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RESEARCH ARTICLE

Deciphering the signature of cuticular lipids with contact sex pheromone function in a parasitic wasp

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SUMMARY

The surface of insects is covered by a complex mixture of cuticular hydrocarbons (CHCs) to prevent desiccation. In many species these lipids also have communicative functions, but often it is unknown which components are crucial for the behavioural response. Furthermore, it is often ignored that polar lipids also occur on the insects' cuticle and might interact with CHCs. In the parasitic wasp *Lariophagus distinguendus*, CHCs function as a contact sex pheromone eliciting wing-fanning in males. Interestingly, not only females but also newly emerged males have the pheromone, resulting regularly in homosexual courtship. However, males deactivate the pheromone within the first two days after emergence. This deactivation is accompanied by the disappearance of 3-methylheptacosane (3-MeC27) and some minor components from the CHC profile of males. Here we show that 3-MeC27 is a key component of the contact sex pheromone which, however, triggers courtship behaviour only if an olfactory background of other cuticular lipids is present. Males responded to (*S*)-3-MeC27 enantioselectively when applied to filter paper but on three-dimensional dummies both enantiomers were behaviourally active, suggesting that physical stimuli also play a role in sexual communication of the wasps. Finally, we report that triacylglycerides (TAGs) are also essential components of the pheromone, and present evidence that TAGs actually occur on the cuticle of *L. distinguendus*. Our data provide novel insights into the semiochemical function of cuticular lipids by showing that the bioactivity of CHCs may be influenced by the stereochemistry and a synergetic interaction with long time ignored TAGs.

Key words: cuticular hydrocarbons, *Lariophagus distinguendus*, 3-methylheptacosane, contact sex pheromone, triacylglycerides.

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INTRODUCTION

The epicuticle of insects is protected against desiccation and pathogen attack by a hydrophobic layer of lipids (Gibbs and Rajpurohit, 2010). This layer is typically composed of a complex mixture of straight-chain and methyl-branched alkanes and alkenes (commonly referred to as cuticular hydrocarbons, CHCs) (Howard and Blomquist, 2005; Blomquist and Bagneres, 2010) as well as a number of more polar compound classes like wax esters, long-chain fatty alcohols and aldehydes (Buckner, 2010). Apart from their protective function, cuticular lipids are also involved in the communication of many insect species. The non-polar CHCs have been extensively investigated over the past three decades with respect to their role as semiochemicals (Howard, 1993; Singer, 1998; Howard and Blomquist, 2005). A multitude of studies have shown that CHCs are not only involved in the diverse interactions of social insects (Greene and Gordon, 2003; Endler et al., 2004; van Zweden and d'Ettorre, 2010) but also used by many solitary insects as contact stimuli when searching for oviposition sites (Colazza et al., 2007; Rostas and Wolfling, 2009; Darrouzet et al., 2010) or sexual mates (Syvertsen et al., 1995; Sullivan, 2002; Steiner et al., 2005; Steiner et al., 2006; Sugeno et al., 2006; Geiselhardt et al., 2009; Ferveur and Cobb, 2010; Ginzel, 2010; Ruther et al., 2011).

Many studies dealing with the function of CHCs as semiochemicals share a general problem in that they are merely correlative, i.e. they conclude behavioural activity of CHCs from their apparently exclusive presence in bioactive insect extracts (Millar, 2010). It is often neglected, however, that those extracts may contain further compounds that might contribute to the behavioural activity of cuticular lipids without being detectable by standard GC-MS methods (Cvacka et al., 2006; Millar, 2010). Such a compound class is represented by triacylglycerides (TAGs). These mostly non-volatile lipids are ubiquitous in insects as the most important storage form of energy, and have been occasionally found in whole-body extracts from insects (Baker et al., 1979; Brey et al., 1985). However, it has been unclear whether they are actually present on the epicuticle or merely co-extracted from the fat body or other internal tissues (Buckner, 1993). Recent studies using ultraviolet laser desorption/ionization mass spectrometry demonstrated the presence of TAGs on the cuticle of *Drosophila* flies (Yew et al., 2011), but a communicative function has never been shown so far.

Even in studies that have demonstrated behavioural activity of purified CHC fractions, the importance of individual compounds or compound classes has often remained unclear (Sullivan, 2002; Steiner et al., 2006; Ruther et al., 2011). In particular, it is not always fully understood whether the CHC bouquet is perceived as a whole or whether single key components are sufficient alone or in combination to elicit a full behavioural response (Ginzel et al., 2003; Sugeno et al., 2006; Lacey et al., 2008; Silk et al., 2009). Recent studies on plant volatiles showed that key components of complex mixtures are often behaviourally active only in the presence of the natural odour background (Mumm and Hilker, 2005; Schroeder and

Hilker, 2008). However, this concept has never been considered in studies addressing the communicative function of cuticular lipids.

