip} is the transformed area of peak i for beetle p (Aitchison, 1986; Geiselhardt, Otte, & Hilker, 2012). As the logarithm is not defined for zero values, the constant 0.01 was added to each relative peak area to uniformly apply the transformation to samples that did not contain all compounds (Geiselhardt et al., 2009). A canonical discriminant analysis was performed to determine whether sex and maturation status affected CHC profiles. Canonical discriminant analysis with all of the 12 groups was also performed to determine whether the variation in CHC profile between species was greater than that observed within species. The quality of the resulting classification was tested using the 'leaving one-out cross-validation' procedure (Efron, 1983). All analyses were implemented in SPSS 18.0 (IBM, Armonk, NY, U.S.A.).

Both contact CHCs and (semi-) volatiles were identified from the cuticular surface of *Altica* beetles (Table S1). Because the (semi-) volatiles may also play a role in male mate choice, we also tested whether there was a significant difference in volatile profiles between mature and immature males and females for each species. We used MANOVA and canonical discriminant analysis to assess these differences.

Mating Experiments

Upon eclosion, beetles were isolated by sex to obtain virgin males and females for mating assays. Sex was determined by examining the shape of the terminal abdominal segment which is smooth in females and has a transverse groove in males (Wang, Cui, Li, & Zhang, 2005). A series of mate choice experiments were carried out in a temperature-controlled room held at 25–27 °C under natural light conditions. We constructed mating arenas using petri dishes (9.0 × 1.2 cm) lined with moistened filter paper (Fig. 1). In all experiments, a mating was considered successful when the male inserted his intromittent organ for longer than 5 min (cf. Xue et al., 2014). For each of the following bioassays, 40–108 replicates were conducted (see Tables 1–6 in the Results), and each individual was used only once. Mate choice and the number of copulating pairs were recorded over a period of three 3 h (cf. Xue et al., 2014). For these experiments, we conducted parallel mating assays for each of the three *Altica* species. In each assay, a focal male was presented with two mate options. Six different sets of mating experiments were conducted for each beetle species.

(1) To test whether males were able to discriminate between the sexes, a focal male was given a choice between a male and a female that were alive and free to move about the mating arena. In this assay, the focal male was the first male to make a mate choice decision.

(2) To test whether a behavioural mating signal was necessary for mate recognition in experiment 1, we presented a focal male

Table 1
Results of experiment 1 in which males of *Altica* flea beetles were offered live conspecific males and females in choice mating trials

	No. of males that mated	No. choosing females	No. choosing males
A. cirsicola ♂(*N*=50)	22	22	0
A. fragariae ♂ (*N*=80)	57	42	15
A. viridicyanea ♂ (*N*=40)	24	24	0

Table 2
Results of experiment 2 in which males of *Altica* flea beetles were presented with dead conspecific males and females

	No. of males that mated	No. choosing females	No. choosing males
A. cirsicola ♂ (*N*=50)	23	23	0
A. fragariae ♂ (*N*=50)	25	22	3
A. viridicyanea ♂ (*N*=65)	24	23	1

Table 3
Results of experiment 3 in which males of *Altica* flea beetles were allowed to choose between conspecific males and females (dead) with exchanged cuticular hydrocarbons

	No. of males that mated	No. choosing females with male CHCs	No. choosing males with female CHCs
A. cirsicola ♂ (*N*=75)	28	12	16
A. fragariae ♂ (*N*=108)	57	40	17
A. viridicyanea ♂ (*N*=90)	31	22	9

Table 4
Results of experiment 4 in which males of *Altica* flea beetles were offered live sexually mature (>10 days) and immature (2–3 days) conspecific females

	No. of males that mated	No. choosing sexually mature females	No. choosing sexually immature females
A. cirsicola ♂ (*N*=64)	32	27	5
A. fragariae ♂ (*N*=53)	35	33	2
A. viridicyanea ♂ (*N*=68)	31	31	0

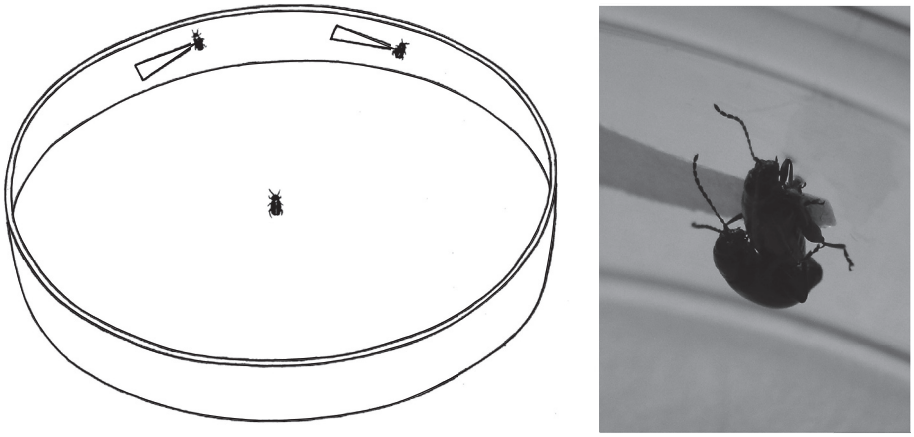

Figure 1. Mating arenas were constructed from a petri dish (9.0 × 1.2 cm) containing moistened filter paper. In mating experiments 2, 3, 5 and 6, two potential mates (dead) were glued on a small piece of triangular filter paper (length=1 cm), and then glued to the wall of the petri dish. A test male was released in the centre of the dish at the beginning of the trial.

