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

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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

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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