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SHORT COMMUNICATION

Analysing small insect glands with UV-LDI MS: high-resolution spatial analysis reveals the chemical composition and use of the osmeterium secretion in *Themira superba* (Sepsidae: Diptera)

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Abstract

For many insect species, pheromones are important communication tools, but chemical analysis and experimental study can be technically challenging because they require the detection and handling of complex chemicals in small quantities. One drawback of traditional mass spectrometry methods such as gas chromatography mass spectrometry is that whole-body extractions from one to several hundred individuals are required, with the consequence that intra- and interindividual differences cannot be detected. Here, we used the recently introduced UV-LDI MS (ultraviolet laser desorption/ionization mass spectrometry) to profile the 'osmeterium' of the sepsid fly *Themira superba* that is located on the edge of the hind tibia of males. Based on analyses of individual legs, we established that the gland produced a secretion that consisted of oxygenated hydrocarbons and putative isoprenoids. The secretion was first detected 24 h after eclosion, and its transfer to the wings of females during mating was demonstrated using UV-LDI MS. We then tested whether the secretion had an anti-aphrodisiac function, but experimental transfer of the secretion to virgin females did not affect mating success or copulation duration. Throughout the study, UV-LDI MS proved invaluable, because it allowed tracking the natural and experimental transfer of small quantities of pheromones to specific body parts of small flies.

Introduction

In many species of insects, cuticular lipids serve as pheromones and influence complex social behaviours such as mate choice and species recognition (Howard & Blomquist, 2005). The study of these pheromones requires precise identification of the targeted chemical compounds, their production, storage sites and fate upon usage (Eberhard, 2001a; Schlick-Steiner *et al.*, 2006; Oliveira *et al.*, 2011; Ramirez *et al.*, 2011; van Wilgenburg *et al.*, 2011; Yew *et al.*, 2011a). Gas chromatography–mass spectrometry (GC-MS) is the most commonly used method for these tasks. Recently, an alternative analytical technique, Direct Analysis in Real Time (DART) MS, was introduced (Cody *et al.*, 2005; Yew *et al.*, 2008), but like GC-MS, DART MS has lower sensitivity for certain classes of epi-cuticular chemicals (e.g. higher molecular weight molecules) and the origin and spatial distribution of the compounds on the body is either lost or poorly resolved due to sample preparation and sampling methods. As a consequence, compounds that play important roles in intra/interspecific

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interactions may be overlooked (Yew *et al.*, 2009, 2011a; Bruschini *et al.*, 2011; Girotti *et al.*, 2012; Kuhbandner *et al.*, 2012).

Ultraviolet laser desorption/ionization (UV-LDI) MS was recently introduced for fine-scale chemical profiling of insect cuticles (Yew *et al.*, 2009, 2011b). It provides improved spatial resolution and higher detection sensitivity by using a 200-μm-beam UV laser to desorb and ionize compounds directly from the cuticles of intact structures. No chemical extraction is required, and single insects or their body parts can be analysed with minimal sample preparation. In addition, UV-LDI MS analysis extends the accessible mass range of GC-MS and DART MS and provides simultaneous detection of long-chain and more polar lipids including hydrocarbons, fatty acids and triglycerides. We here use UV-LDI MS for studying the secretion of a small sepsid gland ('osmeterium') that is found on the male hind tibia of many species (Pont & Meier, 2002; Ang *et al.*, 2013).

Sepsids are models for sexual selection research (e.g. Blanckenhorn *et al.*, 2000, 2002, 2004; Eberhard, 2003, 2005; Ang *et al.*, 2008; Puniamoorthy *et al.*, 2010, 2012a,b; Bowsher *et al.*, 2013), and in many species, exchange of chemicals is thought to take place during copulation (Sŭlc, 1929; Eberhard, 2001a). Male sepsids use their mid legs to rub them against their hind tibiae before stroking the female body and/or wings with the mid legs (Puniamoorthy *et al.*, 2009). It is known that males of some sepsid species have a gland, the osmeterium, on their hind tibia that has been hypothesized to produce a secretion that is transferred to the females via the leg-rubbing behaviour (Sŭlc, 1929; Eberhard, 2001a,b, 2002; Puniamoorthy *et al.*, 2009). Evidence for a transfer of chemicals is based on male behaviour, but the secretion can also be seen on scanning electron microscopy (SEM) images that reveal fluid residues on those wing areas that had been touched by male legs during mating (Eberhard, 2001a). Not all species of Sepsidae have an obvious, externally visible osmeterium (Pont & Meier, 2002; Su *et al.*, 2008; Ang *et al.*, 2013; Zhao *et al.*, 2013), and one of our ongoing studies with UV-LDI MS is to establish which species are capable of producing a secretion.