Identification of putative key components within CHC profiles is often hampered by the complexity of these profiles, the lack of synthetic reference compounds, and the fact that many CHCs are methyl-branched and thus chiral (Hefetz et al., 2010). Hitherto, enantiomers of long-chain methylalkanes cannot be separated with state-of-the-art analytical tools, and thus the enantiomeric composition of these natural products still remains an open question (Hefetz et al., 2010; Millar, 2010). Consequently, although methyl-branched CHCs have been identified as contact pheromones in some species (Sugeno et al., 2006; Ginzel et al., 2003; Lacey et al., 2008; Silk et al., 2009), it is unknown whether insects respond to these compounds stereoselectively (Hefetz et al., 2010), as is the case in many volatile insect pheromones (Mori, 2007).

The parasitic wasp *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae) parasitizes larvae and pupae of grain-infesting beetles. Prior to mating, males perform a stereotypic courtship behaviour starting with a high-frequency wing-fanning (Ruther et al., 2000). Surprisingly, this behaviour is not only triggered by cuticular lipids of virgin females but also of newly emerged males and pupae of either sex. It has been suggested that this might be a strategy of developing males within the grain kernels to distract their earlier emerged competitors away from females (Steiner et al., 2005). After emergence, however, female odour becomes detrimental for young males because of homosexual courtship displayed by other males (Ruther and Steiner, 2008). Therefore, males but not females deactivate the pheromone within the first 32 h after emergence and are no longer taken for females. This deactivation correlates with the disappearance of 3-methylheptacosane (3-MeC27) and some minor components from the CHC profile of males (Steiner et al., 2005). It is, however, unclear whether this modification of the male CHC profile is causally involved in the observed deactivation of the contact sex pheromone and whether compounds other than CHCs are also important for the pheromone function.

The present study aimed at investigating the putative key function of those CHCs for pheromonal activity correlating with bioactivity in *L. distinguendus* and the interaction of these compounds with the remaining CHCs and the TAG fraction, respectively. Furthermore, we asked whether the lost bioactivity of male cuticular lipids can be re-installed by adding synthetic 3-MeC27 and tested whether males respond to this compound enantioselectively. Finally, we analysed the epicuticle of the wasps for detecting the non-volatile TAGs within the cuticular lipids.

MATERIALS AND METHODS

Insects

Lariophagus distinguendus wasps were reared on pupae of the granary weevil *Sitophilus granarius* as described elsewhere (Ruther et al., 2000). Wasp cultures were kept in Petri dishes at 25°C on a 12 h:12 h light:dark photoperiod. Newly emerged males were isolated and kept under the same conditions for 2 days until they were used in the bioassay. Virgin females were deep-frozen shortly after emergence and stored at –23°C until they were used for extraction.

Pheromone extracts

Batches of 50 virgin females were extracted for 1 h with 300 µl of dichloromethane (DCM) each. The wasps were gently squeezed with a spatula during extraction. Afterwards, the supernatant was removed and the wasps were washed again with 100 µl DCM. The extracts were pooled and filtered through clean cotton wool. The solvent was evaporated under a gentle stream of nitrogen and re-dissolved in 20 µl of DCM for fractionation.

Fractionation by size exclusion high performance liquid chromatography

In order to evaluate the putative key compounds and the remaining lipids separately, we fractionated female extracts according to molecular size using a size exclusion high performance liquid chromatography technique (SE-HPLC) originally developed for sample preparation in environmental and pesticide analysis (Rimkus et al., 1996). For this purpose we used a 300×7.5 mm PLgel SE-HPLC column (particle size 5 µm, pore size 100 Å, Agilent Technologies Deutschland, Waldbronn, Germany) operated with HPLC grade dichlormethane (Fisher Scientific, Schwerte, Germany) as mobile phase. The eluent was pumped through the column using a LC-20AD HPLC pump (Shimadzu Europe, Duisburg, Germany) at 1.8–2.0 MPa resulting in a flow rate of 1.00 ml min^{-1}. Twenty microlitres of the raw pheromone extracts (representing 50 female equivalents) were injected into a Rheodyne model 7125 HPLC injector equipped with a 20 µl sample loop (Rheodyne, Cotati, CA, USA). Four fractions (F0–F3; retention times given in the Results) were collected manually using 4 ml glass vials and concentrated under a stream of nitrogen to 50 µl each, representing one female equivalent per microlitre. To ensure a constant quality of the fractions F2 and F3, we analysed them by GC-MS as described below. Cholesterol was used as a marker substance for the separation of fraction F3 (containing the putative pheromone candidates) and the remaining long-chain hydrocarbons, because in a previous study we found that all hydrocarbons correlating with bioactivity eluted before cholesterol on a non-polar stationary GC phase (Steiner et al., 2005). The elution of TAGs in fraction F0 was confirmed by the analysis of safflower oil TAGs using the same procedure.

Solid phase microextraction analysis of cuticular TAGs

To investigate whether the TAGs present in F0 also occur on the insects' cuticle we used a solid phase microextraction (SPME) technique, which we modified for the analysis of fatty acid methyl esters (FAME) after *in situ* transesterification with trimethylsulphonium hydroxide (TMSH) (Butte, 1983). For this purpose we soaked a polydimethylsiloxane-coated SPME fibre (PDMS, Supelco, Bellefonte, PA, USA) repeatedly with a TMSH solution (Sigma-Aldrich, Steinheim, Germany) and conditioned it at 280°C in the GC injector for 90 min until no more FAME were detected in the subsequent GC-MS run. Subsequently, we rubbed the conditioned SPME fibre for 5 min over the abdomen of five female wasps, soaked it with the TMSH solution again and desorbed it for 1 min in the injector of our GC-MS instrument. Resulting FAME were analysed by GC-MS using the instrumentation and methods given below. For control, we also analysed the surface lipids of females without the transesterification reactant. The absence of free fatty acids in these analyses confirmed that the FAME did not result from the esterification of free fatty acids.