Table 5
Results of experiment 5 in which males of *Altica* flea beetles were presented with dead sexually mature (>10 days) and immature (2–3 days) conspecific females

	No. of males that mated	No. choosing sexually mature females (dead)	No. choosing sexually immature females (dead)
A. cirsicola ♂ (*N*=65)	31	23	8
A. fragariae ♂ (*N*=80)	37	31	6
A. viridicyanea ♂ (*N*=61)	25	22	3

Table 6
Results of experiment 6 in which males of *Altica* flea beetles were offered a choice between dead sexually mature (>10 days) and immature (2–3 days) conspecific females with exchanged cuticular hydrocarbons

	No. of males that mated	No. choosing mature females with immature female CHCs	No. choosing immature females with mature female CHCs
A. cirsicola ♂ (*N*=78)	26	11	15
A. fragariae ♂ (*N*=80)	24	12	12
A. viridicyanea ♂ (*N*=80)	30	19	11

with dead specimens rather than living beetles. The focal male was given a choice between one dead male and female that were affixed to the side of the mating arena (Fig. 1).

(3) To test whether CHCs play a role in recognition of the sexes, we exchanged the CHCs between dead males and females and presented one of each to the focal male. As in experiment 2, the dead beetles were affixed to the side of the mating arena.

(4) One sexually mature and one immature female were presented to the focal male, to test whether males can recognize a female's sexual maturity status. As in experiment 1, all beetles were alive and free to move about the arena.

(5) We examined whether a behavioural signal by the female was necessary for mate recognition by using dead females affixed to the side of the mating arena. One sexually mature female and one immature female were presented to a male.

(6) We exchanged the CHCs of sexually mature and immature females (dead) to test the role of CHCs in male assessment of sexual maturity status. One sexually mature female and one immature female were affixed to the side of the mating arena and presented to a male.

For experiments 1 and 4, the test beetles' sex or sexual maturity status was tracked by marking the beetles with enamel paint of different colours on the elytra (Wood, Tilmon, Shantz, & Harris, 1999; Xue et al., 2014). The beetles were initially placed at even intervals around the perimeter of the mating arena and then were allowed to move about freely during the 3 h trial. For experiments 2 and 5, the proffered mates were first killed by freezing at −30 °C for 20 min, and then mounted on a small piece of triangular filter paper (length = 1 cm) that was then glued to the wall of the petri dish (9.0 × 1.2 cm) mating arena (Fig. 1). For experiments 3 and 6, 10 beetles per treatment were frozen and then dipped in 0.4 ml hexane for 30 min to obtain the CHC extracts. These specimens were subsequently rinsed four times in 4 ml of hexane for 30 min. We then recoated the beetles by placing all 10 washed beetles into the appropriate CHC crude extract. The crude extract was allowed to evaporate at room temperature before mounting the mates on small pieces of triangular filter paper as in experiments 2 and 5. To determine whether the washes removed most of the CHCs on our test beetles, we ran GC-FID analysis on randomly selected hexane-washed beetles (five female individuals of both *A. fragariae* and *A. viridicyanea*) and compared these profiles to that of the original beetle extract (i.e. each individual dipped in 40 µl of hexane). Furthermore, because the crude extracts contained both contact CHCs and (semi-) volatile compounds, we also tested whether the putative volatile compounds evaporated during the cuticular chemical exchange procedure. To accomplish this, we used GC-FID to compare the chemical profiles of the crude extract and the crude extract after allowing the volatile compounds to evaporate. Because some of the putative volatile compounds remained after evaporation (see Results), we examined the effect of the presence of these compounds on male mate choice behaviour using *A. viridicyanea* as a model. We first fractionated the polar (putative volatiles) and nonpolar (contact CHCs) cuticular chemicals with silica gel and then confirmed the separation using GC-FID. We then coated these fractions onto washed (using hexane and methyl alcohol) female beetles, mounted the coated beetles onto filter paper triangles and affixed them to petri dish mating arenas as before. Males of *A. viridicyanea* were presented with two females, one coated with the polar fraction and the other with the nonpolar fraction, to test whether males cue in on the volatiles versus contact CHCs. For all of the experiments described above, male mate preference was assessed using chi-square tests.

Ethical Note

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 by hand, gently placing them into plastic containers and immediately transferring them to a growth chamber in the laboratory. Experimental beetles were kept under laboratory conditions similar to their natal climate and were fed fresh leaves from their natal host plant. No beetles showed signs of stress as they behaved normally and fed, mated and successfully reproduced in the laboratory. For some experiments, it was necessary to euthanize beetles by placing them in a −30 °C freezer for 20 min. At the end of the study, experimental beetles were euthanized by freezing overnight at −30 °C.

RESULTS

We identified 18 (*A. cirsicola*), 25 (*A. fragariae*) and 19 (*A. viridicyanea*) nonvolatile CHC components with a mean relative percentage of more than 0.5% in at least one group (immature female, immature male, mature female and mature male; Table S1). A MANOVA of the quantitative CHC profiles showed significant effects due to sexual maturity (*A. cirsicola*: Wilks's $\lambda = 0.025$, $F = 256.504$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.056$, $F = 76.693$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.002$, $F = 2800.783$, $P < 0.001$), sex (*A. cirsicola*: Wilks's $\lambda = 0.180$, $F = 30.341$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.314$, $F = 9.895$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.420$, $F = 8.280$, $P < 0.001$) and the sexual maturity∗sex interaction (*A. cirsicola*: Wilks's $\lambda = 0.207$, $F = 25.533$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.475$, $F = 4.988$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.465$, $F = 6.894$, $P < 0.001$) for all three species. Within species, discriminant analysis clearly separated beetles by sex and sexual maturity (*A. cirsicola*: Wilks's $\lambda = 0.002$, $X^2 = 803.690$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.009$, $X^2 = 586.284$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.0004$, $X^2 = 952.442$, $P < 0.001$; Figs. 2–4). The first canonical root accounted for 87.6% (*A. cirsicola*), 84.8% (*A. fragariae*) and 99.5% (*A. viridicyanea*) of the total variance of the CHC profiles and separated samples according to sexual maturity (mature versus immature). The second canonical root explained 10.0% (*A. cirsicola*), 11.2% (*A. fragariae*) and 0.4% (*A. viridicyanea*) of the total variance and clearly separated beetles according to sex. Among them, 95.7% (*A. cirsicola*), 94.3% (*A. fragariae*) and 91.9% (*A. viridicyanea*) of the