Eberhard (2001a) proposed that the secretion could be produced by the cuticle of the abdomen and then be collected in the osmeterium by rubbing it against the abdomen. Alternatively, Sŭlc (1929) suggested that the secretion could be produced and stored in the osmeterium. Currently, we cannot discriminate between these hypotheses. Here, we use UV-LDI MS to first test whether a specific secretion is produced by the osmeterium. We then determine whether the secretion is transferred during mating to the areas of the female body that are touched by male mid legs. Lastly, we test whether the secretion has an anti-aphrodisiac function; that is, whether the experimental transfer of the secretion to virgin females reduces mating success.

Materials and methods

Themira superba individuals from laboratory cultures were used in this analysis. This species has a well-developed osmeterium (Fig. 1), is easy to breed and has a short development time (9 days from egg to adult).

Sample preparation of males

Newly emergent virgin males were isolated to avoid any sexual interaction with females. Ten individuals from each of the following age groups were analysed: 0–24, 24–48, 48–72 and 72–96 h. Individuals were anesthetized with CO_2, and the structures of interest were excised and fixed to a glass cover slip with double-sided tape. The following structures were analysed (Fig. 1): (i) the hind tibia, which contains the osmeterium; (ii) the left hind tarsus, (iii) the left mid tibia, (iv) the abdominal surface, including the lateral and ventral area and the surface of the sternite brushes, (v) the surface of the surstyli. Up to 10 different samples were loaded simultaneously (Fig. S1), and different parts of each structure were individually analysed (Fig. 1).

Behavioural analysis

Mating trials were performed by placing a virgin couple in a small Petri dish and recording their interactions for 40 min or until mating ended. All mating trials were recorded with a Canon LEGRIA HF S30 digital camcorder. Virgin females aged 4 days were mated with virgin males of the same age ($n = 10$). Immediately after mating, the females were anesthetized with CO_2 and dissected. Ten virgin females of the same age class were used as control. The following structures were dissected and attached to a glass cover slip (Fig. 1): (i) base, middle and tip of the wings; (ii) the left mid leg and (iii) the left hind leg. The femur, tibia and tarsus were individually scanned for chemical secretions from each of the legs. To prevent potential contamination, tools were cleaned with alcohol prior to use.

Testing for an anti-aphrodisiac function: experimental transfer of secretion

To transfer the secretion from the osmeterium of a male to the wings of virgin females, we removed the hind tibiae of virgin males and used an unused fine-tipped brush to topically apply the secretion onto the wings of anesthetized virgin females ('brush+'). This method was chosen because initial trials to extract the secretion using hexane were unsuccessful. We manually transferred the secretion from 10 legs (four brushstrokes each) to each female wing (again four brushstrokes) to ensure sufficient signal. Of the perfumed females, three were randomly selected and