GC-MS analysis

Aliquots (1 µl injected in splitless mode) of the extracts were analysed on a Shimadzu GCMS-QP2010 Plus quadrupole MS equipped with an AOC20i auto sampler (Shimadzu, Tokyo, Japan) and a 30 m × 0.32 mm I.D. BPX5 forte capillary column (film thickness 0.25 µm) (SGE Analytical Science Europe, Milton Keynes, UK). Helium was used as carrier gas at a constant flow of 1.73 ml min^{-1}. The GC effluent was ionized by electron impact

ionization (EI) at 70 eV; the mass range (*m*/*z*) ranged from 35 to 600. The oven programme for the hydrocarbon analysis (fractions F2 and F3) started at 150°C and was increased at 3°C min^{-1} up to 300°C (held for 20 min). The oven programme for the fatty acid analysis (fraction F0) started at 50°C (held for 4 min) and was increased at 3°C min^{-1} up to 280°C (held for 15 min). Before GC-MS analysis, TAGs of the fraction F0 were converted into FAME as described by Blaul and Ruther (Blaul and Ruther, 2011).

Relative linear retention indices (LRI) of methyl-branched and unsaturated hydrocarbons and FAME were estimated by co-injection of straight-chain hydrocarbons (van den Dool and Kratz, 1963). Identification of FAME was done by comparing LRI and MS data with those of synthetic reference chemicals. Methyl-branched hydrocarbons were identified by diagnostic ions resulting from the favoured fragmentation at the branching points (Nelson, 1993) and by comparing LRI values with literature data (Carlson et al., 1998; Steiner et al., 2005; Steiner et al., 2006; Steiner et al., 2007). Positions of the double bonds of unsaturated hydrocarbons were determined by iodine-catalysed methylthiolation using dimethyl disulphide (Howard, 1993).

General procedures for bioassays

Bioassays were performed in a round test arena (10 mm diameter × 3 mm height) (Ruther et al., 2000). Aliquots of fractions, mixtures of fractions representing five wasp equivalents, or synthetic 3-MeC27 enantiomers were applied onto pieces of filter paper (3×3 mm, Sartorius Stedim Biotech, Aubagne, France) and offered to males in the middle of the test arena after the solvent had evaporated. Behaviour of the wasps was observed under a stereo microscope at 16-fold magnification (Wild M 38, Heerbrugg, Switzerland). The duration of wing-fanning behaviour was recorded for 5 min using The Observer XT 9.0 scientific software (Noldus Information Technology, Wageningen, The Netherlands). Males that did not show wing-fanning when confronted with an extract were put into an arena together with a freshly killed female. If the males did not perform wing-fanning towards the female (positive control), it was excluded from further analysis. For each series of experiments, we additionally performed control assays with the same amount of solvent instead of fractions.

Experiment 1: Bioactivity of pheromone fractions

The fractions F0, F2 and F3 obtained by SE-HPLC were tested singly in the bioassay. For control we also tested the ternary mixture of all three fractions (positive control) and the pure solvent (negative control). F1 was not included because no chemicals were detected in this fraction (*N*=30).

Experiment 2: Subtractive approach

In this experiment we investigated the impact of the three lipid fractions F0, F2 and F3 by omitting each of them from the bioactive ternary mixture. Again, the ternary mixture and the pure solvent were used as controls (*N*=30).

Experiment 3: Bioactivity of synthetic 3-MeC27

3-MeC27 is the major compound of those CHCs correlating with bioactivity in *L. distinguendus*. Therefore, we tested whether one of the 3-MeC27 enantiomers alone elicited male wing-fanning. Synthetic reference samples of (*R*)- and (*S*)-3-MeC27 were synthesized by K.M. as described elsewhere (Marukawa et al., 2001). Doses of 750 ng representing approximately five female equivalents (Steiner et al., 2005) were applied to pieces of filter paper and tested in the bioassays as described above (*N*=20).

Experiment 4: Bioactivity of synthetic 3-MeC27 with a cuticular lipid background

This experiment was carried out to test if the observed reduction of bioactivity found after omission of fraction F3 (containing the putative key components) from the ternary mixture can be re-installed by the addition of synthetic 3-MeC27. This was done by adding 750 ng of synthetic (*R*)- or (*S*)-3-MeC27 to the binary mixture of fractions F0 and F2 (*N*=30). As controls we used the binary mixture of F0 and F2 without 3-MeC27 (negative control) and the ternary mixture (positive control).