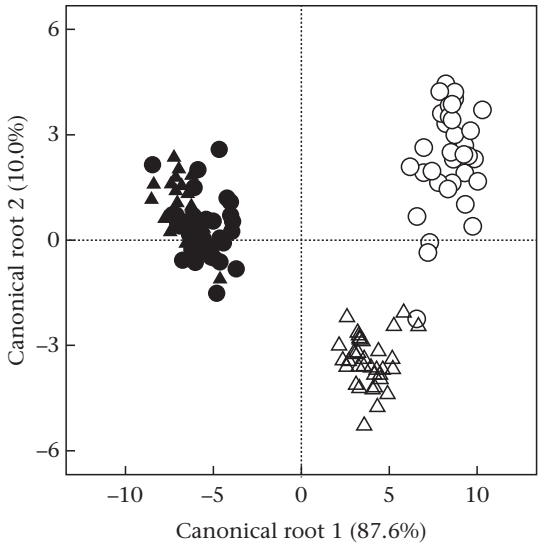

Figure 2. Discriminant analysis of four groups of *A. cirsicola* individuals based on the relative proportions of cuticular hydrocarbons. Sexually mature females (filled circles, $N = 36$), immature females (open circles, $N = 35$), sexually mature males (filled triangles, $N = 35$), immature males (open triangles, $N = 35$).

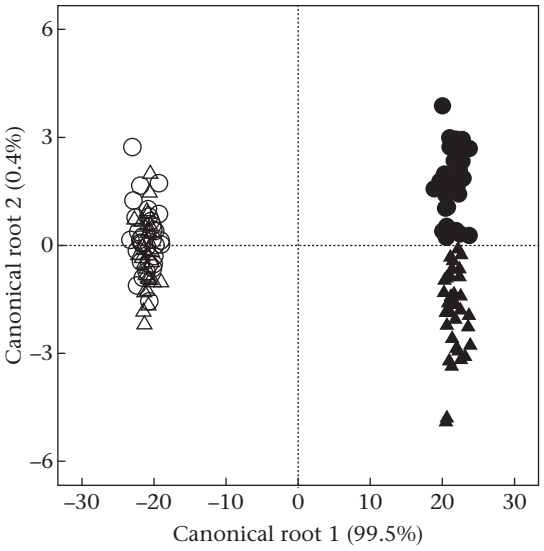

Figure 4. Discriminant analysis of four groups of *A. viridicyanea* individuals based on the relative proportions of cuticular hydrocarbons. Sexually mature females (filled circles, $N = 34$), immature females (open circles, $N = 34$), sexually mature males (filled triangles, $N = 33$), immature males (open triangles, $N = 35$).

individuals were correctly classified by the original discriminant function, and 92.2% (*A. cirsicola*), 90.1% (*A. fragariae*) and 84.6% (*A. viridicyanea*) of the cross-validated cases were correctly classified. Discriminant analysis also clearly showed that the variance CHCs is much greater between than within species. The first and second canonical roots accounted for 70.0% and 21.9% of the total variance of the data (Fig. 5).

For the analysis of the (semi-) volatile compounds, we identified seven (*A. cirsicola*), eight (*A. fragariae*) and six (*A. viridicyanea*) components (Table S1). Similar to the results for the CHCs, a MANOVA also showed significant effects due to sexual maturity (*A. cirsicola*: Wilks's $\lambda = 0.078$, $F = 219.729$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.194$, $F = 67.445$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.075$, $F = 262.066$, $P < 0.001$), sex (*A. cirsicola*: Wilks's $\lambda = 0.135$, $F = 119.776$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.469$, $F = 18.412$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.597$, $F = 14.308$, $P < 0.001$) and the sexual maturity∗sex interaction (*A. cirsicola*: Wilks's $\lambda = 0.161$, $F = 97.516$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.452$, $F = 19.679$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.603$, $F = 13.922$, $P < 0.001$) for all three species. Within species, discriminant analysis clearly separated beetles (*A. cirsicola*: Wilks's $\lambda = 0.021$, $X^2 = 522.102$, $P < 0.001$; *A. fragariae*: Wilks's $\lambda = 0.052$, $X^2 = 395.048$, $P < 0.001$; *A. viridicyanea*: Wilks's $\lambda = 0.040$, $X^2 = 418.029$, $P < 0.001$). The first canonical root accounted for 95.4% (*A. cirsicola*), 73.9% (*A. fragariae*) and 94.7% (*A. viridicyanea*) of the total variance of the CHC profiles and separated samples

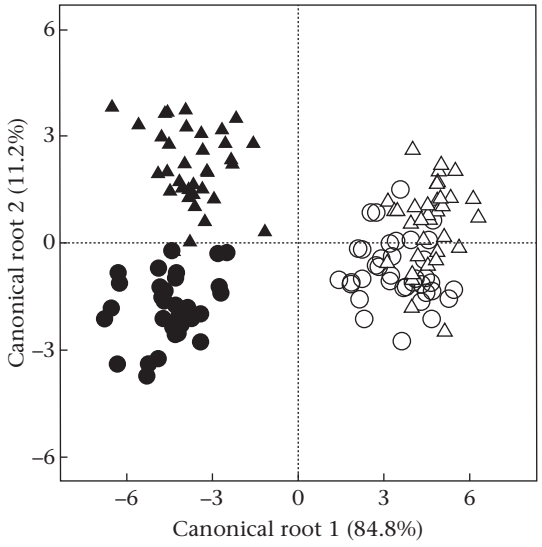

Figure 3. Discriminant analysis of four groups of *A. fragariae* individuals based on the relative proportions of cuticular hydrocarbons. Sexually mature females (filled circles, $N = 35$), immature females (open circles, $N = 37$), sexually mature males (filled triangles, $N = 35$), immature males (open triangles, $N = 34$).