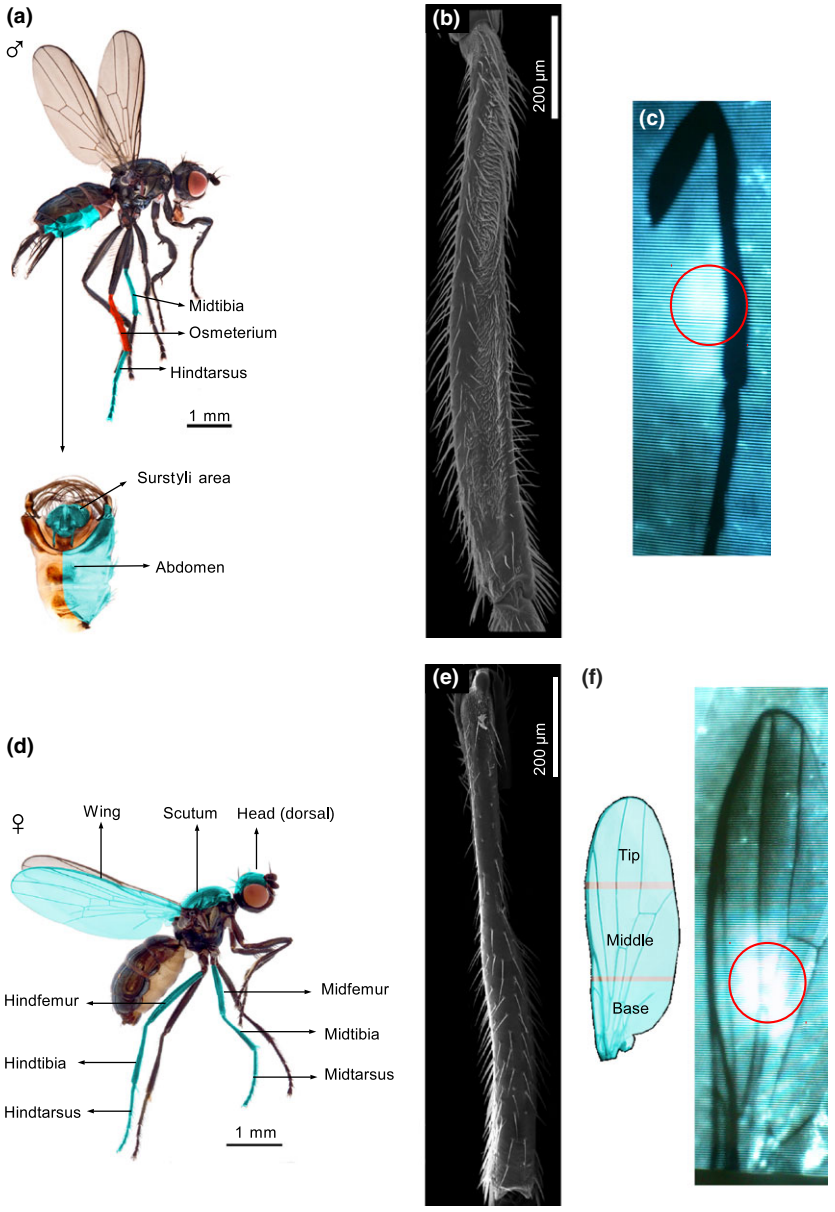


Fig. 1 UV-LDI MS analysis provides spatially resolved profiling of *Themira superba* cuticles. (a) Male structures analysed: in blue, the lateral and ventral surface of the abdomen including the sternite brush, surstyli area, mid tibia, and hind tarsus and, in red, hind tibia with the osmeterium. (b) SEM image of the hind tibia with osmeterium. (c) Dissected leg during UV-LDI MS analysis. The site of laser irradiation is circled in red. (d) Female structures analysed; blue = wing, scutum, head and different regions of the leg. (e) SEM image of the female hindleg shows absence of osmeterium. (f) The three different regions of the wing were analysed by UV-LDI MS; the right panel shows laser beam irradiation of wing.

analysed with UV-LDI MS to confirm the successful transfer of secretion. UV-LDI MS spectra of brush+ females were similar to those of naturally mated females and that similar amounts were found on all perfumed females (Fig. 2d). The remaining perfumed virgin females were given at least 40 min to recover before being paired with virgin males of the same age in mating trials. As a control, a group of females was anesthetized and brushed without the secretion ('brush-') before being tested in mating trials. To determine whether there was an overall effect from brushing and anaesthesia, mating trials were also conducted for virgin pairs that were not brushed or anesthetized ('no brush'). We conducted 33, 32 and 31 mating trials for the brush+, brush- and no brush groups, respectively. Each female and male was used only once in a mating trial. All mating trials were reviewed frame by frame to determine mating success and copulation duration.

To determine whether grouping and body size ratio (female over male) influenced mating success, we used a general linear model with binomial errors (GLM) in R (R Core Team, 2013). The contrasts were set to compare brushed and nonbrushed flies, as well as between brush+ and brush- flies. To test the influence of the same variables on mating duration, an analysis of covariance (ANCOVA) was also run in R.