Experiment 5: Bioactivity of 3-MeC27 on old males

This experiment was performed to test whether the pheromone activity of male wasps, which ceases with increasing age (Steiner et al., 2005), can be re-installed by adding 3-MeC27. For this purpose we tested first the response of males to cadavers of 4-day-old males to make sure that they did not elicit wing-fanning. Subsequently, we applied 150 ng of (*R*)- or (*S*)-3-MeC27 dissolved in dichloromethane to behaviourally inactive cadavers of 4-day-old males and tested them as described above. As positive and negative controls we used cadavers of newly emerged and 4-day-old males that had been treated with dichloromethane only (*N*=20).

Statistical analysis

The duration of wing-fanning behaviour shown by male wasps in each experiment towards differently treated paper disks and cadavers, respectively, was compared by a Kruskal–Wallis *H*-test followed by Mann–Whitney *U*-tests for individual comparisons. The proportion of males that showed wing-fanning towards the differently treated objects was analysed by Chi-square tests. All statistical analyses were performed with PAST 2.10 scientific software (Hammer et al., 2011).

RESULTS

Chemical analyses

All pheromone candidate hydrocarbons eluted in fraction F3 (retention time 7:15–8:00 min), and were thus fully separated from the TAGs (F0, retention time 6:15–6:35 min) and the hydrocarbons with higher molecular masses eluting in fraction F2 (retention times 6:48–7:15 min, Fig. 1A,B); the identities of all CHCs present in *L. distinguendus* have been published elsewhere (Steiner et al., 2005; Steiner et al., 2007). Fraction F1 (retention time 6:35–6:48 min) did not contain any detectable substances and was discarded. FAME analysis of the TAG fraction revealed the presence of common fatty acids with chain lengths of 16 and 18 carbon atoms. We got the same results when analysing trans-esterified cuticular extracts that were obtained by short-term (30 s) extraction of wasps using hexane or chloroform, respectively (results not shown). This suggested that the TAGs are present on the cuticle of the insects rather than being extracted from internal tissues. Our SPME analyses of cuticular lipids with subsequent *in situ* trans-esterification using TMSH confirmed this assumption because we found FAME profiles identical to those of F0 (Fig. 1A). In contrast, neither free fatty acids nor other fatty acid derivatives of sufficient volatility for direct GC analysis were detectable when performing SPME analyses without derivatization. We therefore concluded that non-volatile TAGs were present on the insect's cuticle.

Behavioural bioassays

All statistical data for the bioassays are given in Table 1. None of the SE-HPLC fractions was behaviourally active when tested alone, but almost 60% of the males showed wing-fanning towards a ternary

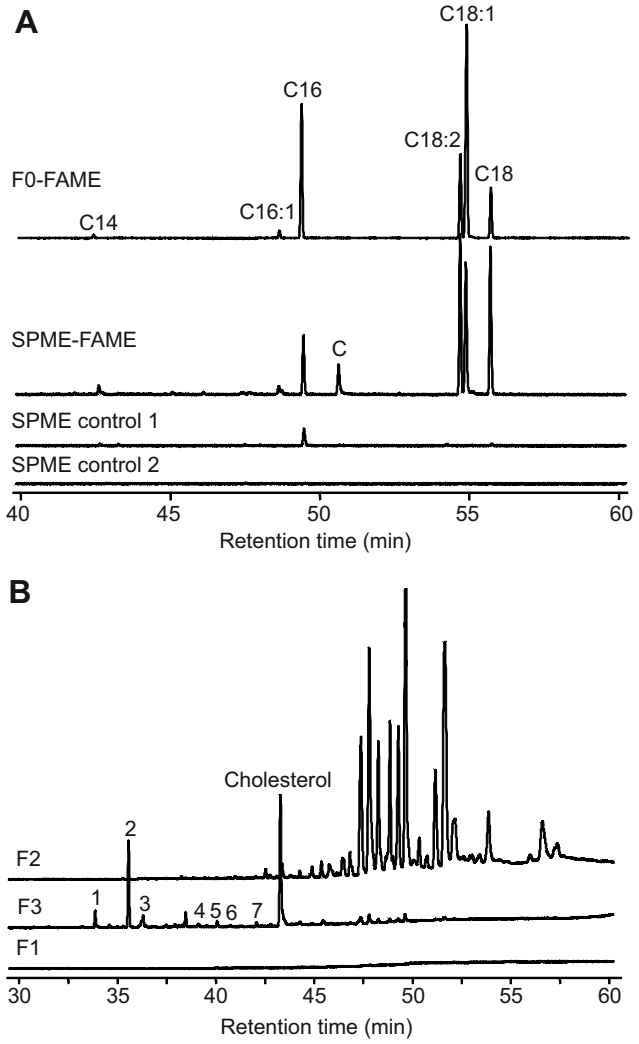

Fig. 1. (A) GC-MS analysis of fatty acid methyl esters (FAME) obtained by trans-esterification of TAGs in the lipid fraction F0 (F0-FAME) and by *in situ* trans-esterification of cuticular lipids sampled by solid phase microextraction (SPME-FAME). A conditioned SPME fibre was rubbed over the surface of female *Lariophagus distinguendus* wasps and desorbed in the GC injector after soaking the fibre in trimethylsulphonium hydroxide (TMSH). For control, the fibre was treated with TMSH without rubbing the insects' surface (SPME control 1), and the fibre was rubbed over the insects' surface without treating it with TMSH (SPME control 2). C16:1, methyl palmitoleate; C16, methyl palmitate; C18:2, methyl linoleate; C18:1, methyl oleate; C18, methyl stearate. (B) GC-MS analyses of lipid fractions F2 and F3 obtained after size exclusion HPLC of a whole-body extract from female wasps. Numbers indicate pheromone candidate hydrocarbons with relatively short-chain lengths eluting before the marker substance, cholesterol (1, C27; 2, 3-MeC27; 3, 3,7-DiMeC27; 4, 13- + 11- + 9-MeC29; 5, 3-MeC29; 6, 3,7-DiMeC29; 7, C31:1(9).