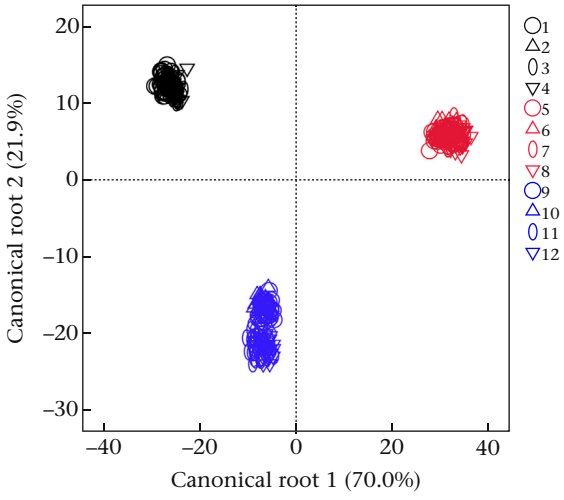

Figure 5. Discriminant analysis of 12 groups of *Altica* individuals based on the relative proportions of cuticular hydrocarbons. Numerals in the key indicate (1) sexually immature females of *A. cirsicola*, (2) immature males of *A. cirsicola*, (3) mature females of *A. cirsicola*, (4) mature males of *A. cirsicola*; (5) sexually immature females of *A. fragariae*, (6) immature males of *A. fragariae*, (7) mature females of *A. fragariae*, (8) mature males of *A. fragariae*, (9) sexually immature females of *A. viridicyanea*, (10) immature males of *A. viridicyanea*, (11) mature females of *A. viridicyanea*, (12) mature males of *A. viridicyanea*.

according to sexual maturity (mature versus immature). The second canonical root explained 4.5% (*A. cirsicola*), 18.2% (*A. fragariae*) and 4.7% (*A. viridicyanea*) of the total variance and clearly separated beetles according to sex. Among them, 80.1% (*A. cirsicola*), 90.8% (*A. fragariae*) and 85.3% (*A. viridicyanea*) of the individuals were correctly classified by the original discriminant function, and 79.4% (*A. cirsicola*), 90.8% (*A. fragariae*) and 81.6% (*A. viridicyanea*) of the cross-validated cases were correctly classified.

GC-FID analysis of hexane-washed beetles confirmed that nearly all of the CHCs were removed during the wash step. Only 1.4% (*A. fragariae*) and 1.7% (*A. viridicyanea*) remained following the hexane rinses, indicating that the washed beetles were appropriate to use as dummies in experiments 3 and 6. Surprisingly, when we examined the chemical profile of the crude extract following evaporation, we found that a considerable amount of the putative volatiles remained. We found that 25.8% (*A. fragariae*) and 11.9% (*A. viridicyanea*) of the volatiles were left in the evaporated extracts. We separated the components via polarity and found that most of the putative volatiles (No. 1–9; Table S1) separated into the polar fraction whereas most of the contact CHCs separated into the nonpolar fraction (*A. viridicyanea*, compounds No. 1–9 account for 3.8% in the nonpolar fraction, CHCs account for 6.1% in the polar fraction, $N = 5$). Two-choice mating tests examining male mate choice of females painted with either the polar or nonpolar fractions showed that only the nonpolar compounds elicited mating behaviour (all matings occurred with females painted with nonpolar compounds; number of males that mated = 16, number of replicates = 49).

Behavioural assays showed that males of these three beetle species can distinguish males from females (for *A. cirsicola* and *A. viridicyanea*, males always chose females; for *A. fragariae*: $X^2 = 12.789$, $P < 0.001$; Table 1) even when presented with dead specimens that lacked behavioural and acoustic cues (*A. cirsicola*: males always chose females; *A. fragariae*: $X^2 = 14.440$, $P < 0.001$; *A. viridicyanea*: $X^2 = 20.167$, $P < 0.001$; Table 2). We also tested whether this observed preference for female mates was reduced when we used dead females versus live ones. The results showed no difference in preference strength for mating trials conducted with dead versus living specimens across all three species (for *A. cirsicola*: males always chose females in both cases; for *A. fragariae*: $X^2 = 2.079$, $P = 0.149$; for *A. viridicyanea*: $X^2 = 1.021$, $P = 0.312$). Interestingly, the males of *A. cirsicola* showed no preference when we exchanged the CHCs of males and females ($X^2 = 0.133$, $P = 0.450$; Table 3), and the strength of preference changed drastically as compared to trials conducted with dead, unaltered beetles ($X^2 = 19.191$, $P < 0.001$). In contrast, males of *A. fragariae* and *A. viridicyanea* preferred females even when cuticular hydrocarbons were exchanged (*A. fragariae*: $X^2 = 9.281$, $P = 0.002$; *A. viridicyanea*: $X^2 = 5.452$, $P = 0.020$; Table 3); however, the strength of preference was significantly reduced in *A. viridicyanea* ($X^2 = 5.622$, $P = 0.018$) and marginally reduced in *A. fragariae* ($X^2 = 2.994$, $P = 0.084$) as compared to trials conducted with dead, unaltered beetles.