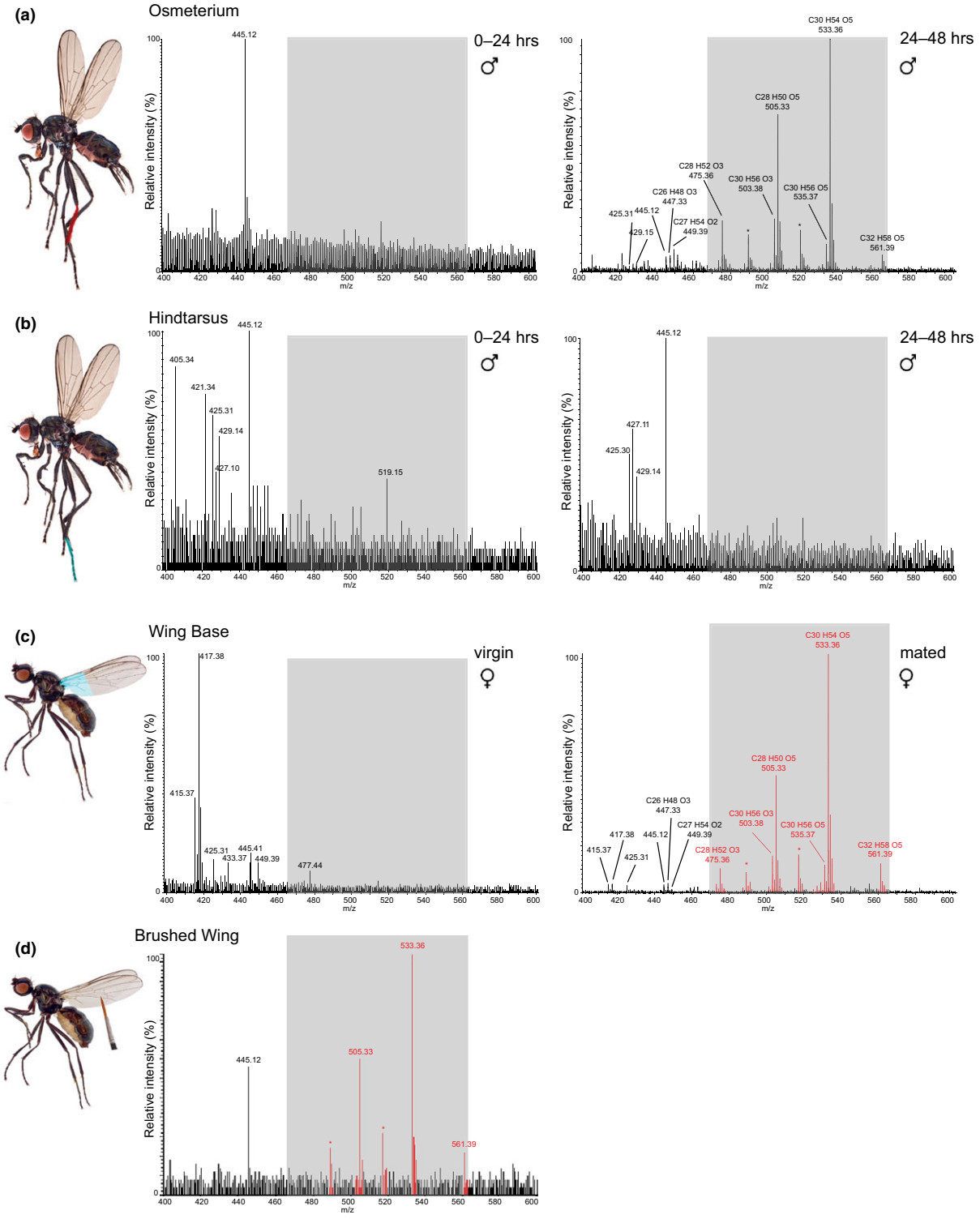


Fig. 2 Representative mass spectrum acquired from *Themira superba* using UV-LDI MS. Anatomical regions that were interrogated are highlighted in colour. (a) Mass spectra recorded from the osmeterium and (b) hindtarsus of newly enclosed males (no secretion) and 24 h post-eclosion (with secretion); (c) Analysis of virgin and mated females shows the presence of ion signals corresponding to the male secretion only on mated females. (d) Ion signals from a female wing with experimentally transferred male secretion (in red). Ion signals corresponding to chitin (m/z 445.12) and other cuticular lipids are found in both males and females. Labelled signals correspond to potassiated molecules $[M+K]^+$. Signals with * indicate sodiated molecules $[M+Na]^+$.