mixture of all three fractions (experiment 1, Fig. 2A,B). Omission of either fraction decreased male wing-fanning behaviour significantly when compared with the ternary mixture (experiment 2, Fig. 3A,B). Binary mixtures resulting from the omission of F0 and F3, respectively, were still more active than the solvent control, whereas omission of F2 completely stopped male wing-fanning. These results demonstrated that both hydrocarbon fractions (F2 and F3), as well as the TAG fraction F0, significantly contributed to the elicitation of wing-fanning in *L. distinguendus* males, and the combination of all three is necessary for a full behavioural response.

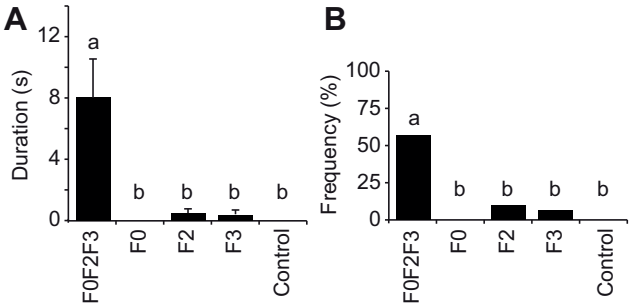

Fig. 2. Results of behavioural experiment 1. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with lipid fractions F0, F2, F3, a ternary mixture of all fractions (positive control) or the pure solvent (Control) during a 5-min observation period. Different lower case letters indicate significant differences at $P<0.001$ (wing-fanning duration analysed by Kruskal–Wallis *H*-test followed by Mann–Whitney *U*-tests, proportion of males analysed by Chi-square tests; $N=30$).

When applied alone to filter paper disks, neither the (*R*)- nor the (*S*)-enantiomer of 3-MeC27 elicited male wing-fanning (experiment 3, Fig. 4A,B). However, when offered in combination with a binary mixture of fractions F0 and F2 (simulating the deactivated cuticular lipid profiles of aged males), (*S*)-3-MeC27 significantly increased male wing-fanning behaviour, while the effect of the (*R*)-enantiomer was not statistically significant (experiment 4, Fig. 5A,B). In fact, synthetic (*S*)-3-MeC27 was able to fully re-install the lost bioactivity associated with the omission of F3 and, thus, could replace the whole fraction. Moreover, bioactivity of 4-day-old dead males was re-installed by applying synthetic 3-MeC27 to their cuticle (experiment 5, Fig. 6A,B). On these three dimensional models both enantiomers were behaviourally active. After this treatment, 90% [(*S*)-3MeC27] and 75% [(*R*)-3-MeC27] of the responding males showed wing-fanning towards 4-day-old male dummies and also wing-fanning duration was significantly increased when compared with the controls. Four-day-old male dummies treated with either enantiomer were as bioactive as newly emerged males.

DISCUSSION

The present study demonstrated that the disappearance of 3-MeC27 from the CHC profile is causally involved in the loss of sexual attractiveness of ageing males to consexuals. Because females retain high proportions of 3-MeC27 throughout their lifetime, this compound has to be considered as the key component of the *L. distinguendus* contact sex pheromone. Application of synthetic 3-MeC27 onto 4-day-old dead males resulted in a full restoration of pheromone activity, but 3-MeC27 did not elicit courtship behaviour in male wasps when offered alone. Rather, our data show that it has to be perceived in combination with the remaining cuticular lipids which, therefore, play an important role as a chemical background (Schroeder and Hilker, 2008). Likewise, Greene and Gordon (Greene and Gordon, 2007) found in a recent study on species and nest-mate recognition in ants that aggression towards alien CHCs was elicited only if a certain degree of CHC complexity was maintained, i.e. if members of different CHC classes (straight chain alkanes, methyl-branched alkanes, monoenes) were present simultaneously.

