Cuticular Lipids of the Parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae): Chemistry and Behavioural Function

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vorgelegt von Stephan Kühbandner
aus München
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Lariophagus distinguendus female (Hymenoptera: Pteromalidae)

Zeichnung: Ruth Kühbandner, 2011

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List of abbreviations

0-d-old male dummies	zero day old male dummies
4-d-old male dummies	four day old male dummies
3-MeC25	3-methylpentacosane
3-MeC27	3-methylheptacosane
5-MeC27	5-methylheptacosane
7-MeC27	7-methylheptacosane
3,7-DiMeC27	3,7-dimethylheptacosane
4,8-DiMeC28	4,8-dimethyloctacosane
3-MeC29	3-methylnonacosane
13-MeC29	13-methylnonacosane
3,7-DiMeC29	3,7-dimethylnonacosane
3-MeC31	3-methylhentriacontane
11,21-DiMeC33	11,21-dimethyltritriacontane
3,7,11,15-TetraMeC33	3,7,11,15-tetramethyltritriacontane
13,17-DiMeC35	13,17-dimethylpentatriacontane
20-E	20-hydroxyecdysone
AT	*Asobara tabida* (Hymenoptera: Braconidae)
Br2	bromine
C25	pentacosane
C27	heptacosane
C27:1(9)	heptacos-9-ene
C29	nonacosane
C29:1(9)	nonacos-9-ene
C31	hentriacontane
$CDCl_3$	deuterated chloroform

CHC	cuticular hydrocarbon
CH_2Cl_2	dichloromethane
CH3(CH2)4COCl	hexanoylchloride
^{13}C-NMR	carbon-13 nuclear magnetic resonance spectroscopy
CoA	coenzyme A
DCM	dichloromethane
DM	*Drosophila melanogaster* (Diptera: Drosophilidae)
DMSO	dimethyl sulfoxide
EI	electron impact ionisation
Et_2O	diethyl ether
EtOAc	ethyl acetate
FAME	fatty acid methyl ester
FFA	free fatty acids
GC	gas chromatography
GC-EAD	gas chromatography coupled with an electroantennographic detector
GC-MS	gas chromatography coupled with mass spectrometry
H_2	hydrogen
HCL	hydrogen chloride
^{1}H-NMR	hydrogen-1 nuclear magnetic resonance spectroscopy
HPLC	high performance liquid chromatography
JH III	juvenile hormone III
K2CO3	potassium carbonate
LD	*Lariophagus distinguendus* (Hymenoptera: Pteromalidae)
LiBH4	lithium borohydride
Li_2CuCl_4	dilithium tetrachlorocuprate (II)
LMC	local mate competition

LRI	relative linear retention indices
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MeI	methyl iodide
MeOH	methanol
Mp	melting point
MS	mass spectrometry
MW	molecular weight
n-BuLi	*n*-butyllithium
Na_2CO_3	sodium carbonate
NaHCO3	sodium bicarbonate
NaHMDS	sodium hexamethyldisilazide
NaI	sodium iodide
Na_2SO_4	sodium sulfate
NH_4Cl	ammonium chloride
NI	*Nicrophorus vespilloides* (Coleoptera: Silphidae)
NMDS	non-metric multidimensional scaling
NPMANOVA	nonparametric multivariate analysis of variance
NV	*Nasonia vitripennis* (Hymenoptera: Pteromalidae)
OBP	odorant binding protein
ODE	odorant degrading enzyme
ODR	odorant receptor neuron
OR	odorant receptor
PA	*Periplaneta americana* (Blattodea: Blattidae)
PBAN	pheromone biosynthesis activating neuropeptide
PBP	pheromone binding protein
PCA	principal component analysis
PDMS	polydimethylsiloxane (coating material for SPME fibre)

Ph_3P	triphenylphosphine
Ph_3PBr_2	triphenylphosphine dibromide
PLS-DA	partial least squares-discriminant analysis
RIO	resource indicating odour
SE-HPLC	size exclusion-high performance liquid chromatography
SIMPER	similarity percentage analysis
SPME	solid phase microextraction
SPME-FAME-GC-MS	SPME of TAGs with *in situ* transesterification into more volatile FAMEs (using TMSH) that are detectable by GC-MS
TAG	triacylglyceride
Tf_2O	trifluoromethanesulfonic anhydride
THF	tetrahydrofuran
TMSH	trimethylsulphonium hydroxide
UPLC-MS	ultra-performance liquid chromatography coupled with mass spectrometry
UV-LDI-o-TOF MS	ultraviolet laser desorption/ionisation orthogonal time-of-flight mass spectrometry

Summary

Animals communicate with each other by means of optical, acoustic, tactile, electrical and chemical signals. This doctoral thesis deals with the contact sex pheromone of the parasitic wasp species *Lariophagus distinguendus* (Hymenoptera: Pteromalidae). Males respond to the cuticular lipids of conspecific females by stereotypic courtship behaviour. Young males and pupae of either sex also produce the cuticular lipids that release courtship behaviour in conspecific males. However, with increasing age, males actively remove 3-MeC27 and some other straight-chain alkanes and methylalkanes from their cuticular lipid profile. The disappearance of these components is accompanied by a loss in their attractiveness towards other males. The capability of the males to actively remove components of their cuticular lipid profile probably rendered a non-communicative barrier against water loss and pathogen attack into a species-specific contact sex pheromone. These findings from previous work led to some questions: is 3-MeC27 actually part of *L. distinguendus* contact sex pheromone? If this is true, is 3-MeC27 alone bioactive or only in combination with other cuticular lipids? Do *L. distinguendus* males respond enantioselectively towards 3-MeC27? Can the addition of synthetic 3-MeC27 onto the cuticle of aged males restore their bioactivity? Can 3-MeC27 be replaced by structurally related methylalkanes that differ in their chain length or the position of the methyl-branch? Or does the application of additional n-alkanes and structurally related methylalkanes interfere with the intact cuticular profile?

During this doctoral thesis, I have shown that 3-MeC27 is the key component of the female contact sex pheromone of *L. distinguendus*. However, unlike in other insects studied so far, the key component 3-MeC27 alone is not attractive to *L. distinguendus* males. For a full behavioural response, 3-MeC27 has to be perceived by the males together with a chemical background consisting of the other cuticular hydrocarbons (CHC) and hitherto widely ignored cuticular triacylglycerides (TAG). The occurrence of cuticular TAGs has probably been underestimated in the past and a behavioural function for TAGs is described here for the first time.

Behavioural experiments with fractionated wasp extracts and synthetic 3-MeC27 and other structurally related components revealed that the cuticular lipid profile of aged males can be rendered attractive again by the addition of synthetic 3-MeC27 in physiological amounts. *L. distinguendus* males responded specifically to even small variations of chain length or methyl-branch position of monomethylalkanes because chemically related components such

as 3-MeC29 or 5-MeC27 could not replace 3-MeC27. On the contrary, the addition of such components rendered behaviourally active cuticular lipid profiles of females and young males unattractive. These results demonstrate that the contact sex pheromone in *L. distinguendus* is perceived as a whole, possibly by means of a specialized sensillum type as used by ants in the context of nestmate recognition.

The males of *L. distinguendus* were able to differentiate between the two enantiomers of 3-MeC27, but this was only behaviourally relevant when fractionated extracts combined with (*R*)- or (*S*)-3-MeC27 were applied onto filter paper and offered to the responding males. In experiments with three-dimensional wasp dummies, however, *L. distinguendus* males did not show a preference for one of the enantiomers implying that a visual component of the dummy also plays a role.

During the development of parasitoids, the host is typically the only available food source. Hence, host species and quality might affect the CHC profiles of parasitoids and thus also its intraspecific communication. In this doctoral thesis it was shown that the CHC profiles of *L. distinguendus* wasps are host specific. CHC profiles of wasp strains reared on different host species were distinguishable by a non-metric multidimensional scaling (NMDS) and a non-parametric multivariate analysis of variance (NPMANOVA). In particular, wasps reared on *Stegobium paniceum* could be significantly distinguished from all wasps reared on other hosts according to their CHC profile. Remarkably, a host shift from *Sitophilus granarius* to *Stegobium paniceum* lead to a significant shift of the CHC profiles within one generation. Provided that these differences actually influence the mate recognition behaviour in *L. distinguendus*, these results suggest that host shifts might result in the reproductive isolation of host races and, in the long-term, end in speciation.

As part of this doctoral thesis, SPME-FAME-GC-MS was developed as a new easy-to-use method for the solvent-free analysis of more polar cuticular TAGs. With this method, the TAGs were sampled from the insect cuticle with the solid phase microextraction (SPME) and transesterified *in situ* with trimethylsulphonium hydroxide into more volatile fatty acid methyl esters (FAME). These can be analysed easily by standard gas chromatography coupled with mass spectrometry (GC-MS) techniques. The occurrence of TAGs on the insect cuticle might have been widely underestimated, because their high melting points make them undetectable with standard GC-MS. Furthermore, the use of solvents for the extraction of cuticular lipids runs the risk of additionally extracting lipids from internal tissues. Six insect species from four different orders were analysed with the new method of SPME-FAME-GC-MS, and

TAGs were detected on the cuticle of all examined species. Thus, TAGs might be far more common cuticular components of insects than usually thought.

Zusammenfassung

Tiere kommunizieren untereinander mittels optischer, taktiler, elektrischer und chemischer Signale. Diese Doktorarbeit beschäftigt sich mit dem Kontakt-Sexualpheromon der parasitoiden Wespenart *Lariophagus distinguendus* (Hymenoptera: Pteromalidae). Die Männchen reagieren auf die kutikulären Lipide artgleicher Weibchen mit einem stereotypen Balzverhalten. Junge Männchen und die Puppen beider Geschlechter produzieren dieselben kutikulären Lipide, welche Paarungsverhalten bei artgleichen Männchen auslösen. Allerdings entfernen die Männchen mit zunehmendem Alter 3-MeC27, sowie andere n-Alkane und methylverzweigte Alkane aktiv aus ihrem kutikulären Lipid-Profil. Das Verschwinden dieser Komponenten von der Kutikula der Männchen geht mit einem Verlust ihrer Attraktivität gegenüber anderen, artgleichen Männchen einher. Die Fähigkeit der Männchen, aktiv und gezielt diese kutikulären Lipide zu entfernen, hat mutmaßlich eine Barriere gegen Krankheitserreger und Wasserverlust, welche vorher keine kommunikative Funktion hatte, in ein artspezifisches Kontakt-Sexualpheromon verwandelt. Diese Erkenntnisse aus vorangegangenen Arbeiten führen zu einer Reihe von Fragen: Ist 3-MeC27 tatsächlich ein Bestandteil des Kontakt-Sexualpheromons von *L. distinguendus*? Wenn das stimmt, ist dann 3-MeC27 alleine verhaltensaktiv oder nur in Kombination mit weiteren kutikulären Lipiden? Reagieren die *L. distinguendus* Männchen enantioselektiv auf 3-MeC27? Kann die Applikation von synthetischem 3-MeC27 auf die Kutikula von älteren Männchen deren Verhaltensaktivität wiederherstellen? Kann 3-MeC27 durch strukturell verwandte Methylalkane, welche sich in der Kettenlänge oder der Position der Methylverzweigung voneinander unterscheiden, ersetzt werden? Oder beeinträchtigt die Applikation zusätzlicher n-Alkane und Methylalkane sogar das intakte Kutikula-Profil?

Im Verlauf dieser Doktorarbeit habe ich herausgefunden, dass 3-MeC27 die Schlüsselkomponente des Kontakt-Sexualpheromons der Weibchen von *L. distinguendus* ist. Allerdings ist 3-MeC27 alleine, im Gegensatz zu Erkenntnissen aus Studien mit anderen Insekten, nicht attraktiv für *L. distinguendus* Männchen. Um das Balzverhalten der Männchen in vollem Umfang auszulösen, müssen die Männchen 3-MeC27 zusammen mit einem chemischen Hintergrund, bestehend aus den anderen kutikulären Kohlenwasserstoffen (CHC) und den bis dato weitgehend vernachlässigten Triacylglycerolen (TAG), wahrnehmen. Das TAGs ein Bestandteil der kutikulären Lipide sind, wurde in der Vergangenheit vermutlich unterschätzt. Eine verhaltensauslösende Funktion der TAGs wurde hier zum ersten Mal gezeigt.

Verhaltensexperimente mit fraktioniertem Wespenextrakt in Kombination mit synthetischem 3-MeC27 und anderen strukturell verwandten Komponenten haben gezeigt, dass die Attraktivität des kutikulären Lipidprofils von älteren Männchen durch den Zusatz von synthetischem 3-MeC27 in physiologischen Mengen wiederhergestellt werden kann. *L. distinguendus* Männchen reagieren sehr spezifisch, selbst auf geringfügige Veränderungen der Kettenlänge oder der Position des Verzweigungspunktes von Methylalkanen, da 3-MeC27 nicht durch chemisch verwandte Substanzen, wie 3-MeC29 oder 5-MeC27 ersetzt werden konnte. Im Gegenteil, die Zugabe solcher Komponenten führte dazu, dass die verhaltensaktiven kutikulären Lipid-Profile von Weibchen und jungen Männchen unattraktiv wurden. Diese Ergebnisse zeigen, dass das Kontakt-Sexualpheromon von *L. distinguendus* als ein Ganzes wahrgenommen wird, mutmaßlich durch den Einsatz eines spezialisierten Sensillums, welches bei Ameisen zur Erkennung von Nestgenossen dient.

Die Männchen von *L. distinguendus* können zwischen den beiden Enantiomeren von 3-MeC27 unterscheiden, was sich aber im Verhalten nur dann auswirkt, wenn fraktionierte Extrakte zusammen mit synthetischem (*R*)- oder (*S*)-3-MeC27 auf Filterpapier aufgetragen und den Männchen angeboten werden. Die *L. distinguendus* Männchen zeigten dagegen in Experimenten mit dreidimensionalen Dummys keine Präferenz für eines der beiden Enantiomere. Dies lässt den Schluss zu, dass der Dummy als optische Komponente ebenfalls eine Rolle spielt.

Während der Entwicklung eines Parasitoiden, stellt dessen Wirt üblicherweise seine einzige Nahrungsquelle dar. Daher können die Spezies und die Qualität des Wirtes einen Einfluss auf das CHC Profil des Parasitoiden und seine Fähigkeiten zur intraspezifischen Kommunikation haben. In dieser Doktorarbeit konnte gezeigt werden, dass die CHC-Profile von *L. distinguendus*-Wespen wirtsspezifisch sind. CHC-Profile von Stämmen, die auf unterschiedlichen Wirten gezogen wurden, konnten mittels der nicht-metrischen multidimensionalen Skalierung (NMDS) und der nichtparametrischen multivariaten Varianzanalyse (NPMANOVA) voneinander unterschieden werden. Insbesondere Wespen, die auf *Stegobium paniceum* gezogen wurden, unterschieden sich in ihrem CHC Profil von allen Stämmen, die auf anderen Wirten gezogen wurden. Bemerkenswerterweise, führte ein Wirtswechsel von *Sitophilus granarius* auf *Stegobium paniceum* zu einem signifikant unterschiedlichem CHC Profil innerhalb von nur einer Generation nach dem Wirtswechsel. Unter der Voraussetzung, dass diese Unterschiede tatsächlich die Erkennung von Paarungspartnern beeinträchtigen, legen meine Ergebnisse nahe, dass solche Wirtswechsel

eine reproduktive Isolation der Wirtsrassen verursachen und langfristig zur Artaufspaltung führen können.