UV-LDI MS analysis and instrumentation

The cover slip containing the dissected structures was attached with adhesive tape onto a customized target plate for the mass spectrometer (Fig. S1). Details of the mass spectrometer ion source and experimental parameters were described previously (Yew *et al.*, 2011b). UV-LDI MS analysis was performed using a QStar Elite (ABSciex) orthogonal time-of-flight equipped with an intermediate pressure oMALDI2 source and a N_2 laser (λ = 337 nm, 40 Hz repetition rate, 200 μm beam diameter). Ions were generated in a buffer gas environment using 2–4 mbar of N_2. Mass spectra were analysed using MS Analyst software (Analyst QS 2.0, ABSciex). Mass accuracy for the mass spectrometer was approximately 20 ppm.

Results and discussion

Analysis by UV-LDI MS of dissected *T. superba* legs confirmed the presence of several lipid compounds that are exclusively found in the osmeterium. UV-LDI MS also provided preliminary identification of the elemental composition. Furthermore, we found evidence for an age-dependent expression and mating-related transfer of the osmeterium-specific chemicals.

Analysis of the hind leg osmeterium revealed a distinct chemical profile that was not observed in other cuticular structures. A series of high-intensity signals corresponding to several oxygen-containing hydrocarbons and two major compounds with mass-to-charge ratio (m/z) of 505.33 ($[M+K]^+$) and 533.35 ($[M+K]^+$) were detected in every osmeterium (n = 10; Fig. 2 and Table 1). Based on exact mass measurement, the elemental compositions of the two major compounds are predicted to be $C_{28}H_{50}O_5$ and $C_{30}H_{54}O_5$, respectively. Based on the degree of unsaturation (4 double bond equivalents each) and the number of oxygen atoms, it is likely that these compounds are isoprenoids. In interpreting these results, it is important to consider the technical limitations of UV-LDI MS. It cannot distinguish branched and linear hydrocarbons, and saturated hydrocarbons can only be detected with the aid of a chemical matrix (Dreisewerd, 2014), which is also required in conventional matrix-assisted LDI (MALDI) MS for the detection of peptides, proteins, sugars and lipids. Our list of compounds is thus a preliminary list, and additional components may be present. The production of the identified compounds in the osmeterium was only detected in males older than 24 h (Fig. 2). No trace of a secretion could be detected in younger males (n = 10).

Transfer of secretion

To determine whether the osmeterium secretions may play a role in courtship behaviour, we first analysed video recordings of sepsid mating behaviour (Movie S1). Consistent with previous observations (Puniamoorthy *et al.*, 2009), males used their mid legs to rub their hind tibiae before stroking the wings of the female with their mid legs. UV-LDI MS analysis of recently mated females revealed that males transferred the osmeterium secretion to females during mating. The chemicals of the male osmeterium were detected on all mated females, but only on those areas that are touched by the male mid legs according to our video evidence (Fig. 2; Movie S1), that is, the base and middle area of the wing (n = 10). The chemicals were not detected on any of the virgin females (n = 10).

Obtaining these data was fast because several samples could be loaded simultaneously and measurements required only 30–60 s per sample. In addition, the ability to scan structures surrounding the targeted body parts allowed for the detection of lipids and sugars on the rest of the insect cuticle, thus generating structure-specific chemical characters for comparative analyses

Table 1 Main chemical signals found in *Themira superba*. For whole-body compounds, we list the ones with highest intensity.

m/z detected*	Predicted elemental composition	Structure of origin
445.12	$C_{16}H_{26}N_2O_{10}$ (chitin dimer)	Whole body (male and female)
447.33	$C_{26}H_{48}O_3$	
449.38	$C_{27}H_{54}O_2$	
475.36	$C_{28}H_{52}O_3$	Osmeterium secretion
503.38	$C_{30}H_{56}O_3$	
505.33	$C_{28}H_{50}O_5$	
533.36	$C_{30}H_{54}O_5$	
535.38	$C_{30}H_{56}O_5$	
561.39	$C_{32}H_{58}O_5$	

*Corresponds to the potassiated ion $[M+K]^+$.

Table 2 Results of generalized linear model for perfuming experiment.