Although 3-MeC27 was able to fully re-install bioactivity, 3-MeC29 and the other minor components disappearing from the CHC profiles of aging males may contribute to bioactivity. This aspect

Table 1. Statistical analyses of wing-fanning duration (analysed by Kruskal–Wallis *H*-test and Mann–Whitney *U*-tests, upper right triangle) and proportion of responding *Lariophagus distinguendus* males (analysed by Chi-square tests, lower left triangle) in behavioural experiments 1–5

Experiment 1 (*N*=30; *H*=21.09)	F0F2F3	F0	F2	F3	Control
F0F2F3	–	*U*=195 *P*<0.001	*U*=226.5 *P*<0.001	*U*=220 *P*<0.001	*U*=195 *P*<0.001
F0	χ^2=23.72 *P*<0.001	–	*U*=405 *P*=0.082	*U*=420 *P*=0.16	*U*=0 *P*=1
F2	χ^2=14.7 *P*<0.001	χ^2=31.58 *P*=0.2	–	*U*=437 *P*=0.70	*U*=405 *P*=0.082
F3	χ^2=17.33 *P*<0.001	χ^2=2.07 *P*=0.36	χ^2=0.22 *P*=0.89	–	*U*=420 *P*=0.16
Control	χ^2=23.72 *P*<0.001	χ^2=§ *P*=§	χ^2=31.58 *P*=0.2	χ^2=2.07 *P*=0.35	–
Experiment 2 (*N*=30; *H*=19.76)	F0F2F3	F0F2	F0F3	F2F3	Control
F0F2F3	–	*U*=278.5 *P*=0.0043	*U*=202.5 *P*<0.001	*U*=265 *P*=0.0018	*U*=195 *P*<0.001
F0F2	χ^2=6.94 *P*=0.03	–	*U*=356.5 *P*=0.02	*U*=433 *P*=0.74	*U*=345 *P*<0.001
F0F3	χ^2=20.32 *P*<0.001	χ^2=5.19 *P*=0.075	–	*U*=374 *P*=0.045	*U*=435 *P*=0.33
F2F3	χ^2=8.53 *P*=0.014	χ^2=0.098 *P*=0.95	χ^2=4.04 *P*=0.13	–	*U*=360 *P*=0.01
Control	χ^2=23.72 *P*<0.001	χ^2=7.92 *P*=0.019	χ^2=1.017 *P*=0.60	χ^2=6.67 *P*=0.035	–
Experiment 3 (*N*=20; *H*=0.098)	(*S*)-3MeC27	(*R*)-3-MeC27	Control		
(*S*)-3MeC27	–	*U*=190 *P*=0.34	*U*=190 *P*=0.34		
(*R*)-3-MeC27	χ^2=1.03 *P*=0.60	–	*U*=0 *P*=1		
Control	χ^2=1.03 *P*=0.60	χ^2=§ *P*=§	–		
Experiment 4 (*N*=30; *H*=23.08)	F0F2F3	F0F2	F0F2 + (*S*)-3MeC27	F0F2 + (*R*)-3MeC27	
F0F2F3	–	*U*=298.5 *P*=0.014	*U*=278.5 *P*=0.01	*U*=398 *P*=0.42	
F0F2	χ^2=4.34 *P*=0.11	–	*U*=142.5 *P*<0.001	*U*=349 *P*=0.089	
F0F2 + (*S*)-3MeC27	χ^2=3.77 *P*=0.15	χ^2=15.15 *P*<0.001	–	*U*=227 *P*<0.001	
F0F2 + (*R*)-3MeC27	$\chi^2$0.60 *P*=0.74	χ^2=1.76 *P*=0.41	χ^2=7.18 *P*=0.03	–	
Experiment 5 (*N*=20; *H*=29.77)	Male 0D	Male 4D	Male 4D + (*S*)-3MeC27	Male 4D + (*R*)-3MeC27	
Male 0D	–	*U*=22.5 *P*<0.001	*U*=187 *P*=0.74	*U*=151.5 *P*=0.19	
Male 4D	χ^2=20.42 *P*<0.001	–	*U*=33 *P*<0.001	*U*=72.5 *P*<0.001	
Male 4D + (*S*)-3MeC27	χ^2=0.36 *P*=0.84	χ^2=17.29 *P*<0.001	–	*U*=151 *P*=0.19	
Male 4D + (*R*)-3MeC27	χ^2=3.14 *P*=0.21	χ^2=10 *P*<0.001	χ^2=1.56 *P*=0.46	–	

§No Chi-square (χ^2) possible, all values=0. 0D, 0-day-old; 4D, 4-day-old.

needs further investigation. The only cuticular contact pheromone components identified in a parasitic wasp so far are two long-chain alkadienes [(*Z*,*Z*)-7,13-heptacosadiene and (*Z*,*Z*)-7,15-hentriacontadiene] in the braconid *Cardiochiles nigriceps*. As in the present study, the compounds did not elicit complex courtship elements when tested alone, but were synergized by other, non sex-specific hydrocarbons (Syvertsen et al., 1995).

The present study shows that the response of an insect to non-volatile monomethylalkanes is enantioselective. When applied to filter paper, only (*S*)-3-MeC27 was behaviourally active, suggesting that (*S*)- might be the natural configuration. However, the natural stereochemistry of a molecule is not always reflected by a higher bioactivity. In the German cockroach, for instance, the natural (3*S*,11*S*)- stereoisomer of 3,11-dimethylnonacosan-2-one was less active than the non-natural ones (Eliyahu et al., 2004). Therefore, analytical tools separating the enantiomers of long-chain monomethylalkanes are necessary to finally establish the stereochemistry of 3-MeC27 in *L. distinguendus* (Hefetz et al., 2010;

