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Love at first sniff: a spermatophore-associated pheromone mediates partner attraction in a collembolan species

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Mate choice is essential in most animals, as a good choice of mating partner largely determines reproductive success. Much evidence shows that olfactory cues play an important role in mate choice. However, the integration of chemical, visual and acoustic cues, often used when both partners meet, makes it hard to test whether olfaction alone can mediate reproductive decisions. Interestingly, several invertebrates have adopted a mating system where males deposit their sperm (packed in spermatophores) in the environment for females to pick up with no visual contact between the sexes. In this case the male cue is conveyed by the spermatophore only. Earlier studies on a species with indirect sperm transfer, the soil arthropod *Orchesella cincta*, showed that, even in these animals, female choice exists. In this study, we tested whether chemical cues provided by the spermatophores mediate this female choice. Chemical analysis of spermatophore extracts revealed that (Z)-14-tricosenol is the main compound in the male spermatophores and this compound attracted females in olfactometer bioassays. Our finding suggests that (Z)-14-tricosenol is thus a pheromone component, which is sufficient for female attraction. This is the first report of a spermatophore-associated sex pheromone in a species performing indirect sperm transfer.

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The ability to rapidly detect a mating partner is crucial for an animal's fitness (Andersson, 1994; Andersson & Simmons, 2006; Weddle, Hunt, & Sakaluk, 2013). Usually, males and females use a range of sensory systems for sexual communication, including visual, acoustic and chemical cues to detect information about the potential mate (Candolin, 2003; Leonard & Hedrick, 2009). Most species in the animal kingdom rely to some extent on chemical cues for mating decisions (Wyatt, 2003); however, chemical cues are more difficult to observe than other signals (Penn, 2006). Moreover, in most mating systems with internal fertilization, reproduction involves pair formation, making it hard to test the hypothesis that a chemical cue alone mediates reproductive decisions. Even though

there is growing evidence that olfactory cues provide information on the individual quality of a potential partner (see e.g. Chemnitz, Jentschke, Ayasse, & Steiger, 2015; Johansson & Jones, 2007; Rantala, Kortet, Kotiaho, Vainikka, & Suhonen, 2003; Ruther, Matschke, Garbe, & Steiner, 2009; Thomas, 2011), the functional integration of multiple sensory modalities during mating contact makes it hard to disentangle the relative contribution of chemical attractants.

Interestingly though, throughout the animal kingdom, the degree of contact during mating is highly variable and shows a continuum from full bodily contact to no contact at all (Zizzari, Jessen, & Koene, 2016; Zizzari, Smolders, & Koene, 2014) and can broadly be categorized as follows: (1) direct transfer of sperm involving intimate contact (i.e. copulation); (2) paired indirect transfer, in which a male courts a particular female before, during or after deposition of sperm in the environment (e.g. salamanders: Arnold, 1976; soil arthropods: Proctor, 1998; Schaller, 1971); (3) dissociated indirect sperm transfer, where males deposit their

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sperm in the environment for females to pick up and use for internal fertilization without meeting the male (e.g. soil and aquatic arthropods: Barazandeh, Davis, Neufeld, Coltman, & Palmer, 2013; Proctor, 1998; Zizzari, van Straalen, & Ellers, 2013; sessile marine invertebrates: Bishop & Pemberton, 2006).

In the latter case, there is no physical or visual contact between the sexes and, most likely, a female relies solely on chemical cues for reproductive decisions. Animals performing dissociated sperm transfer represent a common and widespread group, but are neglected in behavioural studies. Yet, the study of organisms that rely solely on sperm-associated chemical cues for reproductive decisions could deepen our insights in the evolution of chemical signals in general and shed light on their role as sperm competitive traits.

In this study, we used the soil arthropod *Orchesella cincta* (Collembola: Entomobryidae) to investigate the role of olfactory communication when males and females never have contact during reproductive decisions. *Orchesella cincta* displays a mating system widespread among soil arthropods (Proctor, 1998; Walter & Proctor, 2013), i.e. males deposit their sperm (packed in spermatophores) in the environment irrespective of the presence of a female, and females pick up a spermatophore for internal fertilization without the presence of the male. Earlier work has provided evidence that female choice for spermatophores does exist in *O. cincta* (Zizzari, Braakhuis, van Straalen, & Ellers, 2009; Zizzari & Ellers, 2011; Zizzari et al., 2013), suggesting that chemical cues related to the spermatophore are involved in mate assessment. Here, we tested whether olfactory cues associated with spermatophores produced by *O. cincta* males attract conspecific females from a distance.