Contrasts	a ± SE	z-Value	P*
Intercept	−0.60006 ± 0.22443	−2.674	**0.0075**
Brush vs. No brush	−0.39711 ± 0.15312	−2.593	**0.0095**
Brush+ vs. Brush−	0.05889 ± 0.28419	0.207	0.8358

a, estimated coefficient; SE, standard error.
*Significant P-values are in bold.

Table 3 Number of trials performed and mated with their average copulation time and standard deviation recorded.

Group	No. of trials	No. of trials mated	Copulation time (mean ± SD)
Brush+	33	8	13 min ± 47 s
Brush−	32	8	13 min ± 20 s
No brush	31	17	14 min ± 24 s

(Fig. S2). In contrast to chemical extractions of whole insects where the tissues are usually destroyed, samples that were used for UV-LDI MS could be recovered from the adhesive tape for additional morphological analysis and vouchering (Fig. S1).

Does the secretion have an anti-aphrodisiac function?

When testing for an anti-aphrodisiac function of the secretion, we found that brushing lowered mating success (Table 2, GLM, contrasts brush vs. no brush; d.f. = 91 and $P = 0.0095$). Despite there being an effect of brushing, the mating success rates of the brush+ and brush- groups were not significantly different from each other (Table 2, GLM, contrasts brush+ vs. brush-; d.f. = 91 and $P = 0.8358$). We also found no significant differences in copulation time between all three groups (Table 3, ANCOVA, $F_{2,31} = 1.627$, $P = 0.2128$). The body size ratio also did not significantly affect either mating success or duration (mating success, GLM, d.f. = 90, $P = 0.3557$; mating duration: ANCOVA, estimated coefficient = 84.56, SE = 293.01, $P = 0.7749$). Thus, these findings indicate that the osmeterium secretion did not significantly affect both mating success and duration and is unlikely to be an anti-aphrodisiac.

Given that the secretion is sexually dimorphic and transferred by males during mating, one could suspect that the secretion is a nuptial gift (Gwynne, 2008). However, we have studied the mating behaviour of many sepsid species (Puniamoorthy *et al.*, 2009; Tan *et al.*, 2010, 2011) and never observed cleaning/feeding behaviour of females after copulation although the wing membrane with the secretion is easily accessible to the females' legs, which could be used to transfer the secretion to the flies' proboscis. Some known, alternative functions of isoprenoid pheromones in insects include sexual attraction or aggregation (e.g. social insects: Hick *et al.*, 1999), but none of these functions is likely in Sepsidae because perfumed females were not more attractive to either males or other flies. The evolutionary significance of the males' secretion thus remains a mystery.

Conclusion

Here, we use UV-LDI MS to shed light on the chemistry and function of the osmeterium of male sepsids, which for several decades had been suspected to produce a secretion that is transferred during mating. A combination of behavioural analysis with high-resolution video and chemical analysis confirms the presence of a gland-specific secretion. We can also confirm its transfer from male to female, but the ultimate function of the secretion remains unclear. Given the small size of the structures, the use of UV-LDI MS was critical because it provided the necessary micrometre-scale resolution. This property makes UV-LDI MS an attractive new tool despite some drawbacks related to quantification and ability to detect certain classes of molecules.

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References

Ang, Y., Puniamoorthy, N. & Meier, R. 2008. Secondarily reduced foreleg armature in *Perochaeta dikowi* sp.n. (Diptera: Cyclorrhapha: Sepsidae) due to a novel mounting technique. *Syst. Entomol.* **33**: 552–559.

Ang, Y., Puniamoorthy, J., Pont, A.C., Bartak, M., Blanckenhorn, W.U., Eberhard, W.G. *et al.* 2013. A plea for digital reference collections and other science-based digitization initiatives in taxonomy: Sepsidnet as exemplar. *Syst. Entomol.* **38**: 637–644.

Blanckenhorn, W.U., Muhlhauser, C., Morf, C., Reushc, T. & Reuter, M. 2000. Female choice, female reluctance to mate and sexual selection on body size in the dung fly *Sepsis cynipsea*. *Ethology* **106**: 577–593.

Blanckenhorn, W.U., Hosken, D.J., Martin, O.Y., Reim, C., Teuschl, Y. & Ward, P.I. 2002. The costs of copulating in the dung fly *Sepsis cynipsea*. *Behav. Ecol.* **13**: 353–358.