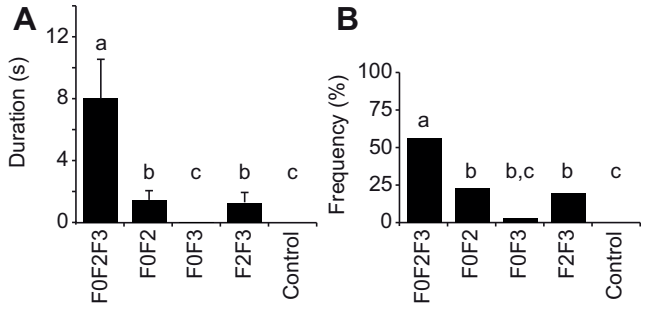

Fig. 3. Results of behavioural experiment 2. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with binary or ternary mixtures of lipid fractions F0, F2 and F3 or the pure solvent (Control) during a 5-min observation period. Different lower case letters indicate significant differences at *P*<0.05 (wing-fanning duration analysed by Kruskal–Wallis *H*-test followed by Mann–Whitney *U*-tests, proportion of males analysed by Chi-square tests; *N*=30).

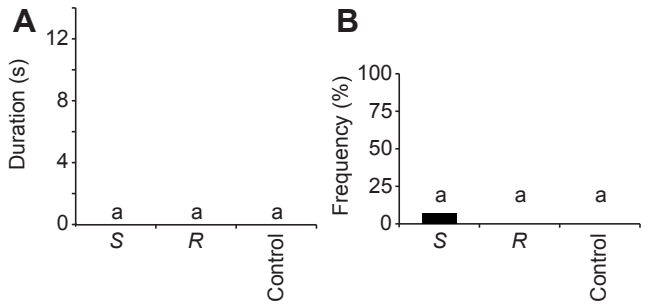

Fig. 4. Results of behavioural experiment 3. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with synthetic (*S*)-3-MeC27, (*R*)-3-MeC27 or the pure solvent (Control) during a 5-min observation period. Columns with the same lower case letters are not significantly different (*P*>0.05, wing-fanning duration analysed by Kruskal–Wallis *H*-test, proportion of males analysed by Chi-square tests; *N*=20).

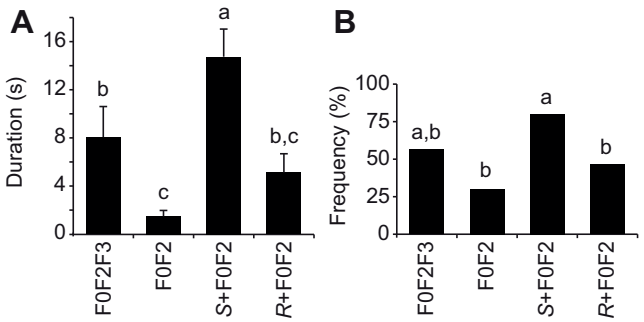

Fig. 5. Results of behavioural experiment 4. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with a binary mixture of lipid fractions F0 and F2 plus synthetic (*S*)-3-MeC27 or (*R*)-3-MeC27 during a 5-min observation period. For control, a ternary mixture of all three lipid fractions (positive control) and a binary mixture of F0 and F2 was tested (Control). Different lower case letters indicate significant differences at $P<0.05$ (wing-fanning duration analysed by Kruskal–Wallis *H*-test followed by Mann–Whitney *U*-tests, proportion of males analysed by Chi-square tests; $N=30$).

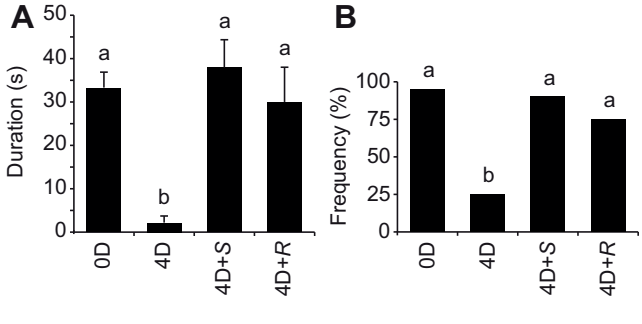

Fig. 6. Results of behavioural experiment 5. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards newly emerged (0D) or 4-day-old (4D) male dummies treated with synthetic (*S*)-3-MeC27, (*R*)-3-MeC27, or the pure solvent during a 5-min observation period. Different lower case letters indicate significant differences at $P<0.001$ (wing-fanning duration analysed by Kruskal–Wallis *H*-test followed by Mann–Whitney *U*-tests, proportion of males analysed by Chi-square tests; $N=20$).

Millar, 2010), particularly when considering that males did not distinguish between the enantiomers on three-dimensional models. This result suggests that also visual and/or tactile cues are involved in sex recognition of *L. distinguendus*, as previously shown for instance in the jewel wasp *Nasonia vitripennis* (Steiner et al., 2006). Interestingly, males of the two sympatric geometrid moths *Lambdina athasaria* and *L. pellucidaria* also responded only to the (*S*)-enantiomer of 7-methylheptadecane (Duff et al., 2001). In this case, however, the monomethylalkane is a component of a blend of volatile sex attractants.