By performing gas chromatography coupled to high-resolution mass spectrometry (GC–MS) analyses of spermatophore extracts, we first identified the chemical compounds from the spermatophores. Subsequently, in olfactometer bioassays we measured the response of females *O. cincta* to the identified compounds. To our knowledge, this study is the first report of female attraction to a male spermatophore-associated pheromone.

METHODS

Study Species and Care

The *O. cincta* strain used in this study was obtained from a large outbred laboratory population that originated from a pine forest in The Netherlands (Roggebotzand; 52°34.40N, 05°47.90E). This stock population had been maintained at 20 °C in a climate-controlled room (70% relative humidity, 12:12 h light:dark) in the laboratory for over 30 years at the time of the experiments. In the experiments, animals were kept in Perspex vials with a moistened bottom of plaster of Paris, which kept humidity inside the vials at nearly 100%. Pine tree twigs naturally covered with green algae (*Desmococcus* sp.) were provided for food.

Natural History

Adult *O. cincta* (Fig. 1) alternate reproductive and nonreproductive periods (instars) separated by moults (Gols, Ernsting, & van Straalen, 2004; Stam, Isaaks, & Ernsting, 2002; Zizzari et al., 2009). Each instar lasts 4–5 days. Spermatophore deposition (Fig. 2) occurs only during the reproductive instar and males usually deposit dozens of spermatophores within a reproductive instar (mean = 58; Zizzari, van Straalen, & Ellers, 2016). In this species, as in several soil arthropods, the reproductive success of males depends on placing a large number of spermatophores, to

Figure 1. Image of *Orchesella cincta*. Photo: Z.V. Zizzari.

increase the probability of detection by females and counterbalance spermatophore destruction by rivals (Dallai, Zizzari, & Fanciulli, 2009; Proctor, 1998; Stam et al., 2002; Zizzari, Jessen, et al., 2016). Female receptivity lasts 48 h after the onset of a reproductive instar (Gols et al., 2004; Zizzari et al., 2009), during which receptive females locate the spermatophores, and take up only one with the genital opening to fertilize all their eggs (Dallai, Zizzari, & Fanciulli, 2008; Gols et al., 2004). A female's reproductive instar can only be assessed by offering her a spermatophore and subsequently checking for eggs (Zizzari et al., 2009; Zizzari & Ellers, 2014; Zizzari, van Straalen, et al., 2016).

Figure 2. Light microscope view of *O. cincta* spermatophores: sd: apical sperm droplet; s: stalk. Photo: Z.V. Zizzari.

Therefore, to detect the onset of a new instar, careful daily monitoring of the moulting rhythms is needed. Earlier work has provided evidence that *Orchesella cincta* females are more attracted by spermatophores produced by males exposed to a rival (Zizzari et al., 2013) and by patches with a high rather than low density of spermatophores (Zizzari et al., 2009). Moreover, *O. cincta* females can gain indirect benefit by choosing between spermatophores. Specifically, when females are allowed a choice between spermatophores from different males, their sons produce more spermatophores than when females are denied a choice (Zizzari et al., 2009).

Female Olfactory Responses to Natural Pheromone Source

We tested the behavioural responses of *O. cincta* females in a Y-shaped olfactometer (Fig. A1 in the Appendix), where females were given a choice between two odour sources. Only virgin females 7–8 weeks old were used in the bioassays.

Prior to introduction to the choice olfactometer, females were checked for moulting skins indicating the onset of the reproductive instar. The behaviour of each individual in the olfactometer was observed for 15 min. If a female remained in the main arm, it was recorded as 'no-choice'. Females were considered to have made a choice when they walked at least 1 cm into one of the two arms. Each individual was tested only once.

First, the females were given a choice between six fresh spermatophores (sperm droplets) versus nothing ($N = 50$; assay a) and spermatophore extract (20 μ l acetone/80 spermatophores) versus pure acetone solvent ($N = 55$; assay b). Spermatophore extracts were obtained from fresh sperm droplets of males ($N = 120$) 6–7 weeks old in a reproductive instar. Each male was kept in an individual vial and checked daily for spermatophores. If spermatophores were found, their apical sperm droplets (Fig. 2) were collected one by one with fine tweezers and transferred to conical vials filled with acetone. The remaining spermatophores were destroyed to ensure that only freshly laid spermatophores were used for the successive extract. Pure acetone constituted the control treatment. In the assay, 4 μ l of test (16 spermatophore equivalents) and control solutions were applied to a 5 mm diameter filter paper disc and the solvent was allowed to evaporate for 1 min before discs were placed inside the olfactometer. The odour sources were placed at the end of each branch of the olfactometer and were allowed to diffuse into the olfactometer for 5 min before the experiment started. Between trials, the odour sources were swapped to opposite branches to avoid side bias, and after each trial, the olfactometers were cleaned with ethanol and demineralized water. All odour sources were renewed for each tested female. Since the spermatophore extracts were chosen, we then proceeded with the identification of the main compounds in the extracts and tested the female behavioural response to these compounds.