Im Rahmen dieser Doktorarbeit wurde SPME-FAME-GC-MS als eine neue, einfach anzuwendende Methode zur lösungsmittelfreien Analyse der polaren, kutikulären Triacylglycerole (TAG) entwickelt. Mit dieser Methode, können die TAGs mittels der Festphasen-Mikroextraktion (SPME) direkt von der Kutikula gesammelt werden. Anschließend können sie noch auf der SPME-Faser, mittels Trimethylsulfoniumhydroxid in volatilere Fettsäuremethylester umgewandelt werden, welche mittels Standard-Gaschromatographie gekoppelt mit Massenspektrometrie (GC-MS) einfach nachweisbar sind. Das Vorhandensein von TAGs auf der Insekten-Kutikula wurde bisher auch aufgrund der hohen Schmelzpunkte von TAGs, welche eine Analyse ohne Derivatisierung mittels (GC-MS) unmöglich machen, vermutlich weitgehend unterschätzt. Der Einsatz von Lösungsmitteln zur Extraktion von kutikulären Lipiden birgt außerdem das Risiko, dass zusätzlich Lipide aus internen Geweben extrahiert werden. Sechs verschiedene Insektenarten aus vier verschiedenen Ordnungen wurden mit der neuen SPME-FAME-GC-MS Methode getestet, wobei TAGs auf der Kutikula von allen untersuchten Arten nachgewiesen werden konnten. TAGs sind daher vermutlich weit häufigere Bestandteile der Kutikula von Insekten als bisher angenommen.

List of publications

This thesis incorporates the following manuscripts

Kühbandner, S., Hacker, K., Niedermayer, S., Steidle, J.L.M. & Ruther, J. 2012. Composition of cuticular lipids in the pteromalid wasp *Lariophagus distinguendus* is host dependent. *Bulletin of Entomological Research*, **102**, 610-617 (**Chapter 2**).

Kühbandner, S., Sperling, S., Mori, K. & Ruther, J. 2012. Deciphering the signature of cuticular lipids with contact sex pheromone function in a parasitic wasp. *Journal of Experimental Biology*, **215**, 2471-2478 (**Chapter 3**).

Kühbandner, S., Bello, J.E., Mori, K., Millar, J.G. & Ruther, J. 2013. Elucidating structure-bioactivity relationships of methyl-branched alkanes in the contact sex pheromone of the parasitic wasp *Lariophagus distinguendus*. *Insects*, **4**, 743-760 (**Chapter 4**).

Chapter 1

General introduction

Animal communication

Biological communication is a complex field and no commonly agreeable definition is available (Wyatt, 2003). Communication occurs within and between species and in various contexts. Animals use optical, acoustic, tactile, chemical and electrical signals for communication with other individuals. According to Endler (1993), evolution has formed animal communication systems that allow individuals to base their decisions on the physiology, behaviour or morphology of others. Animals achieve this by the use of signals. Signals enable information exchange from the 'emitter' to the 'receiver' and can be seen as environmental changes caused by the emitter and detected by the receiver (Endler, 1993). Whereas the evolution of signals leads to more efficient communication and increases the manipulation of the receiver to the emitter's benefit, the evolution of the receiving mechanisms increases the reliability and efficiency of information reception (Endler, 1993).

Smith and Harper (2003) have defined signals as "any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved". The consequences of this definition are that, on the whole, both the emitter and receiver of the signal have to benefit from the signal, because otherwise the communication system would not be stable. However, this does not exclude that the emitter and receiver of the signal have different interests and are involved in an evolutionary arms race (Smith and Harper, 2003).

If one organism alters the behaviour of others, this could also be seen as coercion. This would mean that one organism forces the other to behave in a distinct way. If it is a signal instead of coercion, the receiver benefits, when he/she behaves in a way favourable to the emitter of the signal. Cues can be distinguished from signals on the grounds the latter have evolved because of their effect on others (Smith and Harper, 2003). Smith and Harper (2003) provide within their book some examples and further definitions illustrating the distinction between a cue and a signal. A compound released by one organism without a communicative function like e.g. a

hormone, a cuticular compound or a defensive secretion can become a cue, if another organism evolves the ability to detect this compound and responds to it. This stage, that is only beneficial for the receiver, is also called ‘eavesdropping’ or ‘spying’ stage. A cue can evolve into a signal, if the emitter of the cue benefits from the receivers response to the cue. Then selective forces optimize the signal for a better information exchange (Sorensen and Stacey, 1999; Wyatt, 2003; Steiger et al., 2011; Weiss et al., 2013). A further consequence is that ‘honest’ signals are costly. Those costs can be subdivided into ‘efficacy costs’ and ‘strategic costs’. Efficacy costs are necessary to ensure that the signal is reliably perceived, whereas strategic costs ensure honesty and prevent cheating (Smith and Harper, 2003).

Insect chemical communication

Strictly speaking ‘true communication’ would be limited, according to Smith and Harpers (2003) definition of a signal, to occasions where both, the emitter and the receiver, benefit and the signal has evolved for information exchange. The term ‘semiochemicals’ or ‘infochemicals’ and the following subdivision into different kinds of semiochemicals comprises compounds that contain information about the emitter, but they are not necessarily a signal. Information exchange between individuals is mediated by so-called semiochemicals or infochemicals, which can be divided into intraspecific-acting pheromones and interspecific-acting allelochemicals.

Allelochemicals can be subdivided into compounds that are beneficial for the receiver and costly or neutral for the emitter of the signal (kairomones), beneficial for the emitter and costly or neutral for the receiver of the signal (allomones) and those that are beneficial for both signal emitter and receiver (synomones) (Wyatt, 2003).

The term ‘pheromone’ was first introduced by Karlson and Lüscher (1959) and defined as: “substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process”. Pheromones that cause a developmental process in the receiver are classified as primer pheromones and those that release a specific reaction such as a definite behaviour are termed releaser pheromones (Wyatt, 2003). Pheromones can be further divided according to their behavioural function, for instance as

aggregation, maturation, dispersal, alarm, recruitment (trail) and sex pheromones (Tillman et al., 1999). Semiochemicals that are used as pheromones in one species can be 'eavesdropped' by individuals of another species and, as such, additionally used as kairomones (Wyatt, 2003).

Pheromones

Pheromones are usually effective in minute amounts and their volatility corresponds with their function and effect duration. More volatile pheromones, which are spread via the air, such as alarm pheromones, typically consist of less carbon atoms and have lower molecular weights than, for example, sex pheromones. Larger molecules can be more specific and their lower volatility increases the longevity of the pheromone (Wyatt, 2003). Pheromones can consist of a single substance but, in most cases, they comprise a mixture of compounds with different relative amounts. But stereochemical and synergistic effects may also be important (Tillman et al., 1999; Blomquist and Vogt, 2003). Pheromones can be accompanied by other, for example, visual, tactile or optical signals either to strengthen the signal, because additional sensory channels are necessary for the message, or to modulate the signal intensity (Wyatt, 2003).

Pheromones can be synthesized de novo by the emitting organism, directly sequestered from nutrition or derived from nutritional precursors. Such pheromone precursors are often fatty acid derivatives that are modified enzymatically via chain elongation or shortening, desaturation and reductive modifications of the carbonyl carbon, but also alkaloids and plant isoprenoids can act as pheromone precursors (Tillman et al., 1999). The regulation of pheromone biosynthesis in most insect species studied occurs via one of the three following hormonal messengers and is largely order-specific: juvenile hormone III (JH III; Blattodea, Coleoptera), pheromone biosynthesis activating neuropeptide (PBAN; Lepidoptera) and 20-hydroxyecdysone (20-E; Diptera) (Tillman et al., 1999). JH III and 20-E regulate enzyme synthesis at the transcription level, whereas PBAN affects enzyme activity through second messenger systems during pheromone synthesis (Blomquist and Vogt, 2003). Pheromone biosynthesis can take place in single cells or in tissues such as glands that can be located almost everywhere on/in the insect body (Blomquist and Vogt, 2003; Wyatt, 2003).

Pheromones are detected by olfactory or gustatory cells. Insects detect volatile compounds with olfactory sensilla, whereas they perceive non-volatile substances by gustatory sensilla that have to get into close contact with the source of the pheromone (Ozaki and Wada-Katsumata, 2010). Insect olfactory sensilla are mainly located on the antennae in contrast to gustatory sensilla that can be widely distributed over the insect body (Ebbs and Amrein, 2007). Olfactory cells are directly linked to the brain, while gustatory cells pass on their signal to a sensory nerve fibre (Wyatt, 2003). Gustatory and olfactory stimuli are processed in different parts of the brain. Olfactory cells occur in higher numbers and can perceive a wider range of compounds than gustatory cells (Wyatt, 2003).

Odours are detected by insects with the help of olfactory receptor neurons (ORN) that are located, either as a single cell or as several cells, in olfactory sensilla and that are filled with lymph fluid. Odours, including pheromones, reach the inside (haemolymph) of the sensilla via pores in the cuticle and are thought to be transported to the ORN by odorant-binding proteins (OBP) (Wyatt, 2003; de Bruyne and Baker, 2008). The OBPs solubilize the odour molecules and protect them from degradation by odorant-degrading enzymes (ODE) through encapsulation. When the complex consisting of the odour molecule and the OBP reaches the negatively-charged site of the dendritic membrane, the molecule-OBP complex undergoes a conformational change (Leal, 2005). It is unclear, if the conformational change causes a release of the odour molecule. Either the complex consisting of odour molecule and OBP activates the OR, or the released odour molecule alone. In both cases the ORN generates an electrical signal. The ORN located in the olfactory sensilla innervate the antennal lobe that processes the electrical signals and transmits them to the protocerebrum, which integrates the signals together with other stimuli, makes a decision and activates the motor system (Leal, 2005). After they have activated the OR, the odour molecules are degraded by ODEs. ORs in vertebrate ORNs are G-protein-coupled receptors, while insect ORs are different from typical G-protein coupled receptors (de Bruyne and Baker, 2008). New findings suggest that the insect OR forms together with the co-receptor Or83b a complex that has the function of a ligand-gated ion channel itself (Sato et al., 2008).

Insect cuticular hydrocarbons (CHC)

The cuticle of most insects is covered by a complex mixture of cuticular hydrocarbons (CHCs), which consists of methylalkanes with one to five methyl groups, alkenes with one to three double bonds and straight-chain alkanes (Blomquist and Bagnères, 2010). Usually CHCs are odd-numbered with chain-lengths of about 21-37 carbons, but CHCs with chain-lengths of more than 60 carbons are known (Cvačka et al., 2006b; Blomquist and Bagnères, 2010). Some insect CHC profiles comprise only a few substances, whereas others are complex mixtures of more than 100 compounds (Dani et al., 2001; van Wilgenburg et al., 2010). CHCs are used by many insects, apart from their waterproofing function, for chemical communication with others (Gibbs, 2002; Blomquist and Bagnères, 2010). Social insects use CHCs among others for species, caste and nestmate recognition, as task-specific cues, dominance and fertility cues, and for chemical recruitment, defence and mimicry (Howard and Blomquist, 2005). CHC profiles are often species- and sex-specific and, therefore, many solitary insects including parasitoids use them as low volatile contact sex pheromones that elicit courtship behaviour (Howard and Blomquist, 2005; Blomquist and Bagnères, 2010; Ruther, 2013). Many studies dealing with CHCs as sex pheromones are merely correlative and do not comprise bioassays that support the link between the compound and the behaviour performed by the insect (Howard and Blomquist, 2005). The role of CHCs as part of the female contact sex pheromone of the parasitoid wasp species *Lariophagus distinguendus* has been examined in this work (**Chapter 3** and **Chapter 4**).

Insects synthesize unbranched alkanes and unbranched alkenes by the elongation of fatty acyl-CoAs. The conversion of elongated fatty acyl-CoAs into hydrocarbons includes reduction to the aldehyde, followed by decarbonylation or decarboxylation and ends up in a hydrocarbon that is one carbon shorter than the educts (Jurenka, 2004; Howard and Blomquist, 2005; Blomquist, 2010). Insects seem to regulate the chain-length of their hydrocarbons rather within the fatty acyl-CoA elongase reactions, than by the reductive conversion of acyl-CoAs into hydrocarbons (Howard and Blomquist, 2005).

During the synthesis of alkenes, fatty acyl-CoA desaturases regulate where double bonds are formed (Howard and Blomquist, 2005). Most methyl-branched alkanes are formed by the substitution of methylmalonyl-CoA in place of malonyl-CoA at specific points during the chain elongation (Howard and Blomquist, 2005; Blomquist, 2010). Exceptions are 2-methylalkanes whose synthesis starts from the carbon skeleton of the amino acids valine

and isoleucine via chain-elongation (Blomquist, 2010). Insects with low vitamin B12 levels use the amino acids valine, isoleucine and methionine as precursors for the synthesis of methylmalonyl-CoA, whereas insects with high vitamin B12 levels like termites convert succinate into methylmalonyl-CoA (Howard and Blomquist, 2005; Blomquist, 2010). It has been shown in some insects that the introduction of the methyl-branch occurs during the early steps of chain-elongation.

The synthesis of hydrocarbon takes mainly place in the oenocytes that are associated with the epidermal tissue or with the fat body. The newly synthesized hydrocarbons are bound to the carrier protein lipophorin and selectively transported to their target tissue via the hemolymph (Schal et al., 1998a; Howard and Blomquist, 2005).

Insects synthesize the majority of their CHCs de novo, but also incorporate hydrocarbons from nutritional sources (Blomquist, 2010). Pheromone components may also be derived from modified dietary host precursors like amino acids that are used by insects as starting material for fatty acid derived sex pheromones synthesis (Tillman et al. 1999). Some pheromone precursors cannot be produced by the insects themselves and therefore have to be perceived by dietary intake. Moths, for example, are not able to synthesize linolenic acid or linoleic acid on their own. But many moth species use polyunsaturated hydrocarbons that are derived from linolenic acid or linoleic acid as sex pheromones (Jurenka, 2004). The nutrients that are necessary for pheromone synthesis, the pheromone precursors or the hydrocarbons that are directly incorporated into the CHC profile, all of them have to be perceived by nutrition and therefore the diet of an insect should affect its CHC profile. A dietary influence on the CHC profile is known from workers of the argentine ant *Linepitema humile* that recognize nestmates by their CHC profiles. Former nestmates reared on different insect prey had different CHC profiles and reacted aggressively when encountering each other (Liang and Silverman, 2000). The authors assume that the ants incorporated CHCs from insect prey with little modifications into their own CHC profile. Buczkowski et al. (2005) performed experiments with dissimilar *Linepitema humile* colonies reared on a shared diet and concluded that this may leads to the fusion of the colonies. Further evidence comes from a study with *Drosophila melanogaster* reared on different diets that had effects on the composition of female CHC profiles across age (Fedina et al., 2012) and from *Cephalononmia tarsalis* females whose total CHC quantity and individual CHC composition was affected by their host-feeding status (Howard, 1998).

Insects detect CHCs like other chemical stimuli with their olfactory or gustatory sensilla as described above. Non-volatile long-chain hydrocarbons and sex pheromones that consist of single or a few species-specific CHCs are supposed to be perceived by gustatory sensilla, while bouquets of many CHCs seem to be detected by olfactory sensilla that have far more receptor neurons than gustatory sensilla (Ebbs and Amrein, 2007; Ozaki and Wada-Katsumata, 2010). Ozaki et al. (2005; 2012) found a multiporous sensillum on the antennae of the Japanese carpenter ant *Camponotus japonicus* that responds only to non-nestmate CHCs and seems to perceive the CHC profile as a whole. This type of sensillum is innervated by a high number of receptor neurons, which is unusual for gustatory sensilla.