Monomethylalkanes have been previously reported as key components of contact sex pheromones in beetles. In some of these studies, single female-specific compounds were sufficient to elicit full behavioural response in males without the chemical background of the other CHCs being present (Sugeno et al., 2006; Guedot et al., 2009; Silk et al., 2009). In other studies, individual compounds synergized each other, and full behavioural activity was only elicited by binary or ternary mixtures (Ginzel et al., 2003; Lacey et al., 2008; Spikes et al., 2010). A study on the leaf beetle *Gastrophysa atrocyanea* revealed that both chain length and branching position of monomethylalkanes with pheromone function are slightly variable without losing bioactivity. If one of the parameters differed too much from the structure of the natural pheromone, bioactivity was lost (Sugeno et al., 2006). This nicely matches a recent model suggesting pheromone-binding proteins with two different hydrophobic clefts to be involved in the perception of monomethylalkanes by insects. These clefts are assumed to accommodate the two alkyl groups of differing chain lengths at the branching point, resulting in stereochemically different alkane–protein complexes for the two methylalkane enantiomers with potentially different olfactory properties (Mori, 2011). In a recent learning study, Bos et al. (Bos et al., 2012) found the tendency that methylalkanes are generalized by ants. Workers conditioned for instance with either 3-MeC27 or 3-MeC31 were unable to discriminate these stimuli in a subsequent choice experiment.

Interestingly, 3-methylalkanes are also more abundant or even specific in female CHC profiles in a number of other parasitoid wasps (Howard, 2001; Sullivan, 2002; Darrouzet et al., 2010; Ruther et al., 2011), suggesting a widespread role of these compounds as contact sex pheromones. In the pteromalid *Dibrachys cavus*, proportions of 3-MeC29 and 3-MeC31 increase when females become sexually attractive within the first two days after emergence (Ruther et al., 2011). 3-MeC31 has also been identified as a queen pheromone in the ant *Lasius niger* (Holman et al., 2010).

The exact mechanism behind the pheromone deactivation in *L. distinguendus* males is still unknown, but it is clear that the disappearance of 3-MeC27 and the minor CHCs depends on the male wasps being alive, because newly emerged dead males remain sexually attractive for consexuals (Steiner et al., 2005). The lipoprotein lipophorin is involved in the transport of CHCs from the synthesizing oenocytes to the surface of insects and probably *vice versa*. In the dampwood termite *Zootermopsis nevadensis*, for instance, topically applied radio-labelled 3,11-dimethylnonacosane was internalized and the labelled hydrocarbon was found to be associated with lipophorin (Sevala et al., 2000). The lipophorin transport of hydrocarbons can be quite specific, as demonstrated in *Holomelina* tiger moths. Here, the pheromone component 2-methylheptadecane is synthesized in the abdominal integument and specifically transported by lipophorin to the pheromone gland serving as a reservoir (Schal et al., 1998).

A change in the CHC profiles during ageing and a decreasing sexual attractiveness was recently also reported in *Drosophila melanogaster* (Kuo et al., 2012). As previously shown in our studies on *L. distinguendus* (Steiner et al., 2005; Steiner et al., 2007), the authors found a shift towards compounds with longer chain-lengths and observed a correlation with decreasing sexual attractiveness. It has to be shown, however, that like in the present study the observed chemical changes are causally involved in the behavioural responses.

One surprising result of the present study is that the TAG fraction was essential for a full pheromone response of *L. distinguendus* males, because the omission of the TAG fraction in our bioassays resulted in a significant decrease of male wing-fanning behaviour. We are aware of no other study having shown a communicative function of TAGs before. Until recently, it was not even clear whether TAGs actually occur on the insect cuticle at all because cuticular lipids are typically obtained by whole body washes bearing the danger of co-extracting TAGs from the fat body or other internal tissues (Lockey, 1988; Buckner, 1993). Obviously, the majority of

studies on insect cuticular lipids have ignored the presence of TAGs, as these high molecular lipids are not detected by standard GC-MS techniques without derivatization. We used SPME with *in situ* derivatization to detect TAGs on the cuticle of *L. distinguendus* and to determine their mean fatty acid composition. This approach reliably prevented the danger of analysing internal TAGs, but the exact distribution of fatty acids within the individual TAGs cannot be determined with this technique. Even if unlikely, the presence of other non-volatile fatty acid derivatives in the TAG fraction cannot be fully excluded. In a recent study, Yew et al. (Yew et al., 2011) used ultraviolet laser desorption/ionization mass spectrometry to demonstrate the presence of TAGs on the epicuticle of two *Drosophila* species. They found that some male-derived TAGs are transferred from the anogenital region to the female during copulation, and suggested that these might play a role in the courtship of these flies. More detailed studies using synthetic TAGs are needed to investigate the presumably long underestimated occurrence of TAGs on the insect cuticle and their communicative function in insects. Isolation of TAGs by SE-HPLC and the subtractive bioassay procedure described here could be a promising approach to achieve this goal.

LIST OF ABBREVIATIONS

3-MeC27	3-methylheptacosane
CHC	cuticular hydrocarbon
FAME	fatty acid methyl ester
GC-MS	gas chromatography-mass spectrometry
SPME	solid phase microextraction
TAG	triacylglyceride
TMSH	trimethylsulphonium hydroxide

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