Males of all three species preferred sexually mature females over immature ones in choice mating tests regardless of whether females were alive (*A. cirsicola*: $X^2 = 15.125$, $P < 0.001$; *A. fragariae*: $X^2 = 27.457$, $P < 0.001$; *A. viridicyanea*: all mated with sexually mature females; Table 4) or dead (*A. cirsicola:* $X^2 = 7.258$, $P = 0.007$; *A. fragariae*: $X^2 = 16.892$, $P < 0.001$; *A. viridicyanea*: $X^2 = 14.440$, $P < 0.001$; Table 5). Comparisons of mating trials with living versus dead females showed that the strength of preference for sexually mature females was maintained in *A. cirsicola* ($X^2 = 0.997$, $P = 0.318$) and *A. fragariae* ($X^2 = 2.008$, $P = 0.156$). We observed a significant difference in the strength of preference in *A. viridicyanea* ($X^2 = 3.931$, $P = 0.047$), with a slight reduction in preference in trials using dead females. When we exchanged the CHCs of sexually mature and immature females, males showed no preference (*A. cirsicola*: $X^2 = 0.615$, $P = 0.443$; *A. fragariae*: the mating rate is equal; *A. viridicyanea*: $X^2 = 2.133$, $P = 0.144$; Table 6).

DISCUSSION

Males are generally thought to be indiscriminate with regard to mate choice due to their presumed lower investment in mating (Swierk et al. 2013). However, work on male mate preference in arthropods suggests that males can gain an advantage by being choosy and that reproduction can also be costly for males (reviewed in Scharf et al. 2013). Selection may favour male choosiness in *Altica*, at least in the three species examined here. The results of the present study show that males of *Altica* are able to discriminate between males and females and between sexually mature and immature females. These male preferences have possibly arisen because of energetic costs, time constraints or predation pressures. In *Altica* beetles, males mate multiply with multiple partners during their lifetime. Copulation lasts about 20 min and is followed by a prolonged copulatory mate-guarding period that can last several hours if the mating pair is not harassed. Long duration of copulation and mate guarding can reduce future mating opportunities as well as potentially increase the risk of exposure to predators (Luan, De Barro, Ruan, & Liu, 2013; Magnhagen, 1991).

Multiple cues such as olfactory, visual, acoustical, tactile and behavioural cues may be involved in assessment of potential mates by males (Geiselhardt et al., 2009; Greenspan & Ferveur, 2000; Pureswaran & Poland, 2009; Swierk et al., 2013; Tibbetts, 2002). The combination of experiments employed in the present study allowed us to reject two types of cues in *Altica*: behavioural and acoustic signals. The paired experiments that used alive versus dead potential mates (experiments 1 and 2; experiments 4 and 5) showed that mating preference of males was maintained when dead beetles were offered and that preference strength did not differ during mating trials with dead versus living specimens. Together, these results indicate that mating is initiated by the male and that behavioural cues are not critical in mate recognition. Female behaviour is often a key component of mate choice (Carazo et al., 2004; Elgar & Bathgate, 1996; Hemptinne, Lognay, & Dixon, 1998; Wedell, 2005), yet appears not to play a pivotal role in *Altica*. Furthermore, these results also suggest that acoustic signalling, one of the major modes of communication in insects (Bailey, 1991; Virant-Doberlet & Cokl, 2004), is not required for mate recognition as these cues would also be absent in dead specimens.

In contrast with behavioural and acoustic signalling, cuticular hydrocarbon chemistry appears to be involved in mate choice decisions in *Altica*. If CHCs were being used as the sole signal for mate choice decisions in *Altica*, we would have predicted a complete reversal of preference in the experiments in which CHCs were exchanged. Instead, we observed breakdown of preference, suggesting that males were receiving conflicting signals from multiple sources. For example, the results of experiments 1–3 showed that males of all species preferred females to males, and that when the CHCs of males and females were exchanged, preference in *A. cirsicola* broke down whereas the other species maintained their preference for females. Similarly, the results of the experiments testing male preference between females differing in sexual maturity showed that when the CHCs of sexually mature and immature females were exchanged, the males exhibited no preference. Although it is possible that exchanging the cuticular compounds created a 'mixed' chemical signal, GC analysis showed that the hexane washes were effective, suggesting that this is unlikely.

Furthermore, we have shown that males of *A. fragariae* and *A. viridicyanea* can easily distinguish intact, conspecific females from hexane-washed females that lack CHCs (Xue et al., 2015), and previous studies indicate that our method of exchanging CHCs is a feasible model to test the role of CHC profiles in interspecific identification (Tanigaki, Yamaoka, & Sota, 2007; Zhang et al., 2014). Together, the results suggest that mate choice in *Altica* is determined by multiple cues and that CHCs are one of the elements involved in intraspecific signalling.

Although the intraspecific differentiation observed among CHC profiles was significant (MANOVA and discriminant analysis), these differences were relatively small compared to the observed interspecific differentiation (Fig. 5). This might explain why the CHC cues were not sufficient in and of themselves for males to identify variation in mate quality. Consequently, a remaining issue is to determine the additional signalling mechanisms used by *Altica* in mate recognition. One possibility is that males use volatile compounds to identify high-quality mates. Both contact CHCs and volatiles were detected from the body surface of the three *Altica* beetles examined here (Table S1) and a large proportion remained after evaporation. While other beetle species use both volatile and contact sex pheromones to mediate mate recognition (Barbour, Lacey, & Hanks, 2007; Cervantes, Hanks, Lacey, & Barbour, 2006; Wickham, Xu, & Teale, 2012), the results from our mating trials testing male preference for polar versus nonpolar compounds suggest that *Altica* males are not cuing in on the volatile component. Another possible signalling mechanism is that the males are receiving morphological cues. *Altica* is one of the most difficult chrysomelid genera to handle taxonomically, being referred to as a 'taxonomic nightmare' (Reid & Beatson, 2015). Morphological differences between closely related species are subtle and do not play an important role in species recognition (Xue et al., 2015) and within species, sexual dimorphism is inconspicuous, lacking differentiation between mature and immature females. We currently lack the data, however, to assess whether morphological cues are important in mate recognition in *Altica*.