Blanckenhorn, W.U., Kraushaar, U.R.S., Teuschl, Y. & Reim, R. 2004. Sexual selection on morphological and physiological traits and fluctuating asymmetry in the black scavenger fly *Sepsis cynipsea*. *J. Evol. Biol.* **17**: 629–641.

Bowsher, J.H., Ang, Y.C., Ferderer, T. & Meier, R. 2013. Deciphering the evolutionary history and developmental mechanisms of a complex sexual ornament: the abdominal appendages of Sepsidae (Diptera). *Evolution* **67**: 1069–1080.

Bruschini, C., Cervo, R., Cini, A., Pieraccini, G., Pontieri, L., Signorotti, L. *et al.* 2011. Cuticular hydrocarbons rather than peptides are responsible for nestmate recognition in *Polistes dominulus*. *Chem. Senses* **36**: 715–723.

Cody, R.B., Laramée, J.A. & Durst, H.D. 2005. Versatile new ion source for the analysis of materials in open air under ambient conditions. *Anal. Chem.* **77**: 2297–2302.

Dreisewerd, K. 2014. Recent methodological advances in MALDI mass spectrometry. *Anal. Bioanal. Chem.* **406**: 2261–2278.

Eberhard, W.G. 2001a. Courtship and multi-stage transfer of material to the female's wings during copulation in *Microsepsis armillata* (Diptera: Sepsidae). *J. Kans. Entomol. Soc.* **74**: 70–78.

Eberhard, W.G. 2001b. Species-specific genitalic copulatory courtship in sepsid flies (Diptera, Sepsidae, *Microsepsis*) and theories of genitalic evolution. *Evolution* **55**: 93–102.

Eberhard, W.G. 2002. The relation between aggressive and sexual behavior and allometry in *Palaeosepsis dentatiformis* flies (Diptera: Sepsidae). *J. Kans. Entomol. Soc.* **75**: 317–332.

Eberhard, W.G. 2003. Sexual behavior and morphology of *Themira minor* (Diptera: Sepsidae) males and the evolution of male sternal lobes and genitalic surstyli. *Can. Entomol.* **135**: 569–581.

Eberhard, W.G. 2005. Sexual morphology of male *Sepsis cynipsea* (Diptera: Sepsidae): lack of support for lock-and-key and sexually antagonistic morphological coevolution hypotheses. *Can. Entomol.* **137**: 551–565.

Girotti, J.R., Mijailovsky, S.J. & Juarez, P. 2012. Epicuticular hydrocarbons of the sugarcane borer *Diatraea saccharalis* (Lepidoptera: Crambidae). *Physiol. Entomol.* **37**: 266–277.

Gwynne, D.T. 2008. Sexual conflict over nuptial gifts in insects. *Annu. Rev. Entomol.* **53**: 83–101.

Hick, A.J., Luszniak, M.C. & Pickett, J.A. 1999. Volatile isoprenoids that control insect behaviour and development. *Nat. Prod. Rep.* **116**: 39–54.

Howard, R.W. & Blomquist, G.J. 2005. Ecological, behavioural and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* **50**: 371–393.

Kuhbandner, S., Sperling, S., Mori, K. & Ruther, J. 2012. Deciphering the signature of cuticular lipids with contact sex pheromone function in a parasitic wasp. *J. Exp. Biol.* **215**: 2471–2478.

Oliveira, C.C., Manfrin, M.H., Sene, F.M., Jackson, L.J. & Etges, W.J. 2011. Variations on a theme: diversification of cuticular hydrocarbons in a clade of cactophilic *Drosophila*. *BMC Evol. Biol.* **11**: 179.

Pont, A.C. & Meier, R. 2002. The Sepsidae (Diptera) of Europe. *Fauna Entomol. Scand.* **37**: 1–221.

Puniamoorthy, N., Ismail, M.R.B., Tan, D.S.H. & Meier, R. 2009. From kissing to belly stridulation: comparative analysis reveals surprising diversity, rapid evolution and much homoplasy in the mating behaviour of 27 species of sepsid flies (Diptera: Sepsidae). *J. Evol. Biol.* **22**: 2146–2156.