But it is assumed that in most insect species single compounds or mixtures of several compounds elicit a behavioural response and not the whole CHC profile serves as a recognition cue (Dani et al., 2001; Ginzel, 2010). Most studies investigating the behavioural response of CHCs are merely correlative. This is mainly due to a lack of synthetic reference substances and analytical techniques necessary for the separation of CHCs from a complex mixture (Hefetz et al., 2010). This is particularly the case for methylalkanes that are chiral, but it is largely unknown which enantiomers occur on the cuticle and if they have behaviourally different effects. Enantiomeric pure synthetic reference substances are often not available and the enantiomers cannot be separated with state-of-the-art analytical techniques (Hefetz et al., 2010; Millar et al. 2010). Mori (2011) proposed a model how insects may distinguish the enantiomers of monomethylalkanes by perceiving them with the help of pheromone binding proteins (PBP) that have two different hydrophobic clefts and form stereochemically specific complexes with each stereoisomer. This is possible, because the monomethylalkanes are ‘divided’ by the branching point into two alkyl groups of different chain length that fit into the hydrophobic clefts of the PBP.

Methylalkanes and alkenes are thought to be better perceived by insects than straight-chain alkanes, because they have compared with straight-chain alkane’s additional discrimination features (Dani et al., 2001; van Wilgenburg et al., 2012). Methylalkanes and alkenes do not only occur in various chain lengths, but also the position of their methyl branch and double bond can be different (Dani et al., 2001; Châline et al., 2005; van Wilgenburg et al., 2012). Insects may have series of homolog methylalkanes on their cuticle with the methyl branch at the same position, but variable chain-lengths. It is largely unknown if the insects fail to discriminate those methylalkanes, or whether they regard them as “synonymes” comparable to human language and therefore “generalize” them (van Wilgenburg et al., 2010; Bos et al.,

2012). It is assumed that the occurrence of homolog compounds containing the same amount of information makes CHC profiles far less complex (van Wilgenburg et al., 2010). Chain-length and functional group of methylalkanes may be coded independently from each other (Bos et al., 2012). Not only the composition of a CHC profile matters, but also the relative proportions of the compounds may be important. The augmentation of single compounds naturally occurring on the insects' cuticle may affect the recognition signature, what implies that the relative proportions of some components are perceived (Dani et al., 2001; Dani et al., 2005).

Polar cuticular compounds

The insect cuticle is not solely covered with CHCs, but also with more polar lipids containing oxygenated functional groups like acids, alcohols, aldehydes, ketones, sterol esters and wax esters (esters of long-chain alcohols and long-chain acids) (Lockey, 1988; Buckner, 1993; Buckner, 2010). The introduction of a functional group into a lipid molecule generates a kink in the lipid chain that may result in an increased fluidity and a reduced melting point (Buckner, 2010). Some of those oxygenated cuticular lipids are derived from long-chain hydrocarbons by the introduction of oxygen during biosynthesis (ethers, epoxides, ketones, secondary alcohols, and their esters), while others are not (long-chain fatty acids, primary alcohols, aldehydes, wax esters, sterols, sterol esters, and acylglycerols) (Buckner, 2010). The most common cuticular compounds beside CHCs are esters, free fatty acids, primary and secondary alcohols, ketones and sterols, whereas triacylglycerols and phospholipids are not common cuticular compounds (Blomquist and Bagnères, 2010). The oxygenated cuticular lipids are like the CHCs also part of the protective barrier against desiccation and may also be used for chemical communication with other individuals (Lockey, 1988; Buckner, 1993; Buckner, 2010). But most cuticular lipids that are involved in chemical communication are hydrocarbons. And the majority of polar lipids with known pheromone function are volatiles produced by specialized glands and not part of the cuticular lipids (Buckner, 2010). One reason therefore might be, that the occurrence of oxygenated cuticular lipids is underestimated, because they are often not detectable without derivatisation using standard gas chromatography coupled with mass spectrometry (GC-MS) (Cvačka et al., 2006b; Millar, 2010). Further, studies that found oxygenated lipids on the cuticle of insects using solvent

extraction have to be treated with care, because they run the risk of extracting lipids from internal tissues and not from the cuticle.

Good examples for such oxygenated cuticular lipids that may be underestimated or extracted from internal tissues are triacylglycerols (TAG). Characteristic chemical features of TAGs are the position of the fatty acids on the glycerol backbone, the number and position of the double bonds and the chain length of the fatty acids (Cvačka et al., 2006c; Kofroňová et al., 2009). TAGs have high melting points and thus are not detectable with standard GC-MS technique without derivatisation into more volatile fatty acid methyl esters (FAME) (Cvačka et al., 2006a; Cvačka et al., 2006b; Millar, 2010). Nevertheless, cuticular TAGs have been identified on the cuticle of several insect species (Armold et al., 1969; Baker et al., 1979; Jackson, 1981; Juárez et al., 1984; Brey et al., 1985). But in most cases solvent extraction was used and therefore it is unclear, if the TAGs really originate from the cuticle and not from internal tissues (Hadley, 1980; Jackson, 1981; Jackson et al., 1981; Lockey, 1985; Buckner, 1993). The insects' fat body for example is known to store large amounts of TAGs (Downer and Matthews, 1976; Arrese and Soulages, 2010).

But recently, Yew et al. (2011a) revealed the occurrence of TAGs on the cuticle of *Drosophila mojavensis* and *D. arizonae* using a solvent free method. With ultraviolet laser desorption/ionisation orthogonal time-of-flight mass spectrometry (UV-LDI-o-TOF MS) they could demonstrate that male derived TAGs were transferred to the female anogenital region during copulation. The authors assumed that the TAGs might play a role in courtship of those *Drosophila* species. But a communicative function of TAGs has not been described until now. The potential occurrence of cuticular TAGs on the cuticular of the pteromalid wasp species *Lariophagus distinguendus* (**Chapter 3)** and other insects has been investigated within this work (**Chapter 5**).

Chemical identification of pheromones

The chemical analysis of a pheromone comprises at least three steps. The candidate substances have to be collected, purified and identified. At every step, pheromone functionality has to be checked in behavioural or physiological bioassays (Wyatt, 2003; Bradbury and Vehrencamp, 2013). Pheromones are often mixtures of many components that frequently occur only in amounts at or below the nanogram range and, thus, high resolution separation techniques such as gas chromatography (GC) are necessary to analyse them (Jones and Oldham, 1999).

Candidate pheromone substances are often collected by solvent extraction techniques; this involves the risk of co-extracting non-pheromone compounds or pheromone precursors from glands (Lockey, 1985; Buckner, 1993; Wyatt, 2003). Alternatively, solvent-free extraction methods, such as solid phase micro extraction (SPME) and so-called entrainment methods can be employed (Agelopoulos and Pickett, 1998; Everaerts et al., 2010). The SPME technique uses an inert fibre that is coated with a thin layer of an absorbent polymer and that is inserted into the headspace of a sample or wiped over a probe in order to sample the candidate pheromone molecules (Jones and Oldham, 1999; Wyatt, 2003, Everaerts et al., 2010). Subsequently, the fibre can be desorbed in the injector of a gas chromatograph (GC) (Wyatt, 2003). SPME technique is non-lethal for the analysed insects and can therefore be used to track changes in the pheromone composition (Peeters et al., 1999; Lacey et al., 2008; Ouyang et al. 2011) In **Chapter 3** and **Chapter 5**, a newly developed method for the analysis of cuticular triacylglycerides (TAG) by SPME is described. Entrainment methods (dynamic headspace analysis, closed loop stripping) concentrate volatile candidate pheromone molecules from the air by pumping it over a cold trap or an adsorbent, such as activated charcoal or a porous polymer (Agelopoulos and Pickett, 1998; Jones and Oldham, 1999). The trapped pheromones can be removed from the trap by heating or solvent elution.

As a second step (fractionation) the candidate pheromone molecules have to be separated, purified and concentrated. This is achieved by using chemical or physical features of the candidate pheromone molecules (Bradbury and Vehrencamp, 2013). Frequently used techniques include adsorption chromatography using silicia gel and florisil columns for the purification of crude extracts, size exclusion high performance liquid chromatography (SE-HPLC) and reversed-phase HPLC. Before the samples are analysed by GC techniques, so-called microreactions are sometimes performed in order to alter the chromatographic

properties of the molecules, to alter the functional groups for improved structure determination by mass spectrometry (MS) or to improve the discrimination of stereoisomers (Jones and Oldham, 1999).

For the identification of candidate pheromone molecules, GC is often used, either coupled with mass spectrometry (GC-MS) or with an electroantennographic detector (GC-EAD). If GC-EAD is used, the EAD records the electrical impulses of the chemoreceptors on the insect's antenna (Jones and Oldham, 1999; Wyatt, 2003; D'Ettore et al., 2004; Bradbury and Vehrencamp, 2013). One approach for identifying pheromones is to compare individuals that release a pheromone with non-releasers, e.g. males with females (Wyatt, 2003). Moreover, molecular biological and neurobiological approaches and techniques are used (Wyatt, 2003). If possible, the correct identification of the pheromone should be verified in a behavioural or physiological bioassay by using an artificial pheromone blend consisting of enantiopure synthetic compounds (Mori 2000).

Parasitoids

The term parasitoid refers to an holometabolous insect species whose larvae develop by feeding on the body of other arthropods, usually insects, and kill their host (Godfray, 1994). Approximately 10 % of all described insect species are parasitoids (Eggleton and Belshaw, 1992). Parasitoids are known to occur in the following insect orders: Lepidoptera, Neuroptera, Diptera, Coleoptera and Hymenoptera, which comprise three quarters of all parasitoid species (Eggleton and Belshaw, 1992).

Parasitoids can be divided into 'ectoparasitoids', which develop outside the host body and 'endoparasitoids', which develop within the host body. Further, in solitary species, only one parasitoid develops within a single host, whereas in gregarious species, many parasitoids develop in one host (Godfray, 1994). Idiobiont parasitoids immobilize the host and subsequently stop its further development. They are usually ectoparasitoids. Koinobiont parasitoids do not suppress the further development of the host and can occur as endoparasitoids and ectoparasitoids.

Parasitoids can have an important influence on the population density of their hosts and thus they are successfully used as biological control agents to defend crops/livestock against

agricultural pests (Greathead and Greathead, 1992; Godfray, 1994). Hosts have evolved numerous morphological and behavioural defence strategies against parasitoids (Gross, 1993). Sometimes, parasitoid females parasitize hosts that have previously been parasitized by themselves or by others (superparasitism) (van Alphen and Visser, 1990). Mostly, female parasitoids are aware that the host has previously been parasitized and try to avoid superparasitism, e.g. by the use of marking pheromones, because superparasitized hosts have a negative effect on their offspring number and quality (van Alphen and Visser, 1990). This can be explained on the grounds that parasitoids typically develop within and feed on one single host individual (Godfray, 1994), which only provides a limited amount of nutrition. However, when the number of hosts is limited within a habitat, superparasitism can represent an adaptive strategy of the female parasitoids (van Alphen and Visser, 1990).

The host selection process of female parasitoids can be divided into three steps: host habitat location, host location and host acceptance. Chemical cues are the most important regulation factors at every step of this selection process (Vinson, 1976).

Parasitoid sex pheromones

Parasitoids use semiochemicals for host location (Vinson, 1976), aggregation (Ruther, 2013) and host marking (van Alphen and Visser, 1990) and as sex pheromones (Ruther and Steidle, 2000; Steiner et al., 2007b; Ruther, 2013). Sexual communication in parasitoids can comprise the following three types of pheromones (Ruther, 2013). (1) Volatile compounds emitted by one sex to attract the opposite sex to the site of release. (2) Less volatile contact sex pheromones, e.g. cuticular lipids, mediate mate recognition and elicit stereotypic courtship behaviour. (3) Male parasitoids release, during courtship, an aphrodisiac pheromone from oral or antennal glands in order to elicit female receptiveness. But, oral male courtship pheromones may have a second function. In *Nasonia vitripennis* a mixture of ethyl oleate, ethyl linoleate, and ethyl α-linoleate has been shown to switch off the females response to the male courtship pheromone (Ruther and Hammerl, 2013). Additionally host-associated semiochemicals play often also a crucial role in parasitoid mate finding (Ruther and Steidle, 2000; Steiner et al., 2007b). Not only the composition of a sex pheromone is important, but also the amount of pheromone an individual can release. Females of *Nasonia vitripennis,* for

instance, prefer higher doses of the male sex pheromone, because the pheromone titre correlates with male quality parameters such as fertility and nutritional state (Ruther, 2013).

The Genus *Lariophagus*

The genus *Lariophagus* (Crawford 1909) is taxonomically located within the family of Pteromalidae (Order: Hymenoptera; Superfamily: Chalcidoidea; Family: Pteromalidae) and currently includes the following species: *Lariophagus distinguendus* (Förster 1841), *L. fimbriatus* (Boucek 1965), *L. puncticollis* (Möller 1882), *L. rufipes* (Hedquist 1978), *L. teutonus* (Dalla Torre 1898), *L. dryorhizoxeni* (Ashmead 1886), *L. kuwayamai* (Kamijo 1981), *L. obtusus* (Kamijo 1981) and *L. texanus* (Crawford 1909) (Noyes, 2013). The following *Lariophagus* species occur in Europe: *L. distinguendus* (Förster 1841), *L. fimbriatus* (Boucek 1965), *L. puncticollis* (Moller 1882), *L. rufipes* (Hedquist 1978) and *L. teutonus* (Dalla Torre 1898) (de Jong, 2013).

Lariophagus distinguendus

Lariophagus distinguendus (Förster, 1841; Hymenoptera, Pteromalidae) is a 'quasi-gregarious', 'idiobiont' and 'ectoparasitoid' wasp species that infests larvae and pupae of beetles, which feed on stored products (Steidle and Schöller, 1997; Hansen et al., 2013). The term quasi-gregarious means that single parasitoids emerge from each host-infested grain, whereby the hosts are mostly gregarious (Ruther and Steiner, 2008). *L. distinguendus* wasps are 2-3 mm long, whereby the females are larger than the males. Development from egg to imago is temperature dependent and takes at least 17 days at 25 °C (van den Assem, 1971). Adult female wasps perform host-feeding (Bellows 1985) and live about 3 weeks (van den Assem, 1970). *L. distinguendus* males are haploid and the females are diploid. Unmated females can only produce haploid sons, whereas mated females can produce haploid sons and by using male sperm, diploid daughters (van den Assem, 1971).

L. distinguendus is, with the exception of southern Africa, distributed worldwide and infests typically hosts from the following beetle families: Curculionidae (*Sitophilus granarius*, *S. oryzae*), Anobiidae (*Stegobium paniceum*, *Lasioderma serricorne*), Bostrychidae (*Rhizoperta dominica*), Ptinidae (*Ptinus tectus*, *Gibbium psylloides*), Bruchidae (*Callobruchus chinensis*, *C. maculatus*, *Bruchus brachialis*) (Steidle and Schöller, 1997). The host-parasite relationship between *L. distinguendus* and the granary weevil *Sitophilus granarius* (Linnaeus 1758) is described in Hase (1919), Steidle and Schöller (1997), Steidle (1998) and Ruther and Steidle (2000).