Mate recognition mechanisms aside, the behavioural component of the present study revealed several interesting patterns. For instance, this is the first report of same-sex sexual behaviour in *Altica*. We also observed that males generally performed poorly in our mating trials with only 46% of males successfully copulating within 3 h. This pattern of low mating rate has been generally observed in *Altica* (Xue et al. 2011, 2015) and is probably characteristic of this group. It is possible that our rather large mating arenas (9.0 × 1.2 cm petri dish) may have decreased the encounter rate, resulting in a low mating frequency; however, we have observed some *Altica* males that do not mate even when they encounter a female repeatedly during a mating assay.

Although CHC profiles were not the only component of male mate recognition in *Altica*, we have shown that the chemical signatures could be clearly separated by sexual maturity and sex. Discriminant analysis showed that sexual maturity accounted for more of the variance than sex, and this was reflected in the behavioural assays. There was a consistent pattern of no preference by all three *Altica* species when the CHCs were exchanged between sexually mature and immature females. In contrast, preference was unaltered in two *Altica* species when the CHCs were exchanged between males and females. These results corroborate previous findings showing that CHCs can vary with sex (Cuvillier-Hot, Cobb, Malosse, & Peeters, 2001; Kather & Martin, 2012; Singer, 1998) and breeding status (Everaerts, Farine, Cobb, & Ferveur, 2010; Scott et al., 2008; Steiger, Peschke, Francke, & Müller, 2007; Steiger et al., 2009). In the few studies that have assessed sex-based differences in CHCs using bioassays (Howard & Blomquist, 2005), a majority show that CHCs are used by the insects for sex recognition (Ginzel, Blomquist, Millar, & Hanks, 2003; Howard, 1998; Peterson et al., 2007; Tanigaki et al., 2007). There are a few examples, however, where divergence in CHCs between males and females is not used in mate recognition (Geiselhardt et al., 2009; Hemptinne et al., 1998). *Altica* appears to span the breadth of these findings, where *A. cirsicola* uses sex-specific CHCs (and other cues) whereas *A. fragariae* and *viridicyanea* do not. Together, the results suggest that CHCs provide signals about sex and sexual maturity but do not appear to be the primary mating cue. Instead, we propose that *Altica* males use a combination of CHC cues and other signals to discriminate between potential mates. Very little is known about the cues that males use to assess potential mates (Tuni & Berger-Tal, 2012); thus, understanding more about how insects perceive and interpret intraspecific variation is a worthwhile goal that will contribute broadly to the study of insect mating systems.

Acknowledgments

We thank Xiao-Wei Qin for assisting with the GC analysis, Jocelyn G. Millar for providing reference compounds and David M. Althoff for providing comments on the manuscript. This study was supported by National Natural Science Foundation of China (Grant Nos. 31071951, 31272302) and a grant from the Chinese Academy of Sciences (Grant No. KSCX2-EW-J-2).

Supplementary Material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.anbehav.2015.10.025.

References

Aitchison, J. (1986). *The statistical analysis of compositional data*. London, U.K.: Chapman and Hall.
Andersson, M. (1994). *Sexual selection*. Princeton, NJ: Princeton University Press.
Andersson, M., & Simmons, L. W. (2006). Sexual selection and mate choice. *Trends in Ecology and Evolution, 21*, 296–302.
Aranaud, L., & Haubruge, E. (1999). Mating behaviour and male mate choice in *Tribolium castaneum* (Coleoptera, Tenebrionidae). *Behaviour, 136*, 67–77.
Bagemihl, B. (1999). *Biological exuberance: animal homosexuality and natural diversity*. New York, NY: St. Martin's Press.
Bailey, W. J. (1991). *Acoustic behaviour of insects. An evolutionary perspective*. London, U.K.: Chapman and Hall.
Bailey, N. W., & French, N. (2012). Same-sex sexual behaviour and mistaken identity in male field crickets, *Teleogryllus oceanicus*. *Animal Behaviour, 84*, 1031–1038.
Bailey, N. W., & Zuk, M. (2009). Same-sex sexual behavior and evolution. *Trends in Ecology and Evolution, 24*, 439–446.
Barbour, J. D., Lacey, E. S., & Hanks, L. M. (2007). Cuticular hydrocarbons mediate mate recognition in a species of longhorned beetle (Coleoptera: Cerambycidae) of the primitive subfamily Prioninae. *Annals of the Entomological Society of America, 100*, 333–338.
Ben-Ari, E. T. (2000). Choosy females: exploring the role of cryptic female choice in sexual selection and battles over paternity. *Bioscience, 50*, 8–12.
Blomquist, G. J., & Bagnères, A. G. (2010). *Insect hydrocarbons: Biology, biochemistry, and chemical ecology*. Cambridge, U.K.: Cambridge University Press.
Bonduriansky, R. (2001). The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biological Reviews, 76*, 305–339.
Burgevin, L., Friberg, U., & Maklakov, A. A. (2013). Intersexual correlation for same-sex sexual behaviour in an insect. *Animal Behaviour, 85*, 759–762.
Carazo, P., Sanchez, E., Font, E., & Desfilis, E. (2004). Chemosensory cues allow male *Tenebrio molitor* beetles to assess the reproductive status of potential mates. *Animal Behaviour, 68*, 123–129.
Carlson, D. A., Bernier, U. R., & Sutton, B. D. (1998). Evolution patterns from capillary GC for methyl-branched alkanes. *Journal of Chemical Ecology, 24*, 1845–1865.
Cervantes, D. E., Hanks, L. M., Lacey, E. S., & Barbour, J. D. (2006). First documentation of a volatile sex pheromone in a longhorned beetle (Coleoptera: Cerambycidae) of the primitive subfamily Prioninae. *Annals of the Entomological Society of America, 99*, 718–722.
Cuvillier-Hot, V., Cobb, M., Malosse, C., & Peeters, C. (2001). Sex, age and ovarian activity affect cuticular hydrocarbons in *Diacamma ceylonense*, a queenless ant. *Journal of Insect Physiology, 47*, 485–493.
Dietemann, V., Peeters, C., Liebig, J., Thivet, V., & Hölldobler, B. (2003). Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in