Puniamoorthy, N., Kotrba, M. & Meier, R. 2010. Unlocking the "black box": internal female genitalia in Sepsidae (Diptera) evolve fast and are species-specific. *BMC Evol. Biol.* **10**: 275.

Puniamoorthy, N., Schafer, M.A. & Blanckenhorn, W.U. 2012a. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum*. *Evolution* **66**: 2117–2126.

Puniamoorthy, N., Blanckenhorn, W.U. & Schafer, M.A. 2012b. Differential investment in pre- versus post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphis in *Sepsis punctum* (Diptera: Sepsidae). *J. Evol. Biol.* **25**: 2253–2263.

R Core Team 2013. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL: http://www.R-project.org/.

Ramirez, S.R., Eltz, T., Fujiwara, M.K., Gerlach, G., Goldman-Huertas, B., Tsutsui, N.D. *et al.* 2011. Asynchronous diversification in a specialized plant-pollinator mutualism. *Science* **333**: 1742–1746.

Schlick-Steiner, B.C., Steiner, F.M., Moder, K., Seifert, B., Sanetra, M., Dyreson, E. *et al.* 2006. A multidisciplinary approach reveals cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). *Mol. Phylogenet. Evol.* **40**: 259–273.

Su, K.F.Y., Kutty, S.N. & Meier, R. 2008. Morphology versus Molecules: The phylogenetic relationships of Sepsidae (Diptera: Cyclorrhapha) based on morphology and DNA sequence data from ten genes. *Cladistics* **24**: 902–916.

Sůlc, K. 1929. Dosud neznámá osmeteria u Sepsid (Diptera). *Biol. Listy* **14**: 428–439.

Tan, D.S.H., Ang, Y., Lim, G.S., Ismail, M.R. & Meier, R. 2010. From 'cryptic species' to integrative taxonomy: an iterative process involving DNA sequences, morphology, and behaviour leads to the resurrection of *Sepsis pyrrhosoma* (Sepsidae: Diptera). *Zool. Scr.* **39**: 51–61.

Tan, D.S.H., Ng, S.R. & Meier, R. 2011. New information on the evolution of mating behaviour in Sepsidae (Diptera) and the cost of male copulations in *Saltella sphondylii*. *Org. Divers. Evol.* **11**: 253–261.

van Wilgenburg, E., Symonds, M.R.E. & Elgar, M.A. 2011. Evolution of cuticular hydrocarbon diversity in ants. *J. Evol. Biol.* **24**: 1188–1198.

Yew, J.Y., Cody, R.B. & Kravitz, E.A. 2008. Cuticular hydrocarbon analysis of an awake behaving fly using direct analysis in real-time time-of-flight mass spectrometry. *Proc. Natl. Acad. Sci. USA* **105**: 7135–7140.

Yew, J.Y., Dreisewerd, K., Luftmann, H., Müthing, J., Pohlentz, G. & Kravitz, E.A. 2009. A new male sex-pheromone and novel cuticular cues for chemical communication in *Drosophila*. *Curr. Biol.* **19**: 1245–1254.

Yew, J.Y., Dreisewerd, K., de Oliveira, C.C. & Etges, W.J. 2011a. Male-specific transfer and fine scale spatial differences of newly identified cuticular hydrocarbons and triacylglycerides in a *Drosophila* species pair. *PLoS ONE* **6**: e16898.

Yew, J.Y., Soltwisch, J., Pirkl, A. & Dreisewerd, K. 2011b. Direct laser desorption ionization of endogenous and exogenous compounds from insect cuticles: practical and methodologic aspects. *J. Am. Soc. Mass Spectrom.* **22**: 1273–1284.

Zhao, L., Ang, A.S.H., Srivathsan, A., Su, K.S.F.Y. & Meier, R. 2013. Does better taxon sampling help? A new phylogenetic hypothesis for Sepsidae (Diptera: Cyclorrhapha) based on 50 new taxa and the same old mitochondrial and nuclear markers. *Mol. Phylogenet. Evol.* **69**: 153–164.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Preparation and samples (top) and preservation of samples for future morphological work and vouchering (bottom).

Figure S2 Representative mass spectra from a male osmeterium and a female wing, shown with m/z up to 1000.

Movie S1 Video recording of *T. superba* leg rubbing with secretion transfer.

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