Identification of Spermatophore-associated Compounds

Spermatophore extracts used for identification were obtained from spermatophores of 6–7 weeks old males following the procedure described above (see assay b). All the extracts and controls were stored at -20°C until analysis. Between May and October 2015, we collected and analysed 5200 spermatophores for the identification of pheromone-like compounds. Spermatophore extracts were analysed by GC–MS, on an HP6890 GC

(Agilent, Santa Clara, CA, U.S.A.) equipped with a high-resolution polar capillary column DBWAXetr (Agilent) and connected to an Autospec sector field mass spectrometer (Micromass UK Ltd, Manchester, U.K.) operated in electron ionization (EI) and chemical ionization (CI) mode. The GC–MS was temperature-programmed from 60°C (2 min hold) to 180°C at $30^{\circ}\text{C}/\text{min}$, then to 230°C at $5^{\circ}\text{C}/\text{min}$ and finally to 250°C at $20^{\circ}\text{C}/\text{min}$, and the injector was held at 250°C . Helium was used as the carrier gas. To exclude the possibility that contaminants in the GC–MS or solvents occur at the same retention time as the pheromone compounds, before each GC–MS sequence we measured a blank sample and a sample containing only acetone.

The fragmentation pattern and high-resolution EI and CI measurements indicated two unsaturated straight-chain components in the spermatophore extracts, namely docosenol and tricosenol. Localization of the double-bond positions in docosenol and tricosenol from spermatophore extracts was performed by acetonitrile chemical ionization (ACN-CI) tandem-mass spectrometry on a Varian 240 ion-trap MS coupled to a 450 GC (see Appendix). The deduced double bond positions of the unsaturated alcohols from the Acetonitrile MS/MS fragmentation patterns were confirmed by comparing their fragmentation patterns and retention time with those of authentic standards. The synthetic compounds (Z)-13-docosenol, (E)-13-docosenol, (Z)-14-tricosenol and (E)-14-tricosenol were purchased from Pherobank (Wageningen, The Netherlands).

Z/E-isomer identification in spermatophore extracts was performed by isomer separation on a ZB5HT GC column ($30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$, Phenomenex, Torrance, CA, U.S.A.), comparing retention times of peaks in the spermatophore extract with retention times of the four synthetic compounds. This analysis was again performed on an HP6890 GC coupled with an Autospec sector field mass spectrometer.

The amount of the two identified compounds in the extracts was calculated relative to the 3 ng of internal standard (pentadecane) in each spermatophore extract (30 μ l acetone/200 spermatophores) analysed by a 7890A GC (Agilent) equipped with a high-resolution polar capillary column DBWAXetr and coupled to a flame ionization detector (FID). A solution of 3 μ l was transferred in a crimp-capped vial. Using a 7683 automatic injector, the entire volume of each extract was injected in a splitless inlet of the GC (GC program the same as HP6890 GC above).

Female Olfactory Responses to Synthetic Compounds

After identification of the two main compounds, we conducted a third assay (assay c), using the same procedure as assay b, where we tested the behavioural response of receptive females to the synthetic compounds versus pure acetone solvent: (Z)-13-docosenol ($N = 55$), (Z)-14-tricosenol ($N = 56$) and their mixture ($N = 55$). We estimated that a male spermatophore contains about $2.42 \pm 0.55\text{ ng}$ ($N = 6$) of (Z)-13-docosenol and (Z)-14-tricosenol, based on the peak area of the internal standard pentadecane. The ratio of (Z)-13-docosenol and (Z)-14-tricosenol in the crude pheromone extract was determined to be approximately 3:2 ($N = 6$), based on peak areas in the GC analysis. One equivalent of (Z)-13-docosenol and (Z)-14-tricosenol in a male spermatophore corresponded to $1.41 \pm 0.32\text{ ng}$ and $1.01 \pm 0.23\text{ ng}$, respectively.

In assay c, we used an amount of (Z)-13-docosenol and (Z)-14-tricosenol that corresponded to ca. 25 spermatophore equivalents.