In the laboratory, *L. distinguendus* has successfully been reared on wheat grains (*Triticum aestivum*) infested with larvae of *S. granarius* according to the protocol of Steidle and Schöller (1997). The females of *L. distinguendus* can discriminate wheat grains parasitized by *S. granarius* from those that are unparasitized (Steidle and Schöller, 1997). When *L. distinguendus* females detect an infested grain, they start a behavioural sequence described by Hase (1924) and Steidle (2000). The female begins walking on the grain, followed by antennal drumming on the grain surface (drumming). Subsequently, the female again starts walking or she begins drumming on a small spot on the grain (selecting). Then, the female bends her abdomen forward, between her legs, and begins to tap the preselected spot on the grain surface with her abdominal tip (tapping). She fixes her ovipositor at the selected spot on the grain surface and unsheathes the ovipositor by bending her abdomen backwards. The ovipositor is inserted into the grain accompanied by drilling movements. The female probes the host with her ovipositor, lays one egg beside the host and injects venom into the host. Afterwards she withdraws her ovipositor from the grain, starts cleaning her abdomen with the hind legs in combination with drumming and performs host-feeding and host marking.

S. granarius larvae alone are not attractive to *L. distinguendus* females, whereas the combination of infested wheat grains with *S. granarius* larvae is attractive (Steidle and Schöller, 1997). The faeces of the larvae are a stimulus for both sexes, but learning also plays a role (Steidle and Schöller, 1997; Steidle, 1998; Ruther and Steidle, 2000). The faeces of the hosts can contain the alarm pheromone of the host-associated mould mite *Tyrophagus putrescentiae* (Ruther and Steidle, 2000). The chemical cues from the mites are associatively learnt, being highly attractive to both sexes of *L. distinguendus*, and consist of neral, geranial, neryl formate and tridecane (Ruther and Steidle, 2000).The faeces of mite-free *S. granarius* cultures also have an innate attractiveness to both sexes of *L. distinguendus* and can be regarded as host-associated kairomones (Steiner et al., 2007b). The males of *L. distinguendus*

are not attracted by female volatiles but seem to rely on the scent of the host faeces instead to find conspecific females (Ruther and Steidle, 2000; Steiner et al., 2007b).

L. distinguendus is commercially available and can be used for biological pest control (Steidle and Schöller, 2002; Querner and Biebl, 2011; Niedermayer and Steidle, 2013; Niedermayer et al., 2013). The wasps can locate parasitized grains in grain elevators up to a distance of 4 meters horizontally and vertically from the release point (Steidle and Schöller, 2002).

The contact sex pheromone of *Lariophagus distinguendus*

The mating system of *L. distinguendus* is characterized by local mate competition (LMC) (Werren and Simbolotti, 1989). The sex ratio is female-biased (2/3 female offspring). Males emerge on average 1.5 days before the females, because of their shorter development time (protandry) and sibling mating is common. Mating occurs close to the emerging site (Steiner et al., 2005; Steiner et al., 2008)**.**

When a male *L. distinguendus* wasp encounters an unmated female, it is arrested by a female contact sex pheromone, which is located on the female cuticle, and subsequently starts a stereotypical courtship behaviour. The courtship and mating behaviour of *L. distinguendus* has been described in detail by van den Assem (1970) and Ruther et al. (2000). The courtship begins with a sequence of high-frequency wing-fanning (Benelli et al., 2013) possibly accompanied by abdomen raising. The function of the male wing-fanning behaviour is not fully understood but the vibrations are species-specific and therefore could be a signal for the females or used to spread male pheromones (Ruther et al., 2000). The male then mounts the female and performs, with his antennae, cyclic stroking movements along the female antennae. The female signals her willingness to copulate by lowering her head and withdrawing her antennae. At the same time, she exposes her genital orifice. During antennal stroking, a male aphrodisiac might be transferred to the female antennae, thereby eliciting the female receptivity signal (Ruther et al., 2000), as is known in *Nasonia vitripennis* (van den Assem et al., 1980; Ruther et al., 2010). The male takes the mating position and mating occurs. Afterwards, the male typically shows post-copulatory behaviour, consisting of the same elements as the pre-copulatory courtship, i.e., wing-fanning, mounting, and antennal

stroking. The function of the post-copulatory behaviour is unclear but mate-guarding has been proposed (Ruther et al., 2000).

The majority of females mate only once during their lifetime (monandry) and seem to stop investment into pheromone production after mating (van den Assem, 1970; Ruther et al., 2000; Steiner et al., 2008). Van den Assem (1970) created so-called pseudo-virgin females by disturbing the courtship behaviour after the female had signalled her willingness to copulate, but before mating took place. These females remained in the mating position for a while and afterwards were no longer receptive, despite no mating had occurred.

The female contact sex pheromone is active within a range of 0 to 5 mm and is long-lasting, because females were still attractive 5 days after mating (Ruther et al., 2000). Freeze-killed virgin females even remained attractive to males for 4 months and more (van den Assem, 1970). This indicates that the female contact sex pheromone has a low-volatility. Dichloromethane extracts of *L. distinguendus* females applied to filter paper elicited courtship behaviour in males, whereas male-derived extracts and the pure solvent alone did not (Ruther et al., 2000). Extracts from the abdomen were more attractive than those from thorax and head, indicating that the pheromone is produced in the abdomen (Ruther et al., 2000).

The contact sex pheromone of *L. distinguendus* is produced during the pupal stage by both sexes but is actively degraded by males within 32 hours after emergence (Steiner et al., 2005). The males emerge 1.5 days before the females, because of a shorter development time (protandry), but not all males emerge simultaneously (Steiner et al., 2005). Thus, earlier emerged males have an advantage over their developing conspecifics as they can guard wheat grains containing female pupae. Therefore, developing males have been suggested to produce the contact sex pheromone to distract their earlier emerged rivals away from females about to emerge and thus to increase their own mating chances. The loss of attractiveness in aging males correlates with the disappearance of a few cuticular compounds including straight-chain alkanes and methylalkanes. The main compound that vanishes from the cuticle of aging males is 3-methylheptacosane (3-MeC27) (Steiner et al., 2005). After emergence, the presence of the contact sex pheromone is disadvantageous for young males, because homosexual courtship behaviour of conspecifics has been shown to reduce their mating chances when competing with older males for virgin females (Ruther and Steiner, 2008).

An evolutionary scenario has been proposed to explain the function of cuticular lipids as a contact sex pheromone in *L. distinguendus* (Ruther and Steiner, 2008). According to this

scenario, the cuticular lipids had a non-pheromonal primary function in both sexes (e.g. prevention of desiccation). Subsequently, males have evolved to exploit the species specificity of the cuticular lipid profile for the localization of grains containing females about to emerge (Steiner et al., 2005;). At this stage, however, males were unable to discriminate the gender of the developing wasps (Steiner et al., 2005). In a second step, the males evolved the capability to deactivate the pheromone by removing distinct compounds from their cuticle and, thus, the pheromone specificity shifted from the species to the sex level (Ruther and Steiner, 2008).

Steiner et al. (Steiner et al., 2005, 2007a) identified more than 60 compounds in whole body extracts of *L. distinguendus* by GC-MS analysis. The majority of these compounds were hydrocarbons: straight-chain alkanes, alkenes with one double-bond (monoenes), methyl-branched alkanes with one to four methyl groups and squalene. Moreover, cholesterol was found. The chain-lengths of the identified hydrocarbons was between 25 and 37 carbon atoms (Steiner et al., 2005, 2007a). The most abundant compounds were 3,7,11,15-TetraMeC33, 11,21-DiMeC33 and 13,17-DiMeC35. Pupae, freshly emerged males and females as well as 72-h-old females possessed some hydrocarbons, especially 3-MeC27, which were absent on the cuticle of 72-h-old male wasps (Steiner et al., 2005, 2007a). The disappearance of these compounds correlated with the loss of the behavioural activity of the cuticular lipids and seemed to be an active process, because newly emerged males killed by freezing remained behaviourally active for days whereas live males deactivated the pheromone within the same time period (Steiner et al., 2005, 2007a). The authors suggested that the lipid carrier protein lipophorin is involved in the disappearance of the behaviourally active compounds.

Steiner et al. (Steiner et al., 2005) have found that only the hexane fraction of an behaviourally active extract elicits male arrestment and wing-fanning. Therefore, they suggested that the contact sex pheromone of *L. distinguendus* consists of non-polar hydrocarbons. In a follow-up study Steiner et al. (Steiner et al., 2007a) analysed the composition of whole body extracts of *L. distinguendus* by a principal component analysis (PCA) and a partial least squares-discriminant analysis (PLS-DA). They found that the methylalkanes 3-MeC27, 3,7-DiMeC27, 4,8-DiMeC28, 3-MeC29, 3,7-DiMeC29 and 13-MeC29 and the monoenes C27:1(9) and C29:1(7) had the highest discriminating power.

Open questions – outline of the doctoral thesis

The CHC profiles of insects play an important role in species and gender recognition and CHCs often function as contact sex pheromones (Blomquist and Bagnères, 2010). Because pheromone precursors are taken up by nutrition (Tillman et al., 1999), a host shift in parasitoids may result in different CHC profiles and finally can end up in the reproductive isolation of the host races. The effects of a host shift on the wasps CHC profiles have been tested in **Chapter 2** by comparing the CHC profiles of *L. distinguendus* males and females from spatially isolated strains reared either on the same host species or on different host species. Further, it has been tested, if the CHC profiles of *L. distinguendus* wasps are distinguishable even one generation after a host shift from *Sitophilus granarius* (Curculionidae) to *Stegobium paniceum* (Anobiidae).

As described above, CHCs have a contact sex pheromone function in *L. distinguendus* and elicit courtship behaviour including wing-fanning in male wasps. Pupae and young males of *L. distinguendus* also produce bioactive CHCs but they lose their attractiveness towards conspecific males within 32 h after emergence (Steiner et al., 2005). This loss in attractiveness is accompanied by the disappearance of 3-MeC27 and other minor compounds from the cuticle. This raises several questions (**Chapter 3**): Is 3-MeC27, which disappears from the male cuticle, causally involved in the contact pheromone function of CHCs? Is 3-MeC27 alone able to elicit courtship behaviour in male *L. distinguendus* wasps? If so, do males respond enantioselectively? Can the bioactivity of aged males be re-installed by the addition of synthetic 3-MeC27?

If 3-MeC27 is the key-component of the female contact sex pheromone in *L. distinguendus*, can it be replaced by structurally related methylalkanes that differ in their chain length or the position of the methyl-branch (**Chapter 4**)? On the contrary, does the application of additional structurally related methylalkanes interfere with the intact cuticular profile (**Chapter 4**)?

In addition to CHCs, more polar compounds such as alcohols, aldehydes, ketones, wax-esters and triacylglycerides can occur on the insects cuticle (Buckner, 2010). The occurrence of such compounds is often ignored in studies that analyse insect cuticular lipid profiles, because they are not always detectable with standard GC-MS techniques (Cvačka et al., 2006b; Millar, 2010). Do such polar compounds occur on the cuticle of *L. distinguendus* wasps? If so, which functions do they have (**Chapter 3**)?

The extraction of cuticular compounds with the help of solvents, bears the risk of co-extracting other compounds from deeper layers of the cuticle or from internal glands and tissues (Lockey, 1985; Buckner, 1993; Wyatt, 2003) and thus it is often unclear, if those compounds really occur on the cuticle and are perceptible for other insects. The solid phase microextraction (SPME) technique has been used as an alternative to solvent extraction for the analysis of cuticular CHCs (Jones and Oldham, 1999; Everaerts et al., 2010). However, SPME has not as yet been used for the analysis of more polar cuticular compounds such as long time ignored triacylglycerides (TAGs). In **Chapter 5**, commonly used solvent extraction is compared with SPME-FAME-GC-MS, which is described here as a new solvent-free technique for the analysis of cuticular TAGs following *in situ* transesterification with trimethyl sulfonium hydroxide (TMSH) into more volatile fatty acid methyl esters (FAME) that can be analysed by standard GC-MS.

Chapter 2

Composition of cuticular lipids in the pteromalid wasp *Lariophagus distinguendus* is host dependent

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Authorship: **Stephan Kühbandner**[1], **Kerstin Hacker**[2], **Steffi Niedermayer**[2], **Johannes L.M. Steidle**[2] and **Joachim Ruther**[1]

[1]Institute of Zoology, University of Regensburg, Regensburg

[2]Institute for Zoology, University of Hohenheim, Stuttgart

2.1 Abstract

The insect cuticle is covered by a thin layer of hydrocarbons not only preventing desiccation but also playing an important role in the sexual communication of several species. In the pteromalid wasp *Lariophagus distinguendus*, a parasitoid of grain infesting beetles, female cuticular hydrocarbons (CHCs) elicit male courtship behaviour. We analyzed the CHC profiles of male and female *L. distinguendus* wasps reared on different beetle hosts by coupled gas chromatography-mass spectrometry (GC-MS). Statistical analysis of the data revealed significant differences between strains reared on different hosts, while spatially isolated strains reared on the same host produced similar profiles. CHC profiles of parasitoids reared on *Stegobium paniceum* were statistically distinguishable from those of wasps reared on all other hosts. A host shift from *Sitophilus granarius* to *S. paniceum* resulted in distinguishable CHC profiles of *L. distinguendus* females after only one generation. Considering the role of CHCs as contact sex pheromones, our data suggest that host shifts in parasitic wasps might lead to reproductive isolation of host races due to the modification of the cuticular semiochemistry.

2.2 Introduction

Cuticular hydrocarbons (CHCs) of insects function mainly as a water barrier to avoid desiccation, but also play an important role in intraspecific communication. Because of their low volatility, they act mostly over short distances as contact pheromones (Singer, 1998; Gibbs, 2002; Blomquist and Bagnères, 2010). CHC profiles are complex mixtures of aliphatic long-chain alkanes and alkenes, as well as methylbranched alkanes. Described functions of CHCs comprise the mediation of recognition, aggregation, dispersal, alarm and sexual behaviour in insects (Howard, 1993; Tillmann et al., 1999; Blomquist and Bagnères, 2010). While social insects also use CHCs for recognition and interaction with nestmates and as fertility and dominance signals (Singer, 1998; Liebig, 2010), solitary insects mainly use CHCs for the discrimination of conspecifics and enemies, location of mating partners and the elicitation of courtship behaviour (Ruther et al., 2011). Evidence for solitary insects using CHCs as contact sex pheromones comes from several insect orders, for example the Coleoptera (Buprestidae: Lelito et al., 2009; Silk et al., 2009; Cerambycidae: Ginzel, 2010; Chrysomelidae: Sugeno et al., 2006; Peterson et al., 2007; Geiselhardt et al., 2009), Diptera (Drosophilidae, Glossinidae and Muscidae: Wicker-Thomas, 2007; Ferveur and Cobb, 2010) and Hymenoptera (Syvertsen et al., 1995; Schiestl et al., 2000; Sullivan, 2002; Mant et al., 2005; Steiner et al., 2005, 2006, 2007a). Within the parasitic wasp family Pteromalidae, females of *Roptrocerus xylophagorum* (Sullivan, 2002), *Lariophagus distinguendus* (Steiner et al., 2005, 2007a), *Nasonia vitripennis* (Steiner et al., 2006) and *Dibrachys cavus* (Ruther et al., 2011) produce CHCs which act at short-range as contact sex pheromones eliciting courtship behaviour in males.