the ant *Myrmecia gulosa*. *Proceedings of the National Academy of Sciences of the United States of America, 100*, 10341–10346.
Dodds, E. D., McCoya, M. R., Reac, L. D., & Kennisha, J. M. (2005). Gas chromatographic quantification of fatty acid methyl esters: flame ionization detection vs. electron impact mass spectrometry. *Lipids, 40*, 419–428.
Doolittle, R. E., Proveaux, A. T., Alborn, H. T., & Heath, R. R. (1995). Quadrupole storage mass spectrometry of mono- and dimethylalkanes. *Journal of Chemical Ecology, 21*, 1677–1695.
Dukas, R. (2010). Causes and consequences of male-male courtship in fruit flies. *Animal Behaviour, 80*, 913–919.
Efron, B. (1983). Estimating the error rate of a prediction rule: improvement on cross-validation. *Journal of the American Statistical Association, 78*, 316–331.
Elgar, M. A., & Bathgate, R. (1996). Female receptivity and male mate-guarding in the jewel spider *Gasteracantha minax* Thorell (Araneidae). *Journal of Insect Behavior, 9*, 729–738.
Everaerts, C., Farine, J., Cobb, M., & Ferveur, J. (2010). *Drosophila* cuticular hydrocarbons revisited: mating status alters cuticular profiles. *PLoS One, 5*, e9607.
Geiselhardt, S., Otte, T., & Hilker, M. (2009). The role of cuticular hydrocarbons in male mating behavior of the mustard leaf beetle, *Phaedon cochleariae* (F.). *Journal of Chemical Ecology, 35*, 1162–1171.
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. *Ecology Letters, 15*, 971–977.
Ginzel, M. D., Blomquist, G. J., Millar, J. G., & Hanks, L. M. (2003). Role of contact pheromones in mate recognition in *Xylotrechus colonus*. *Journal of Chemical Ecology, 29*, 533–545.
Greenspan, R. J., & Ferveur, J. F. (2000). Courtship in *Drosophila*. *Annual Review of Genetics, 34*, 205–232.
Hemptinne, J. L., Lognay, G., & Dixon, A. F. G. (1998). Mate recognition in the two-spot ladybird beetle, *Adalia bipunctata*: role of chemical and behavioural cues. *Journal of Insect Physiology, 44*, 1163–1171.
Howard, R. W. (1998). Ontogenetic, reproductive, and nutritional effects on the cuticular hydrocarbons of the host-specific ectoparasitoid *Cephalonomia tarsalis* (Hymenoptera: Bethylidae). *Annals of the Entomological Society of America, 91*, 101–112.
Howard, R. W., & Blomquist, G. J. (2005). Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology, 50*, 371–393.
Johansson, B. G., & Jones, T. M. (2007). The role of chemical communication in mate choice. *Biological Reviews, 82*, 265–289.
Kather, R., & Martin, S. J. (2012). Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. *Physiological Entomology, 37*, 25–32.
Krasnec, M. O., & Breed, M. D. (2013). Colony-specific cuticular hydrocarbon profile in *Formica argentea* ants. *Journal of Chemical Ecology, 39*, 59–66.
Luan, J. B., De Barro, P. J., Ruan, Y. M., & Liu, S. S. (2013). Distinct behavioural strategies underlying asymmetric mating interactions between invasive and indigenous whiteflies. *Entomologia Experimentalis et Applicata, 146*, 186–194.
Magnhagen, C. (1991). Predation risk as a cost of reproduction. *Trends in Ecology & Evolution, 6*, 183–186.
Nelson, D. R., Sukkestad, D. R., & Zaylskie, R. G. (1972). Mass spectra of methyl-branched hydrocarbons from eggs of the tobacco hornworm. *Journal of Lipid Research, 13*, 413–421.
Ozaki, M., Wada-Katsumata, A., Fujikawa, K., Iwasaki, M., Yokohari, F., Satoji, Y., et al. (2005). Ant nestmate and non-nestmate discrimination by a chemosensory sensillum. *Science, 309*, 311–314.
Peterson, M. A., Dobler, S., Larson, E. L., Juárez, D., Schlarbaum, T., Monsen, K. J., et al. (2007). Profiles of cuticular hydrocarbons mediate male mate choice and sexual isolation between hybridising *Chrysochus* (Coleoptera: Chrysomelidae). *Chemoecology, 17*, 87–96.
Pitnick, S., Spicer, G. S., & Markow, T. A. (1995). How long is a giant sperm. *Nature, 375*, 109.
Pomonis, J. G., Nelson, D. R., & Fatland, C. L. (1980). Insect hydrocarbons. 2. Mass spectra of dimethylalkanes and the effect of the number of methylene units between groups on fragmentation. *Journal of Chemical Ecology, 6*, 965–972.
Porco, D., Deharveng, L., & Gers, C. (2004). Sexual discrimination with cuticular lipids in *Schoettella ununguiculata* (Tullberg, 1869) (Collembola: Hypogastruridae). *Pedobiologia, 48*, 581–583.
Pureswaran, D. S., & Poland, T. M. (2009). The role of olfactory cues in short-range mate finding by the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Journal of Insect Behavior, 22*, 205–216.
Reid, C. A. M., & Beatson, M. (2015). Disentangling a taxonomic nightmare: a revision of the Australian, Indomalayan and Pacific species of *Altica* Geoffroy, 1762 (Coleoptera: Chrysomelidae: Galerucinae). *Zootaxa, 3918*, 503–551.