Figure 3. Behavioural responses of virgin *O. cincta* females to (a) natural spermatophore (assay a, six spermatophores), (b) natural spermatophore extract (assay b, 16 spermatophore equivalents) and (c) the synthetic compounds (assay c, 25 spermatophore equivalents) in a Y-olfactometer. Numbers inside the bars indicate the absolute number of females that chose. Total number of tested females is given in the text. Differences in responses for each dual-choice bioassay were assessed by two-tailed binomial tests. Asterisks indicate significant differences at $P < 0.05$.

Statistics

Two-tailed binomial tests were used to evaluate the olfactometer bioassay results. Females that did not choose a particular arm within the observation time were excluded from statistical analysis. Figure 3 was created using R v.2.15.3 (The R Foundation for Statistical Computing, Vienna, Austria, <http://www.r-project.org>). Statistical analyses were performed using IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, U.S.A.).

Ethical Note

Experimental manipulation of springtails conformed to the current legal and ethical requirements for animal welfare of the Netherlands. All springtails were carefully handled during experiments and maintained in the laboratory under appropriate conditions.

RESULTS

In assay a, most of the females preferred the branch of the olfactometer with the natural spermatophores ($N = 46$, $P = 0.026$; Fig. 3). Similarly, when given the choice between the pure solvent and the spermatophore extract (assay b), females were most attracted to the extract ($N = 50$, $P = 0.033$; Fig. 3).

GC–MS analyses of crude spermatophore extracts showed two prominent peaks, which were identified as unsaturated straight-chain alcohols, namely (Z)-13-docosenol and (Z)-14-tricosenol (Figs A2 and A3 in the Appendix). Besides (Z)-13-docosenol and (Z)-14-tricosenol, we detected two more compounds in the spermatophore extracts of *O. cincta* that we could not identify because of their low concentration levels (Fig. A4 in the Appendix).

In the bioassay with (Z)-13-docosenol (assay c), females were not significantly attracted to the synthetic standard ($N = 52$, $P = 0.212$; Fig. 3). However, when given the choice between pure solvent and (Z)-14-tricosenol, females were significantly attracted

by (Z)-14-tricosenol ($N = 52$, $P = 0.018$; Fig. 3). *Orchesella* females also chose the mixture of (Z)-13-docosenol and (Z)-14-tricosenol over the solvent, although this was only marginally significant ($N = 51$, $P = 0.049$; Fig. 2). There was no significant difference between females attracted to the natural spermatophore and females attracted to (Z)-14-tricosenol ($N = 66$, $P = 0.712$), nor between females attracted to (Z)-14-tricosenol and females attracted to the mixture ($N = 68$, $P = 0.904$).

DISCUSSION

We have provided evidence that spermatophore finding is mediated by a volatile pheromone in the collembolan *O. cincta*. To our knowledge, this is the first report of a spermatophore-associated sex pheromone in a species with dissociated sperm transfer. Although analyses of the spermatophore extracts led to the identification of two potential compounds, namely (Z)-13-docosenol and (Z)-14-tricosenol, bioassays showed that (Z)-14-tricosenol alone suffices to attract females. Even though there are no previous reports of (Z)-14-tricosenol being involved in sexual communication, it is a structural analogue of (Z)-9-tricosene, which is a well-known component of sex pheromones in the house fly *Musca domestica* (Carlson et al., 1971) and was recently shown to be a male-produced female attractant in the spider *Pholcus beijingensis* (Xiao, Zhang, & Li, 2010) and in the olive fruit fly, *Bactrocera oleae* (Carpita et al., 2012).

The identification of the major sex pheromone component that elicits spermatophore attraction by *O. cincta* females is an important step towards the elucidation of the interindividual variation in the spermatophore attractiveness suggested by previous studies (Stam et al., 2002; Zizzari et al., 2009; Zizzari et al., 2013). That female attraction to the spermatophores goes beyond species recognition is indicated by the fact that females can benefit from choosing between spermatophores of different males (Zizzari et al., 2009). Second, *O. cincta* males adjust the attractiveness of their spermatophores

under male–male competition, as females prefer spermatophores of males exposed to a competitor (Zizzari et al., 2013).

The female behaviour of probing the spermatophore before uptake (Zizzari et al., 2009) suggests that contact chemoreception might also be involved in the final spermatophore choice. None the less, the presence of several compounds in the spermatophore extracts could indicate variation in the volatile pheromone blend, as shown in the burying beetle *Nicrophorus vespilloides*, in which males differ in the relative composition of the two pheromone components emitted (Chemnitz et al., 2015). On the other hand, in the wasp *Nasonia vitripennis*, male quality (i.e. sperm load) is reflected by the quantity of the male sex pheromone (Ruther et al., 2009). Possibly, the spermatophore-based pheromone system may also have evolved to prevent heterospecific attraction and/or predation. Earlier studies suggest no cross-species attractiveness between the spermatophores of the two closely related species *O. cincta* and *Orchesella villosa* (Stam et al., 2002).