The diet of an insect can be an important factor influencing its pheromone communication (Landolt and Phillips, 1997; Tillmann et al., 1999; Blomquist, 2010). With respect to CHCs, three ways of acquisition are conceivable which are not mutually exclusive: (a) CHCs may be sequestered from the diet after ingestion, (b) absorbed from the environment, or (c) synthesized de novo in oenocytes from dietary precursors (Blomquist and Jackson, 1973; Etges et al., 2006; Bagnères and Blomquist, 2010). In the case of parasitic wasps, the first two ways are of particular interest because, due to their parasitic life cycle, these insects have been suggested to have lost the ability to biosynthesize fatty acids (Visser et al., 2010), i.e. the same machinery involved in CHC biosynthesis (Blomquist, 2010). However, the way how parasitic wasps acquire their CHCs and how the composition is controlled is not well understood. In any case, resources provided by the host should be of crucial importance for the cuticular chemistry.

Because of the influence of diet on the pheromone chemistry of insects, it is reasonable to assume that changes in the diet, e.g. caused by host switches in phytophagous or carnivorous insects, may lead to a breakdown in communication between mating partners and may ultimately contribute to the formation of host races and speciation. In fact, an example for such a host shift-induced breakdown in communication is reported for *Drosophila serrata* and *D. melanogaster*. In these species, the development on different substrates was found to induce differences in the CHC profiles, leading to preferential mating of individuals from the same substrates (Rundle et al., 2005; Sharon et al., 2010). A similar scenario is also thinkable in oligophagous and polyphagous parasitic wasps. Different hosts may provide different pools of precursors for CHC biosynthesis or different CHCs to be sequestered by the wasps. Consequently, feeding on different hosts might lead to differences in the CHC profiles of male and female wasps of one population, causing a breakdown in sexual communication and eventually leading to speciation.

As a first step to study this hypothesis, the present paper examines the influence of hosts on the CHC profile in *Lariophagus distinguendus*, a quasi-gregarious and polyphagous ectoparasitoid of grain infesting beetles (Steidle and Schöller, 1997). Female cuticular hydrocarbons have been shown to arrest males and elicit wing fanning, a typical element of the male courtship behaviour (Steiner et al., 2005). Interestingly, also pupae of both sexes and newly emerged males elicit courtship behaviour in older males. Unlike females, however, males deactivate the behaviourally active chemicals within 32h after emergence (Steiner et al., 2005, 2007a; Ruther and Steiner, 2008). Thus, CHCs evolved to a sex-specific contact pheromone mediating mate recognition in *L. distinguendus*. We analyzed the CHCs of wasps from six strains reared on four different host species and analyzed the relative composition of the profiles by multivariate statistical methods. Our questions were: Are there differences between the CHC profiles from strains reared on different hosts? Do these differences occur in both sexes? Which compounds account for the differences in CHC profile? Can differences in the CHC profiles be caused by a host shift on an alternative host already within one generation? Do the wasps directly sequester significant amounts of host CHCs? The results are discussed with respect to a possible role of CHCs in prezygotic isolation and sympatric speciation.

2.3 Materials and methods

Insects

Six strains of *L. distinguendus* were reared on four different beetle species as hosts as described by Steidle and Schöller (1997) (table 1). The wasp cultures were kept in Petri dishes at 25 °C, L16:D08 photoperiod and 50 % RH. Freshly emerged wasps were isolated and kept under the same conditions for two days. Afterwards, they were deep frozen and stored at -23 °C until they were extracted for chemical analysis.

Table 1. Investigated *Lariophagus distinguendus* strains reared on different beetle hosts.

	Host species	Host substrate	Reared on host since	*n* ♂	*n* ♀	Strain-origin
BerSit	*Sitophilus granarius* (Curculionidae)	wheat-grains	2003	10	12	Berlin
PfoSit	*Sitophilus granarius* (Curculionidae)	wheat-grains	2005	10	14	Pforzheim
StuSteg	*Stegobium paniceum* (Anobiidae)	koi fish pellets	2007	10	15	Stuttgart
RavSteg	*Stegobium paniceum* (Anobiidae)	koi fish pellets	2008	8	14	Ravensburg
BerAca	*Acanthoscelides obtectus* (Bruchidae)	black-eyed peas	2008	10	15	Berlin
BerLas	*Lasioderma serricorne* (Anobiidae)	wheat-grains	2004	10	13	Berlin

n, number of samples in chemical analysis.

Chemical analysis

For the analysis of CHCs, three *L. distinguendus* individuals from the same strain and sex were pooled and extracted for 15min in 30 µl of hexane containing tetracosane (2.6 ng μl^{-1}) as an internal standard. The solvent was evaporated under a gentle stream of nitrogen, and the sample was re-dissolved in 10 µl of hexane. To investigate the possible sequestration of ingested host-derived CHCs by the parasitoid, we also analyzed the CHC profiles of the four beetle hosts both in the larval and in the adult stage following the protocol described above (n=3 for each host species and stage, respectively). Aliquots (1 µl in splitless mode) of these extracts were analyzed by coupled gas-chromatography mass spectrometry (GC-MS) on a Shimadzu GCMS-QP2010 Plus quadrupole MS (Shimadzu, Tokyo, Japan) equipped with 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 column flow of 1.73 ml min^{-1}. The oven program started at 150 °C and was increased at 3° C min^{-1} up to 300 °C (held for 20 min). The GC effluent was ionized by electron impact ionization at 70 eV; the mass range reached from m/z 35 to m/z 600.

Relative retention indices (LRI) of methyl-branched and unsaturated hydrocarbons were estimated by co-injection of straight-chain hydrocarbons (van den Dool and Kratz, 1963). Methyl-branched hydrocarbons were identified by diagnostic ions resulting from the favoured fragmentation at the branching points (Lockey, 1988; Nelson, 1993) and by comparing LRI values with literature data (Carlson et al., 1998a; Steiner et al., 2005, 2006, 2007a). Positions of the double bonds of unsaturated hydrocarbons were determined by iodine-catalyzed methylthiolation using dimethyl disulphide (Francis and Velant, 1981; Howard, 1993). MS and LRI data of identified compounds were used to build a custom MS library allowing automatic analysis of GC-MS runs with the help of a two-dimensional search algorithm (MS+LRI) using the GC-MS Solution scientific software (Shimadzu) of the mass spectrometer.

Host shift experiment

Female wasps from the BerSit strain kept on *Sitophilus granarius* (F0) were reared for one generation on *Stegobium paniceum* (F1). The CHC profiles of the wasps from the F1 generation were analyzed as described above. The resulting data were compared to those from female wasps, which were reared at the same conditions but without a host shift (F1 *S. granarius*).

Statistical analysis

We integrated the 50 largest peaks (by area) within each run (overlapping compounds were calculated together). All peaks larger than 1 % of the whole peak area were selected for further analysis. The absolute amount of each compound was calculated by relating individual peak areas to the internal standard. Statistical analysis was conducted with PAST version 2.01 scientific software (Hammer et al., 2001). We used the non-metric multidimensional scaling (NMDS, Bray- Curtis similarity measure) to visualize the data and the nonparametric MANOVA (NPMANOVA, Bray-Curtis similarity measure of Bonferroni-corrected data) for calculation of the differences between CHC profiles of wasps from the different hosts. Similarity percentage (SIMPER) was used to calculate the individual contribution of each peak to the differences between wasps from different hosts.

2.4 Results

The CHC profiles of the wasps consisted mainly of methylbranched long chain alkanes. For the analysis of female and male profiles, 33 and 30 compounds, respectively, were used (table 2–3). Overall, 83 female and 58 male samples were analyzed. Data of wasps originating from different strains but reared on the same host species (table 1) were pooled for statistical analysis since there were no significant differences in the NPMANOVA analysis between strains reared on the same host-species (*S. granarius* Berlin vs. Pforzheim: P=1 (males), P=0.9285 (females); *S. paniceum* Stuttgart vs. Ravensburg: P=1 (males); permutation n=10,000) with the exception of female wasps grown on *Stegobium paniceum* from the Ravensburg and the Stuttgart strain (P=0.0045).

Differences in CHC profiles of females reared on different host-species

The NMDS analysis of the CHC profiles of *L. distinguendus* females reared on different beetle species as hosts (fig. 1A) showed a distinct separation between a cluster consisting of wasps from *S. granarius*, *A. obtectus* and *L. serricorne* and a cluster of wasps from *S. paniceum*. While wasps from *S. granarius* and *A. obtectus* overlapped fully, *L. serricorne* was concentrated at one edge of the cluster. This is also reflected in the NPMANOVA analysis, which gave significant differences between all hosts ($P<0.05$; permutation n=10,000) with the exception of *S. granarius* and *A. obtectus* (P=0.1566). The SIMPER analysis allowed identification of compounds contributing most to the dissimilarity of the CHC profiles of wasps from different beetle hosts. The compounds with the strongest impact were: 3,7,11,15-tetramethyltritriacontane, 11,21- +11,15-dimethyltritriacontane, 3,7,11-trimethyltritria-contane, and an unknown compound with an LRI of 3089 (table 2).

Differences in CHC profiles of males reared on different host-species

In *L. distinguendus* males reared on different beetle species, NMDS indicated also a separation of a cluster consisting of males from *S. granarius* and *A. obtectus* and a cluster consisting of males from *S. paniceum* (fig. 1B). Males from *Lasioderma serricorne* were located in a third cluster intermediate to the others. While wasps from *S. granarius* and *A. obtectus* overlapped, the other clusters did not. This result was also supported by NPMANOVA analysis, which revealed significant differences between all hosts ($P<0.05$; permutation n=10,000) with the exception of wasps from *S. granarius* and *A. obtectus* ($P=1$). SIMPER analysis of the male profiles revealed 3,7,11,15-tetramethyltritriacontane, 13,17-dimethylpentatriacontane, 3-methyltritriacontane and 11,21- +11,15-dimethyl-tritriacontane as compounds with major influence on the dissimilarity of the CHC profiles of wasps from different beetle hosts (table 3).

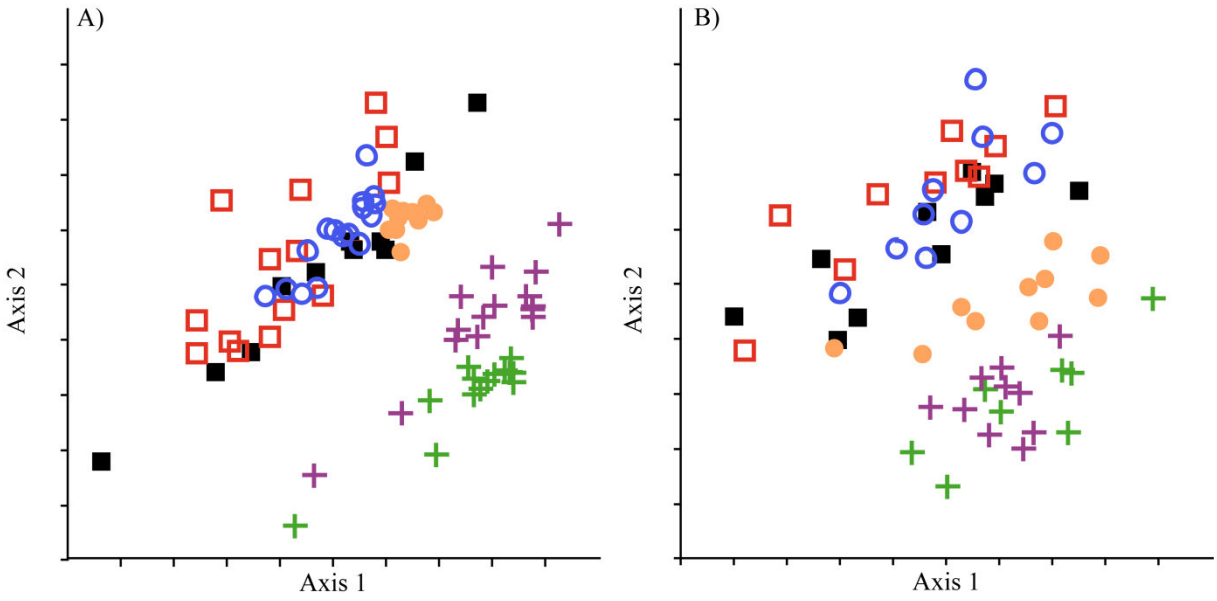

Figure 1 Non-metric multidimensional scaling (NMDS; Bray-Curtis similarity measure) of the CHC profiles of *L. distinguendus* females (A) and males (B). Hosts: □ = *S. granarius* (Pforzheim); ■ = *S. granarius* (Berlin); + = *S. paniceum* (Ravensburg); + = *S. paniceum* (Stuttgart); o = *A. obtectus* (Berlin); ● = *L. serricorne* (Berlin)

Table 2. Similarity Percentage (SIMPER) analysis of the *L. distinguendus* females CHC-profiles (overall average dissimilarity: 29.19)

Compound:	LRI[1]	Contribution	Cumulative %	Mean abund. 1	Mean abund. 2	Mean abund. 3	Mean abund. 4
3,7,11,15-TetraMeC33	3442	3.1	10.46	32.7	50.1	37.1	50.3
11,21- + 11,15-DiMeC33	3350	2.053	17.38	22.5	10.2	25.3	24.5
unknown	3089	1.6	22.78	3.43	14	4.46	15.3
3,7,11-TriMeC33	3425	1.517	27.89	16.8	23.4	17.6	22.6
3,7-DiMeC33	3404	1.451	32.79	18.3	20.9	20.9	22.7
15- + 13- + 11-MeC33	3328	1.433	37.62	17.9	10.6	19	20.2
4,8-DiMeC34	3477	1.277	41.93	6.23	10.5	9.22	8.88
3-MeC33	3374	1.256	46.17	15.5	22.7	18.8	21.8
13,17-DiMeC35	3541	1.256	50.4	21.8	19.4	21.5	22.3
3-MeC27	2773	1.143	54.26	5.35	12.8	3.65	8.61
C33:1(9)	3280	1.106	57.99	5.38	13	5.11	3.45
unknown	3394	0.975	61.28	2.92	5.61	2.65	3.13
3-MeC35 + 5,9DiMeC35	3569	0.9647	64.53	2.85	10.3	3.47	5.03
C27:1(9) + 3-MeC26	2675	0.9599	67.77	0.467	8.46	0	0
3,7-DiMeC27	2805	0.7861	70.42	3.25	7.02	0.911	0.626
unknown	3562	0.775	73.03	3.82	0.696	4.65	4.85
unknown	3411	0.7719	75.64	0.928	4.2	1.68	2.26
17- + 15- + 13- + 11-MeC35	3517	0.7222	78.07	7.87	12.4	8.24	11.5
C33	3300	0.7127	80.48	5.29	1.52	7.55	6.33
3,7,11,15-TetraMeC35	3631	0.6681	82.73	4.94	5.57	6.45	10.4
C25	2500	0.6353	84.87	3.71	5.17	0.655	1.17
Cholesterol	3122	0.4739	86.47	4.67	4.77	3.56	3.04
C27	2700	0.4674	88.05	4.05	4.17	2.11	1.45
15- + 13- + 11-MeC31	3130	0.4286	89.49	4.06	1.89	2.83	5.79
C29:1(9)	2877	0.4049	90.86	0.283	3.6	0	0
unknwon	3606	0.4026	92.22	0.653	3.47	0.739	0.715
C30:1(9)	3017	0.3987	93.56	1.59	1.58	2.13	5.84
C33	3300	0.3958	94.9	1.03	0.376	1.1	4.03
5,9-DiMeC25	2577	0.3336	96.02	0.283	2.81	0	0.138
19- + 17- +15- +13- + 11-MeC37	3711	0.3084	97.06	4	3.92	4.05	4.75
C32 + 3,7-DiMeC31	3204	0.2523	97.91	2.68	3.68	2.44	2.84
3-MeC31	3173	0.2286	98.68	1.87	3.16	1.7	2.59
3,7,11-TriMeC31	3425	0.2191	99.42	2.95	3.42	2.66	3.71