Ruther, J., Sieben, S., & Schricker, B. (2002). Nestmate recognition in social wasps: manipulation of hydrocarbon profiles induces aggression in the European hornet. *Naturwissenschaften, 89*, 111–114.
Scharf, I., & Martin, O. Y. (2013). Same-sex sexual behavior in insects and arachnids: prevalence, causes, and consequences. *Behavioral Ecology and Sociobiology, 67*, 1719–1730.
Scharf, I., Peter, F., & Martin, O. Y. (2013). Reproductive trade-offs and direct costs for males in arthropods. *Evolutionary Biology, 40*, ,169–184.
Schlechter-Helas, J., Schmitt, T., & Peschke, K. (2012). Learning individual signatures: rove beetle males discriminate unreceptive females by cuticular hydrocarbon patterns. *Animal Behaviour, 84*, 369–376.
Scott, M. P., Madjid, K., & Orians, C. M. (2008). Breeding alters cuticular hydrocarbons and mediates partner recognition by burying beetles. *Animal Behaviour, 76*, 507–513.
Singer, T. L. (1998). Roles of hydrocarbons in the recognition systems of insects. *American Zoologist, 38*, 394–405.
Steiger, S., Peschke, K., Francke, W., & Müller, J. K. (2007). The smell of parents: breeding status influences cuticular hydrocarbon pattern in the burying beetle *Nicrophorus vespilloides*. *Proceedings of the Royal Society B: Biological Sciences, 274*, 2211–2220.
Steiger, S., Whitlow, S., Peschke, K., & Müller, J. K. (2009). Surface chemicals inform about sex and breeding status in the biparental burying beetle *Nicrophorus vespilloides*. *Ethology, 115*, 178–185.
Swierk, L., Myers, A., & Langkilde, T. (2013). Male mate preference is influenced by both female behaviour and morphology. *Animal Behaviour, 85*, 1451–1457.
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*. *Zoological Science, 24*, 39–45.
Thomas, M. L. (2011). Detection of female mating status using chemical signals and cues. *Biological Reviews, 86*, 1–13.
Tibbetts, E. A. (2002). Visual signals of individual identity in a wasp *Polistes fuscatus*. *Proceedings of the Royal Society B: Biological Sciences, 269*, 1423–1428.
Tuni, C., & Berger-Tal, R. (2012). Male preference and female cues: males assess female sexual maturity and mating status in a web-building spider. *Behavioral Ecology, 23*, 582–587.
Virant-Doberlet, M., & Cokl, A. (2004). Vibrational communication in insects. *Neotropical Entomology, 33*, 121–134.
Wagner, D., Tissot, M., Cuevas, W., & Gordon, D. M. (2000). Harvester ants utilize cuticular hydrocarbons in nestmate recognition. *Journal of Chemical Ecology, 26*, 2245–2257.
Wang, S. Y., Cui, J. Z., Li, W. Z., & Zhang, Y. (2005). The feeding habits of the genus *Altica* and biological significance. *Chinese Bulletin of Entomology, 42*, 385–390.
Wedell, N. (2005). Female receptivity in butterflies and moths. *Journal of Experimental Biology, 208*, 3433–3440.
Wickham, J. D., Xu, Z. C., & Teale, S. A. (2012). Evidence for a female-produced, long range pheromone of *Anoplophora glabripennis* (Coleoptera: Cerambycidae). *Insect Science, 19*, 355–371.
Wood, T. K., Tilmon, K. J., Shantz, A. B., & Harris, C. K. (1999). The role of host-plant fidelity in initiating insect race formation. *Evolutionary Ecology Research, 1*, 317–332.
Xue, H. J., Egas, M., & Yang, X. K. (2007). Development of a positive preference-performance relationship in an oligophagous beetle: adaptive learning? *Entomologia Experimentalis et Applicata, 125*, 119–124.
Xue, H. J., Li, W. Z., Nie, R. E., & Yang, X. K. (2011). Recent speciation in three closely related sympatric specialists: inferences using multi-locus sequence, post-mating isolation and endosymbiont data. *PLoS One, 6*, e27834.
Xue, H. J., Li, W. Z., & Yang, X. K. (2014). Assortative mating between two sympatric closely-related specialists: inferred from molecular phylogenetic analysis and behavioral data. *Scientific Reports, 4*, 5436.
Xue, H. J., Wang, S. Y., Li, W. Z., Zhang, X. Z., & Yang, X. K. (2007). Bionomics of *Altica fragariae*. *Chinese Bulletin of Entomology, 44*, 69–73.
Xue, H. J., Wei, J. N., Magalhães, S., Zhang, B., Song, K. Q., Liu, J., et al. (2015). *Contact pheromones of two sympatric beetle species are modified by the host plant and affect mating* (submitted manuscript).
Xue, H. J., & Yang, X. K. (2007). Host plant use in sympatric closely related flea beetles. *Environmental Entomology, 36*, 468–474.
Zhang, B., Xue, H. J., Song, K. Q., Liu, J., Li, W. Z., Nie, R. E., et al. (2014). Male mate recognition via cuticular hydrocarbons facilitates sexual isolation between sympatric leaf beetle sister species. *Journal of Insect Physiology, 70*, 15–21.