Future investigation with emphasis on the genus *Orchesella* will help to determine whether congeners produce spermatophores with different olfactory cues. Further work is also necessary to unravel the interindividual variation in spermatophores, link this variation to male quality and assess whether females discriminate between spermatophores after only touching them. However, irrespective of whether the final female choice is due to the pheromone ratio/quantity or to contact chemoreception, our study suggests that male reproductive success in *O. cincta* depends on a volatile pheromone emanating from the spermatophore, as females find the spermatophore using this pheromone. The existence of a spermatophore-associated pheromone has been suggested for species performing dissociated sperm transfer (Thomas & Zeh, 1984; Walter & Proctor, 2013). Our study not only characterizes, for the first time, the main pheromone component behind the female spermatophore-finding behaviour in a species with dissociated sperm transfer, but also expands the evidence that sexual chemical communication plays a crucial role in these animals.

Finally, since most research on sperm competition has been conducted on species that copulate, it is current thinking that sperm competition mainly occurs after mating. Interestingly though, when sperm transfer is indirect sperm competition occurs prior to mating, as males of these species are known to destroy the spermatophores of rivals (Zizzari, Jessen, et al., 2016). Thus, chemical signals may play a crucial role in precopulatory sperm competitive strategies if males tailor spermatophore attractiveness according to male–male competition (Zizzari et al., 2013). Although virtually unexplored, sexual chemical communication as a sperm competition trait may be very common in the great diversity of animals performing indirect sperm transfer (see e.g. sessile marine invertebrates: Bishop & Pemberton, 2006; soil and aquatic arthropods: Barazandeh et al., 2013; Proctor, 1998; Zizzari et al., 2013; salamanders: Arnold, 1976). We emphasize the potential of animals with this unconventional, yet widespread, mode of sperm transfer, as an experimental model system to study the importance of female choice for the evolution of male chemical cues.

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Appendix

Briefly, in ACN-CI, analytes are ionized by acetonitrile gas and the diagnostically useful M+54 adduct ($[C_3H_4N]^+$ ion) is formed from an ion/molecule reaction between $[C_2H_2N]^+$ (m/z 40) and neutral acetonitrile, followed by a loss of HCN (Oldham, 1999). This M+54 adduct is enriched and stored in the ion trap and then fragmented by collision-induced dissociation with helium gas. The double-bond position can be deduced from the resulting fragmentation pattern (Kroiss, Svatos, & Kaltenpoth, 2011). For the determination of the double-bond positions, we applied a CI MS/MS approach using acetonitrile as a reagent gas. The CI MS-MS experiments of the $[M+54]^+$ adducts (Moneti et al., 1997; Oldham & Svatos, 1999) were conducted with the resonant

waveform type for precursor ion excitation (m/z 526), an isolation window of m/z 3, and 20 ms excitation time. The automatic 'q' calculator was used for the determination of precursor ion excitation energy and product ion mass range. The ion trap operated in internal-ionization mode and was kept at 220 °C. Data were analysed using the MS Workstation software (version 6.9.3, Varian Inc., Palo Alto, CA, U.S.A.).

Figure A1. The behavioural response of *O. cincta* females to different odour sources was tested in a Y-shaped olfactometer. This was made of a thick, transparent, Plexiglas, enclosed cylinder (15 cm diameter) with a Y-shaped tunnel (2.5 cm high; 6.5 cm stem; 6 cm each branch; stem-arms angle 120°). Individual females were released into the stem of the olfactometer through a hole in the cylinder.

Figure A2. Gas chromatogram (GC-FID) of crude pheromone spermatophore extract (blue line) and control (red line). (Z)-13-docosenol and (Z)-14-tricosenol in the extract are indicated with Peak 1 at RT 21.03 min and Peak 2 at RT 23.07 min.

Figure A3. EI spectra of Peak 1 and Peak 2 found in the crude pheromone spermatophore extract. (a) Spectra of Peak 1 refers to the EI spectra of (Z)-13-docosenol. (b) Spectra of Peak 2 refers to the EI spectra of (Z)-14-tricosenol.

Figure A4. GC-MS chromatogram of crude pheromone spermatophore extract (black line) and control (grey line). Two additional peaks found in the extract indicated with Peak 3 at RT 19.16 min and Peak 4 at RT 19.28 min could not be identified because of their low concentration levels.