[1]Linear Retention Index according to van den Dool and Kratz (1963)

Table 3. Similarity Percentage (SIMPER) analysis of the *L. distinguendus* males CHC-profiles (Overall average dissimilarity: 30.92)

Compound:	LRI[1]	Contribution	Cumulative %	Mean abund. 1	Mean abund. 2	Mean abund. 3	Mean abund. 4
3,7,11,15-TetraMeC33	3442	3.927	12.7	10.5	24.2	9.22	21.2
13,17-DiMeC35	3541	3.04	22.53	15.5	25.1	12.9	16.2
3-MeC33	3376	2.535	30.73	8.97	19	8.36	12.7
11,21- + 11,15-DiMeC33	3351	2.474	38.73	19.9	13.1	19.2	15.8
3,7,11-TriMeC33	3426	1.916	44.93	5.58	12.5	5.72	8.6
unknown	3567	1.854	50.92	2.25	10.4	2.41	3.73
15- + 13- + 11-MeC33	3330	1.589	56.06	12.2	7.8	8.55	7.02
3,7-DiMeC33	3406	1.462	60.79	8.98	12.3	8.3	11.6
C33	3302	1.294	64.97	6.36	2.19	6.21	7.27
17- + 15- + 13- + 11-MeC35	3520	1.227	68.94	4.26	8.56	3.25	4.65
unknown	3396	0.8648	71.74	0.47	4.29	0.452	0.569
unknown	3126	0.8101	74.36	3.38	0.912	2.51	1.25
C25	2501	0.734	76.73	2.85	1.82	0.986	0.0863
3,7,11,15-TetraMeC35	3632	0.6825	78.94	2.13	3.22	1.6	5
unknown	3816	0.6115	80.92	1.69	0.0115	1.24	2.78
15- + 13- + 11-MeC31	3132	0.6087	82.88	2.88	0.401	1.11	0.813
19- + 17- + 15- + 13- + 11-MeC37	3713	0.597	84.81	4.29	4.81	3.47	4.95
C33:1(9)	3282	0.5588	86.62	2.33	1.11	2.02	0.496
C27	2702	0.4812	88.18	2.01	0.869	0.973	0.164
C32 + 3,7-DiMeC31	3206	0.4105	89.51	0.74	1.91	0.354	0.0949
C31	3102	0.4002	90.8	2.15	0.752	2.06	1.65
unknown	3453	0.3946	92.08	0.201	0.4	0.323	2.53
4,8-DiMeC34	3478	0.3849	93.32	2.06	2.82	2.22	2.37
unknown	3589	0.3541	94.47	0.0913	1.72	0.153	0.331
3-MeC31	3175	0.3456	95.58	1.15	2.27	0.992	0.7
unknown	3150	0.3332	96.66	1.63	0.536	1.47	0.557
unknown	2797	0.3173	97.69	1.23	0.643	1.78	0.889
C29	2902	0.2743	98.57	1.41	0.476	1.16	0.627
3247	3249	0.2208	99.29	1.33	1.07	0.822	0.703
unknown	3340	0.2204	100	1.11	0.667	1.42	0.98

[1]Linear Retention Index according to van den Dool and Kratz (1963)

Host shift experiment

NMDS-analysis of female wasps (fig. 2) showed a clear separation between clusters formed by CHC profiles of wasps reared on *S. granarius* and *S. paniceum* in the F1 generation. This result was supported by NPMANOVA analysis, which revealed significant differences between both strains ($P<0.05$; permutation n=10,000). The compounds with the highest influence on the dissimilarity of profiles were 3,7,11,15-tetramethyltritriacontane, 11,21- +11,15-dimethyltritriacontane, 13,17-dimethylpentatriacontane and the peak belonging to the co-eluting compounds 15- +13- +11-methyltritriacontane (SIMPER analysis; table 4).

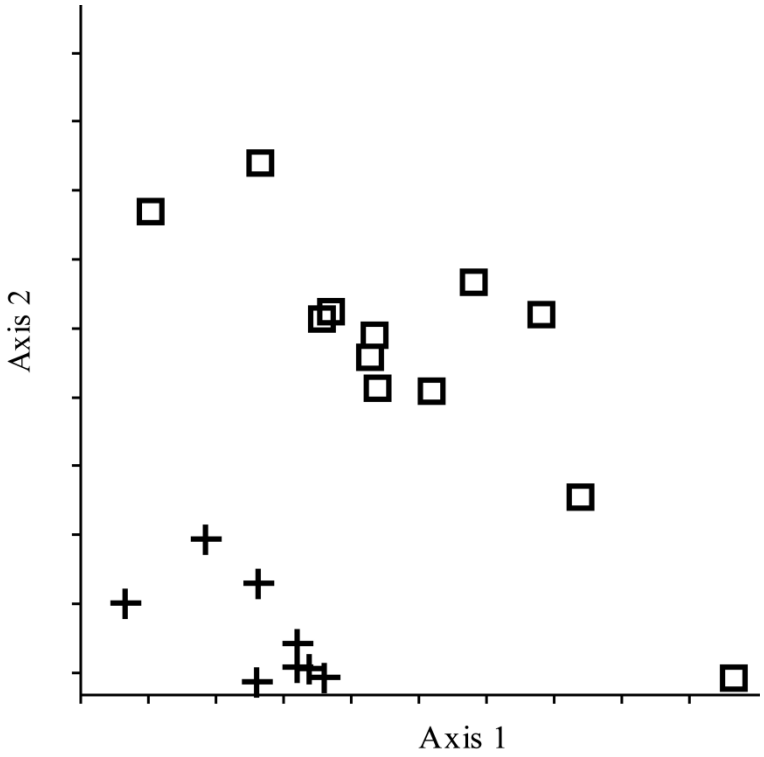

Figure 2 Host shift experiment: Non-metric multidimensional scaling (NMDS; Bray-Curtis similarity measure) of the CHC profiles of *L. distinguendus* females Hosts: □ = *S. granarius* (BerSit; F1); + = *S. paniceum* (former BerSit; reared for one generation on *S. paniceum*; F1)

Table 4. Similarity Percentage (SIMPER) analysis of CHC-profiles of *L. distinguendus* wasps from the host shift experiment (Overall average dissimilarity: 25.76)

Compound:	LRI[1]	Contribution	Cumulative %	Mean abund. 1	Mean abund. 2
3,7,11,15-TetraMeC33	3443	4.507	17.49	62.1	39.9
11,21- + 11,15-DiMeC33	3350	3.206	29.94	38.6	24
13,17-DiMeC35	3542	2.185	38.42	30.9	21.1
15- + 13- + 11-MeC33	3329	1.909	45.83	25.8	18.7
3-MeC33	3374	1.433	51.39	24.2	17.9
3,7,11-TriMeC33	3425	1.412	56.87	24.5	19.1
unknown	3089	1.294	61.89	10.7	4.16
3,7-DiMeC33	3405	0.9706	65.66	21.8	20.4
unknown	3124	0.9154	69.21	0.142	5.26
C30:1(9)	3016	0.8262	72.42	5.74	1.37
C33:1(9)	3280	0.8067	75.55	1.54	5.71
4,8-DiMeC32	3285	0.6666	78.14	3.85	0.712
3-MeC27	2773	0.6603	80.7	5.99	4.48
17- + 15- + 13- + 11-MeC35	3517	0.5934	83	9.96	8.16
unknown	2173	0.5399	85.1	0	3.31
4,8-DiMeC34	3477	0.5168	87.1	2.55	5.27
C33	3300	0.51	89.08	3.63	6.19
15- + 13- + 11-MeC31	3129	0.4221	90.72	4.88	4.06
3,7,11,15-TetraMeC35	3631	0.3923	92.24	5.66	6.26
C25	2499	0.3136	93.46	0.0577	1.83
unknown	2801	0.2619	94.48	0.778	1.88
unknown	3394	0.2507	95.45	1.39	1.9
C27	2700	0.2479	96.41	1.05	2.14
3,7,11-TriMeC31	3227	0.2389	97.34	2.64	2.91
19- + 17- + 15- + 13- + 11-MeC37	3712	0.229	98.23	3.74	3.28
C32 + 3,7-DiMeC31	3204	0.1951	98.99	2.26	2.68
3-MeC31	3173	0.1365	99.52	1.84	1.93
C29	2900	0.1247	100	1.35	1.78

[1]Linear Retention Index according to van den Dool and Kratz (1963)

Comparison of CHC profiles from hosts and parasitoid

To investigate the possible direct sequestration of CHCs from the host into the parasitoid, we compared the CHC profiles of beetle hosts and the respective parasitoids. These analyses revealed that host CHCs cannot account for the observed major differences of the CHC profiles because the CHC profiles of the wasps are generally composed of compounds with higher molecular masses when compared to larval and adult stages of the respective beetle hosts (for comparative fingerprint chromatograms see figs S1–4 in the supplementary material). The major compound of the parasitoids are almost absent in the hosts. Conversely, several major compounds of the beetle hosts occurred only in traces in the CHC profiles of the wasps or were completely absent. Apart from *A. obtectus*, CHCs were hardly present in cuticular extracts from larvae.

2.5 Discussion

The chemical analysis of CHC profiles of female and male *L. distinguendus* wasps reared on different beetle hosts revealed significant quantitative differences. These were not only observed between strains from different hosts but also between individuals from the same strain which were reared on the two hosts, *S. granarius* and *S. paniceum*. In contrast, the profiles of wasp strains reared on the same host species were similar with the exception of female wasps from the Ravensburg and Stuttgart strain reared on *S. paniceum*. Furthermore, some of the compounds with major influence on the differences between wasp strains reared on *S. granarius* or *S. paniceum* were also found to be important in the host-shift experiment. Thus, the CHC profiles of *L. distinguendus* are indeed host dependent.

Remarkably, the differences in CHC profiles between wasps from *S. granarius* and *S. paniceum* were present already after one generation and did not require several generations to develop. This indicates that the presence and quantity of compounds in the CHC profiles do not depend on strain related features but are presumably caused by host-dependent precursors in the diet of the wasps. Although direct incorporation of host CHCs can only be demonstrated by labelling experiments (Blomquist and Jackson, 1973), which, to our knowledge, have never been performed in parasitic wasps, this is unlikely in *L. distinguendus*. Neither larvae nor adults of the four beetle hosts had significant amounts of the parasitoids' major CHCs on their cuticle, and vice versa many major components of the beetle CHCs were absent from the wasps' cuticle or occurred only in traces. This suggests that the host species

influences the wasps' own CHC metabolism rather than serving as a direct source for CHC sequestration. The published literature on parasitoid/host CHCs does not provide a clear picture of whether parasitoids are able to sequester significant amounts of host CHCs or not. Some species share major components with their hosts (see for instance Howard and Liang, 1993; Howard and Infante, 1996); whereas, in other studies, host and parasitoid profiles differed clearly (Howard and Perez-Lachaud, 2002). Like in the present study, the qualitative composition of the CHC profile was largely independent from the host in the bethylid wasp *Cephalonomia hyalinipennis* and the pteromalid wasp *Pteromalus cerealellae*, whereas the relative quantities of the components differed (Howard, 2001; Howard & Perez-Lachaud, 2002). The biosynthesis of CHCs is closely associated with the fatty acid metabolism. For the synthesis of methyl-branched compounds, considerable amounts of valine, leucine, isoleucine, and methionine are also needed (Blomquist, 2010). These amino acids are among the essential dietary resources for insects, which cannot be biosynthesized de novo by themselves (Behmer, 2006). Hence, differing pools of limiting primary nutrients provided by the different hosts might account for the observed differences in the CHC profiles.

Interestingly, the CHC profiles of *L. distinguendus* wasps reared on *A. obtectus* and *S. granarius* overlapped in NMDS analysis, and the CHC profiles of wasps reared on *L. serricorne* cluster close to this group and are well separated from the CHC profiles of wasps reared on *S. paniceum*. Thus, the CHC profiles of wasps from these non-related hosts are more similar than the CHC profiles of those reared on *L. serricorne* and *S. paniceum*, which belong to the same family. It is most likely that *S. granarius*, *A. obtectus* and *L. serricorne* represent similar food substrates and provide qualitative and quantitative similar precursors for the CHCs of *L. distinguendus*, despite their phylogenetic differences.

In conclusion, our data demonstrate that the composition of CHC profiles in parasitic wasps depend on the host on which the wasps have developed. Because these differences arise already within one generation on a specific host, the composition of the CHC profiles is most likely determined by host-dependent precursors in the diet of the wasps. Since CHCs are known to play an important role in the recognition of conspecifics and mating partners in these insects (Sullivan, 2002; Steiner et al., 2005, 2006, 2007a; Ruther, et al., 2011), it is possible that the differences in CHC profiles caused by different hosts represent a reproductive barrier and may finally contribute to the formation of host-races and eventually to new species. This scenario might be more common in parasitic wasps, which could explain the high diversity in this group of insects. Future studies will have to address the question if the observed effects on the cuticular chemistry actually influence the courtship behaviour of

L. distinguendus. Furthermore, it will be interesting to study which differences in host chemistry are responsible for the differences in the CHC profiles of *L. distinguendus*.

2.6 Acknowledgements

We are grateful to C. Schmid for technical assistance and J. Stökl for his help with statistical analyses. Two anonymous reviewers gave helpful comments on an earlier draft of the manuscript. This research was funded by the Deutsche Forschungsgemeinschaft (DFG, grant RU-717/8-2.). S.K. was supported by a doctoral scholarship of the Universität Bayern e.V.

2.7 Supplementary Material

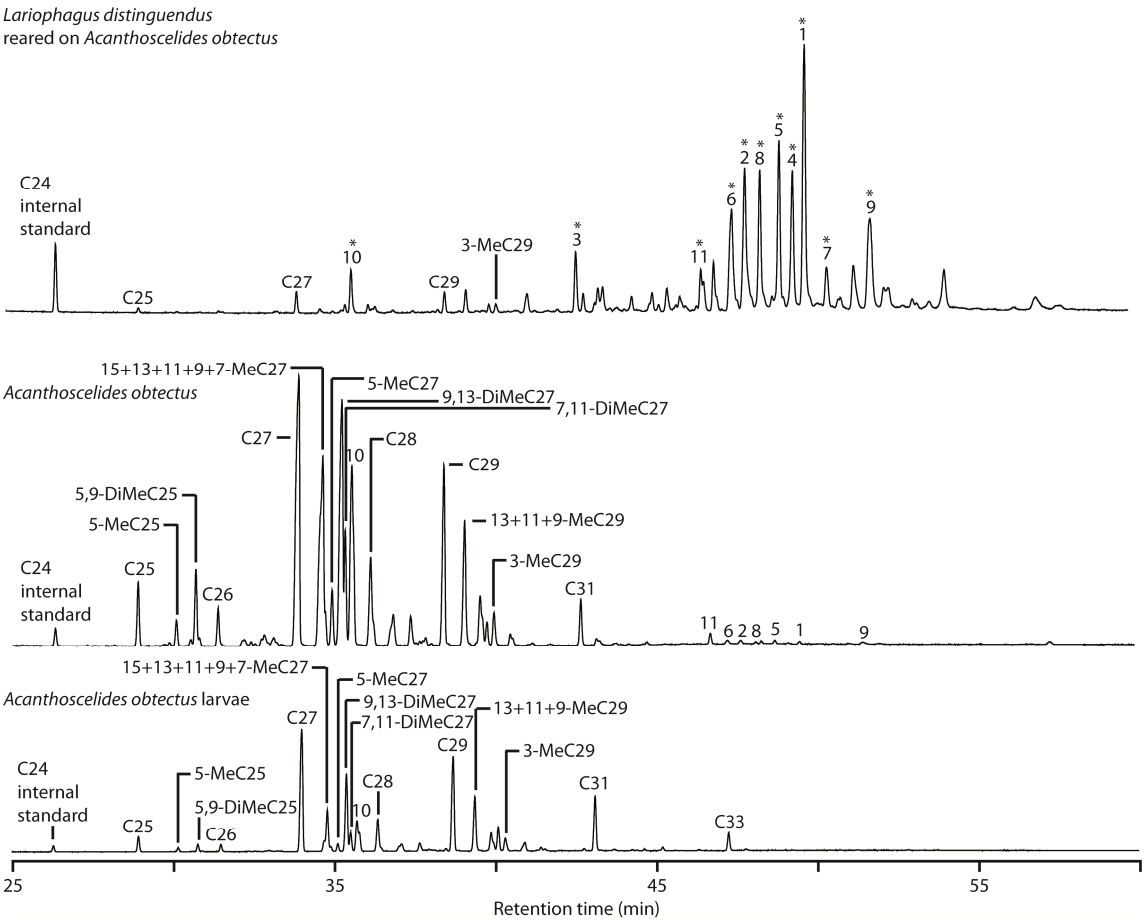

Figure S1 Chromatograms obtained by GC-MS of cuticular extracts from females of the parasitic wasp *Lariophagus distinguendus* reared on the host *Acanthoselides obtectus* and for comparison chromatograms of the respective host (adult and larval stage). Numbers and asterisks indicate in descending order the 11 compounds with contributions > 1 to the average dissimilarity (Table 2) of the CHC profiles as revealed by similarity percentage analysis (SIMPER).

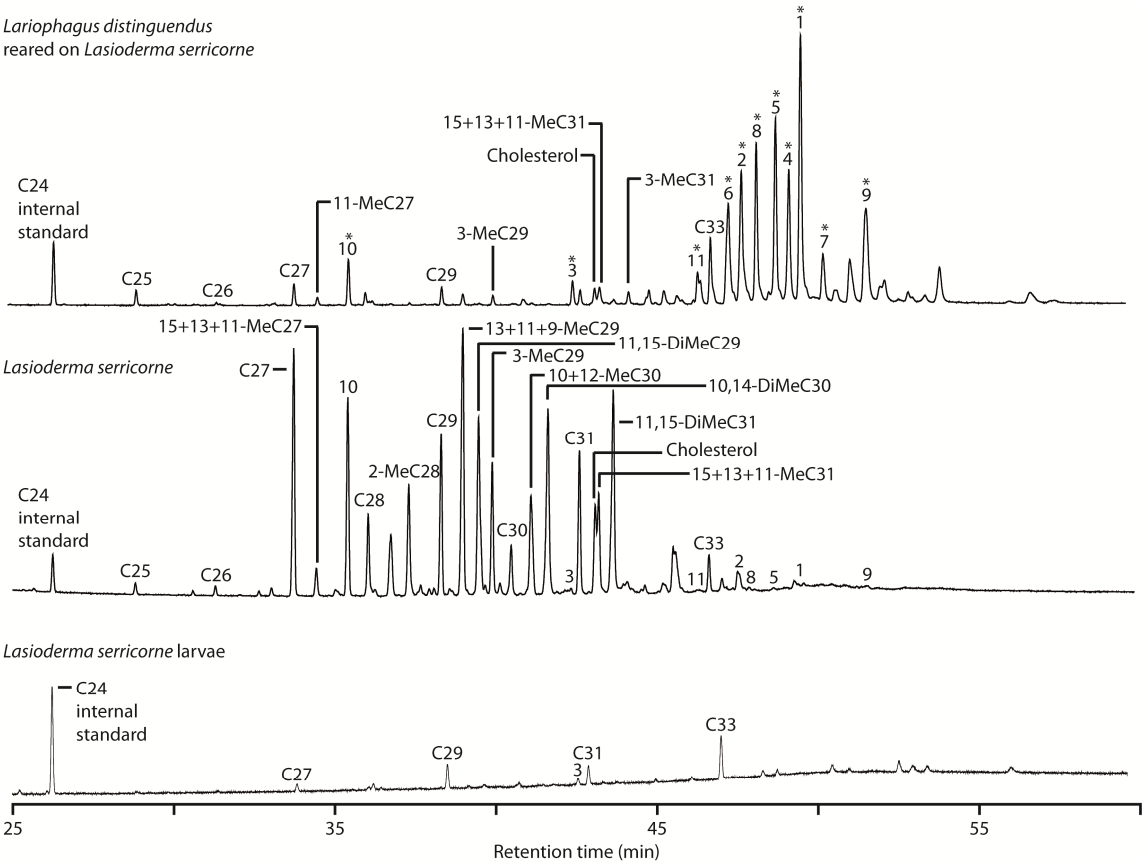

Figure S2 Chromatograms obtained by GC-MS of cuticular extracts from females of the parasitic wasp *Lariophagus distinguendus* reared on the host *Lasioderma serricorne* and for comparison chromatograms of the respective host (adult and larval stage). Numbers and asterisks indicate in descending order the 11 compounds with contributions > 1 to the average dissimilarity (Table 2) of the CHC profiles as revealed by similarity percentage analysis (SIMPER).

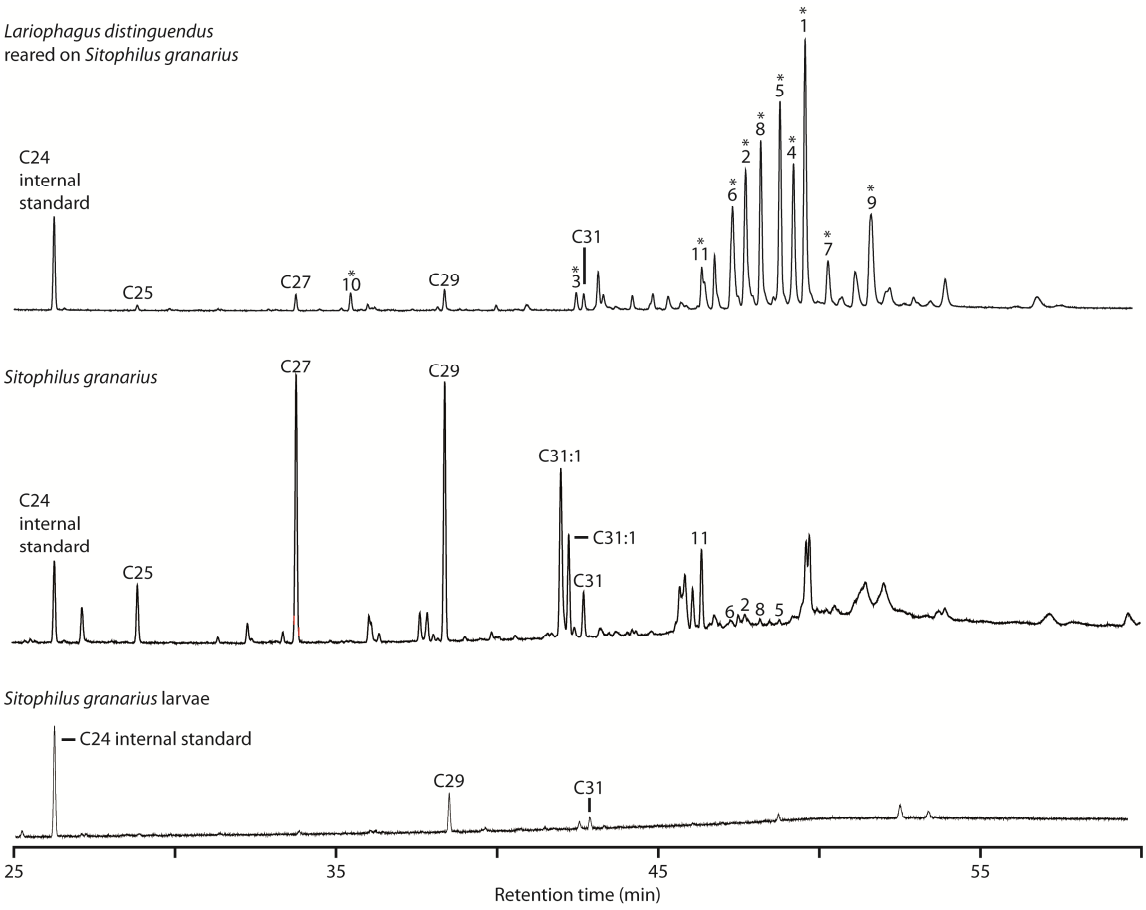

Figure S3 Chromatograms obtained by GC-MS of cuticular extracts from females of the parasitic wasp *Lariophagus distinguendus* reared on *Sitophilus granarius* and for comparison chromatograms of the respective host (adult and larval stage). Numbers and asterisks indicate in descending order the 11 compounds with contributions > 1 to the average dissimilarity (Table 2) of the CHC profiles as revealed by similarity percentage analysis (SIMPER).

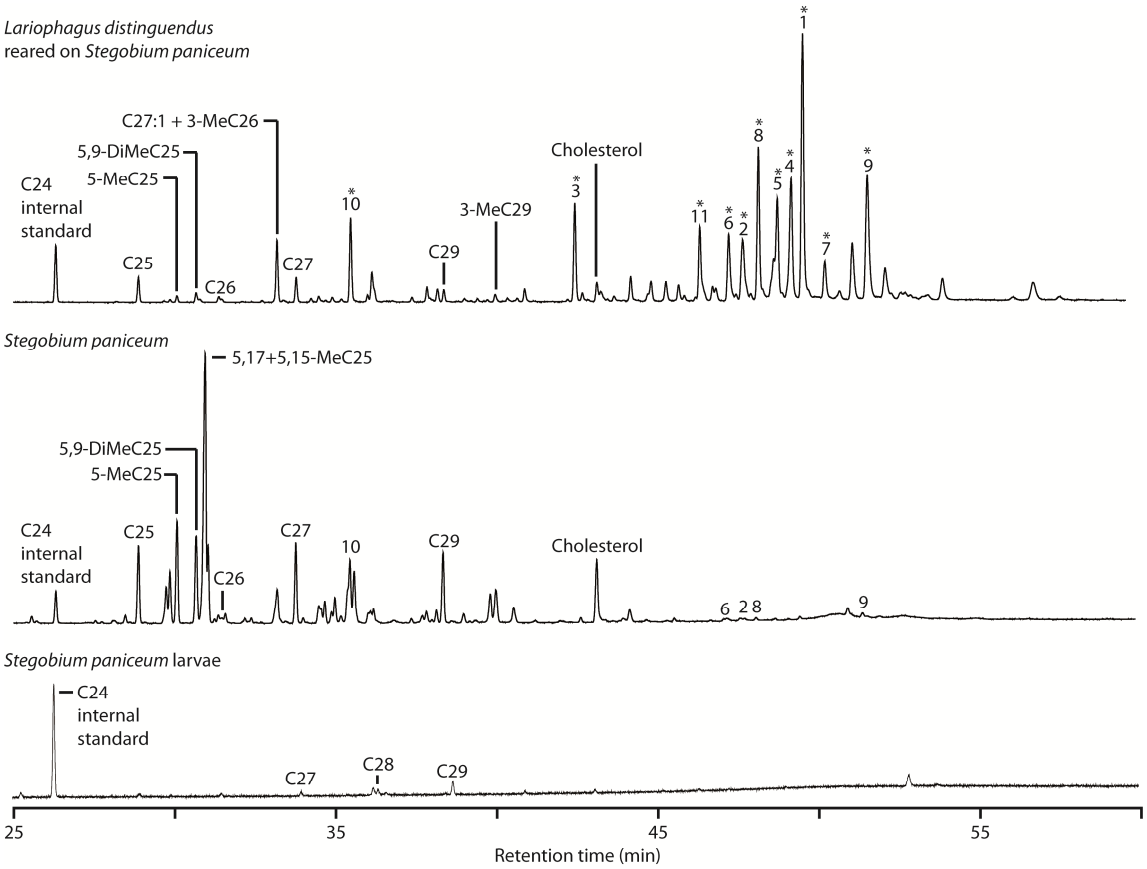

Figure S4 Chromatograms obtained by GC-MS of cuticular extracts from females of the parasitic wasp *Lariophagus distinguendus* reared on *Stegobium paniceum* and for comparison chromatograms of the respective host (adult and larval stage). Numbers and asterisks indicate in descending order the 11 compounds with contributions > 1 to the average dissimilarity (Table 2) of the CHC profiles as revealed by similarity percentage analysis (SIMPER).

Personal contribution:

I established the experiments of this paper and carried out the experimental work by myself. Christoph Schmid partly assisted me with the sample preparation for GC-MS analysis. Johannes Stökl gave advice with the statistical analysis of the data. I prepared the data for statistical analysis, created the figures and wrote the text of this paper as a first version.

Chapter 3

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

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Authorship: **Stephan Kühbandner**[1], **Sergej Sperling**[1], **Kenji Mori**[2] and **Joachim Ruther**[1]
[1]Institute of Zoology, University of Regensburg, Regensburg
[2]Photosensitive Materials Research Center, Toyo Gosei Co., Chiba, Japan

3.1 Abstract

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.

3.2 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., 2006b; 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., 2011a), 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.

3.3 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 SEHPLC 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 GCMS 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., 1998a; Steiner et al., 2005; Steiner et al., 2006; Steiner et al., 2007a). 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 reinstalled 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).

3.4 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., 2007a). 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.

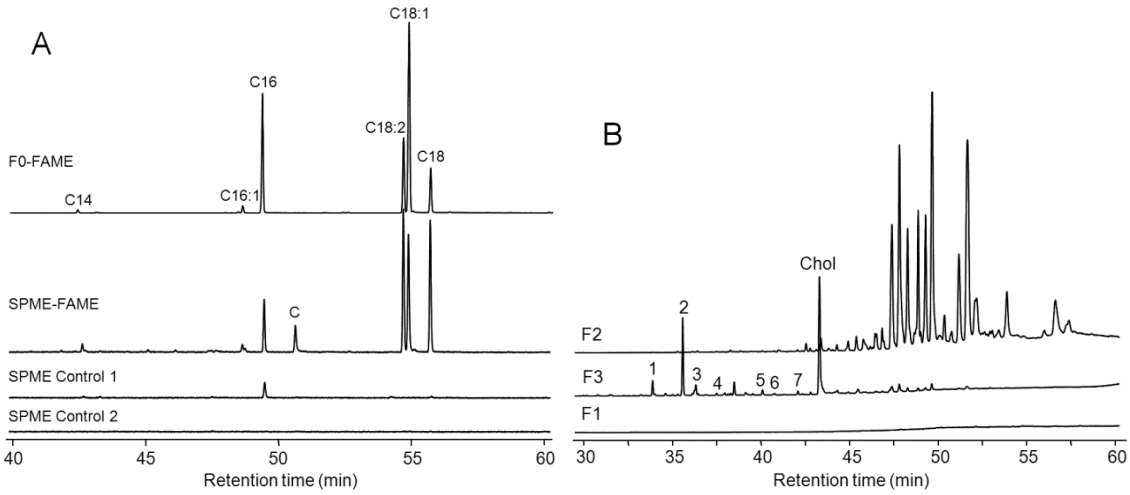

Fig. 1. (A) GC-MS analysis of fatty acid methyl esters (FAME) obtained by trans-esterification of TAGs in the lipid fraction F0 (F0-FAME) and by *in situ* trans-esterification of cuticular lipids sampled by solid phase microextraction (SPME-FAME). A conditioned SPME fibre was rubbed over the surface 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).

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 mixture of all three fractions (experiment 1, Fig. 2A,B). Omission of either fraction decreased male wing-fanning behavior 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.

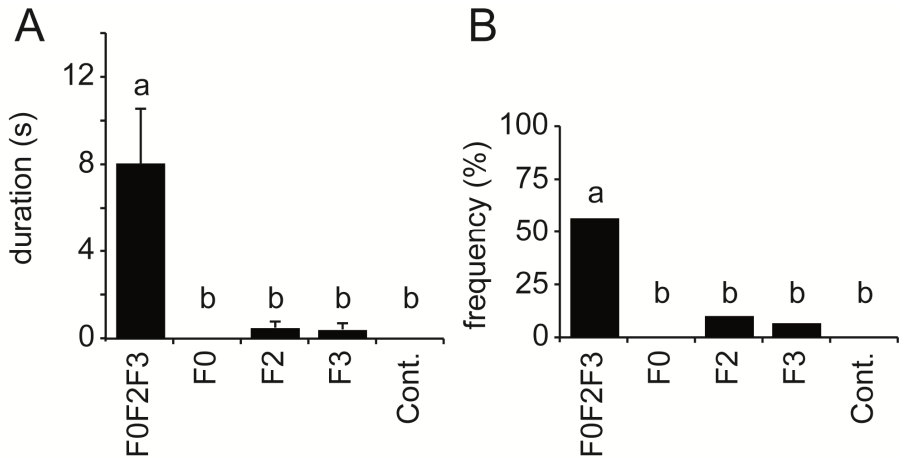

Fig. 2. Results of behavioural experiment 1. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with lipid fractions F0, F2, F3, a ternary mixture of all fractions (positive control) or the pure solvent (Control) 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; $N=30$).

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	$\chi^2 = 23.72$ $P < 0.001$	-	$U = 405$ $P = 0.082$	$U = 420$ $P = 0.16$	$U = 0$ $P = 1$
F2	$\chi^2 = 14.7$ $P < 0.001$	$\chi^2 = 31.58$ $P = 0.2$	-	$U = 437$ $P = 0.70$	$U = 405$ $P = 0.082$
F3	$\chi^2 = 17.33$ $P < 0.001$	$\chi^2 = 2.07$ $P = 0.36$	$\chi^2 = 0.22$ $P = 0.89$	-	$U = 420$ $P = 0.16$
Control	$\chi^2 = 23.72$ $P < 0.001$	$\chi^2 =$ [§] $P =$ [§]	$\chi^2 = 31.58$ $P = 0.2$	$\chi^2 = 2.07$ $P = 0.35$	-
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	$\chi^2 = 6.94$ $P = 0.03$	-	$U = 356.5$ $P = 0.02$	$U = 433$ $P = 0.74$	$U = 345$ $P < 0.001$
F0F3	$\chi^2 = 20.32$ $P < 0.001$	$\chi^2 = 5.19$ $P = 0.075$	-	$U = 374$ $P = 0.045$	$U = 435$ $P = 0.33$
F2F3	$\chi^2 = 8.53$ $P = 0.014$	$\chi^2 = 0.098$ $P = 0.95$	$\chi^2 = 4.04$ $P = 0.13$	-	$U = 360$ $P = 0.01$
Control	$\chi^2 = 23.72$ $P < 0.001$	$\chi^2 = 7.92$ $P = 0.019$	$\chi^2 = 1.017$ $P = 0.60$	$\chi^2 = 6.67$ $P = 0.035$	-
Experiment 3 (n=20; H=0.098)	S-3MeC27	R-3-MeC27	Contr.		
S-3MeC27	-	$U = 190$ $P = 0.34$	$U = 190$ $P = 0.34$		
R-3-MeC27	$\chi^2 = 1.03$ $P = 0.60$	-	$U = 0$ $P = 1$		
Contr.	$\chi^2 = 1.03$ $P = 0.60$	$\chi^2 =$ [§] $P =$ [§]	-		
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	$\chi^2 = 4.34$ $P = 0.11$	-	$U = 142.5$ $P < 0.001$	$U = 349$ $P = 0.089$	
F0F2 + S	$\chi^2 = 3.77$ $P = 0.15$	$\chi^2 = 15.15$ $P < 0.001$	-	$U = 227$ $P < 0.001$	
F0F2 + R	$\chi^2 = 0.60$ $P = 0.74$	$\chi^2 = 1.76$ $P = 0.41$	$\chi^2 = 7.18$ $P = 0.03$	-	
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	$\chi^2 = 20.42$ $P < 0.001$	-	$U = 33$ $P < 0.001$	$U = 72.5$ $P < 0.001$	
Male 4d + S	$\chi^2 = 0.36$ $P = 0.84$	$\chi^2 = 17.29$ $P < 0.001$	-	$U = 151$ $P = 0.19$	
Male 4d + R	$\chi^2 = 3.14$ $P = 0.21$	$\chi^2 = 10$ $P < 0.001$	$\chi^2 = 1.56$ $P = 0.46$	-	

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

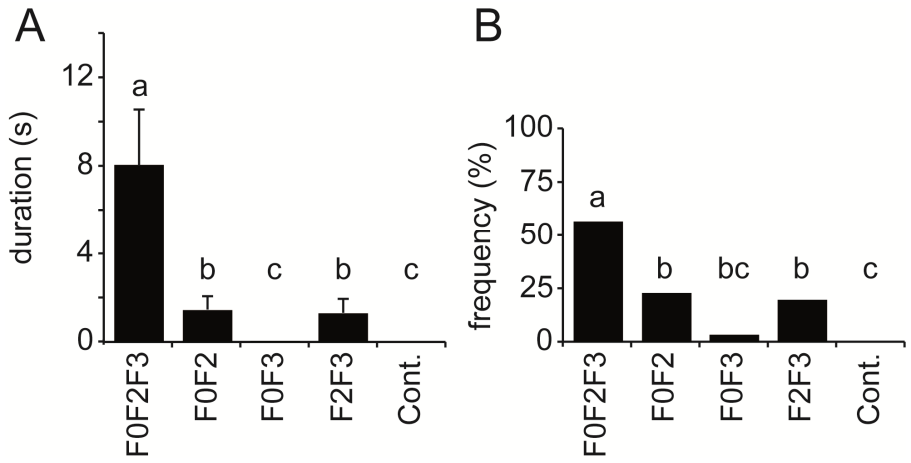

Fig. 3. Results of behavioural experiment 2. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with binary or ternary mixtures of lipid fractions F0, F2 and F3 or the pure solvent (Control) during a 5-min 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; $N=30$).

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 reinstalled 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.

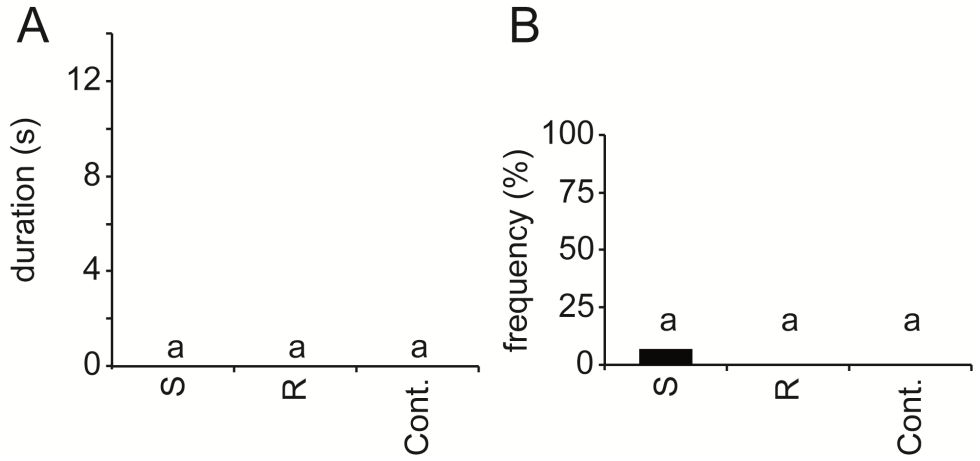

Fig. 4. Results of behavioural experiment 3. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with synthetic (*S*)-3-MeC27, (*R*)-3-MeC27 or the pure solvent (Control) during a 5-min observation period. 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; $N=20$).

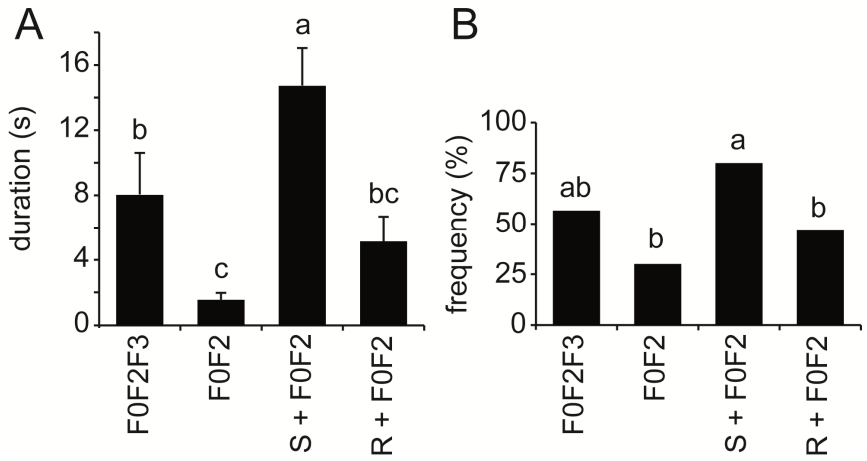

Fig. 5. Results of behavioural experiment 4. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards paper disks treated with a binary mixture of lipid fractions F0 and F2 plus synthetic (*S*)-3-MeC27 or (*R*)-3-MeC27 during a 5-min 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; $N=30$).

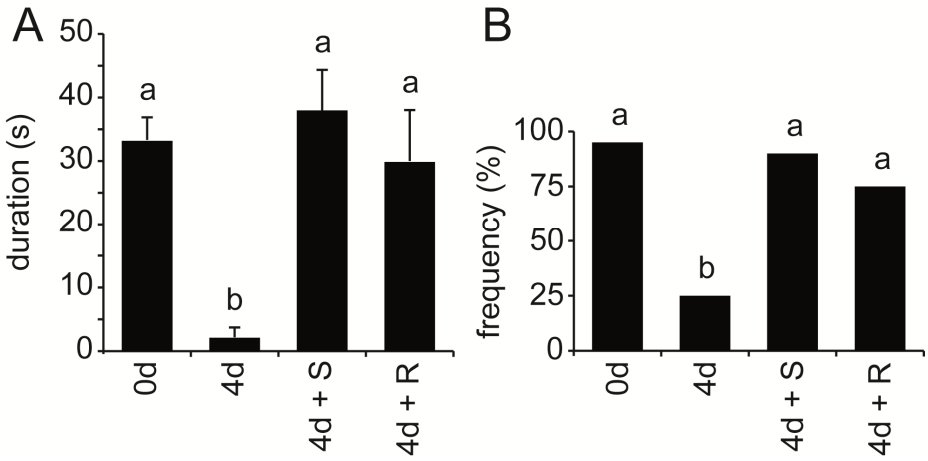

Fig. 6. Results of behavioural experiment 5. (A) Mean wing-fanning duration (±1 s.e.m.) of *L. distinguendus* males and (B) proportion of males showing wing-fanning towards newly emerged (0D) or 4-day-old (4D) male dummies treated with synthetic (*S*)-3-MeC27, (*R*)-3-MeC27, or the pure solvent during 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; $N=20$).

3.5 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 behavior 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 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 sexspecific hydrocarbons (Syvertsen et al., 1995).

The present study shows that the response of an insect to nonvolatile 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; 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., 1998b).

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., 2007a), 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., 2011a) 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.

3.6 Acknowledgements

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Personal contribution:

I established the bioassays of this paper and carried out the experimental work together with Sergej Sperling. I analyzed the data, created the figures and wrote the text of this paper as a first version.

Chapter 4

Elucidating structure-bioactivity relationships of methyl-branched alkanes in the contact sex pheromone of the parasitic wasp *Lariophagus distinguendus*

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Authorship: **Stephan Kühbandner**[1], **Jan E. Bello**[2], **Kenji Mori**[3], **Jocelyn G. Millar**[2,4], **Joachim Ruther**[1]

[1]Institute of Zoology, University of Regensburg, Regensburg

[2]Department of Chemistry, University of California, Riverside, CA, USA

[3]Photosensitive Materials Research Center, Toyo Gosei Co., Chiba, Japan

[4]Department of Entomology, University of California, Riverside, CA, USA

4.1 Abstract:

The exoskeletons of insects are covered by complex mixtures of cuticular hydrocarbons (CHCs) which are involved in social and sexual communication. However, little is known about the relationship between the structures of CHCs and their behavioral activity. The key component of the contact sex pheromone of the parasitoid *Lariophagus distinguendus* is 3-methylheptacosane (3-MeC27), which is present in CHC profiles of both females and newly emerged males. The CHCs of females and young males elicit wing-fanning behavior in older males. However, as young males age, 3-MeC27 disappears from their CHC profiles and they no longer elicit wing-fanning responses from other males. We applied enantiopure 3-MeC27 and structurally related CHCs (with respect to chain length or methyl-branch position) to the cuticle of aged male dummies and recorded the wing-fanning behavior of responding males. Only the two enantiomers of 3-MeC27 restored the dummies' attractiveness. The addition of structurally related CHCs or various n-alkanes to bioactive dummies of young males and females significantly decreased wing-fanning by test males. Hence, *L. distinguendus* males respond specifically but not enantioselectively to 3-MeC27, and perceive the CHC profiles as a whole. Both removal (as is the case with 3-MeC27 in aging males) and addition of individual compounds may disrupt the behavioral response.

4.2 Introduction

Insects utilize chemical signals and cues in all aspects of their life histories and ecologies, and thus possess an innate ability to detect and discriminate different chemicals and associate them with the correct biological context. It has been well established that insects employ volatile substances for long-range communication, and more recently it has become clear that many insects also utilize non-volatile compounds as short-range or contact pheromones (Howard and Blomquist, 2005). These compounds are components of the protective layer of cuticular lipids covering the insect exoskeleton. This lipid layer consists primarily of a complex blend of n-alkanes, methyl-branched alkanes, and alkenes, typically with chain lengths of about 21-37 carbons (referred to as cuticular hydrocarbons, CHCs), as well as more polar compounds such as long-chain fatty acids, alcohols, aldehydes, wax esters, and triacylglycerides (Buckner, 2010; Yew et al. 2011a; Kühbandner et al., 2012; Mori, 2012). CHCs function primarily as a water barrier preventing desiccation (Gibbs 2002), but components of this protective layer are also utilized in insect communication (Howard and Blomquist, 2005). Solitary insects use CHCs to recognize conspecifics and to determine gender, and thus identify potential mates (Howard and Blomquist, 1982; Singer, 1998; Howard and Blomquist, 2005). CHCs are also employed as kairomones, fertility signals, and to mark territories (Howard and Blomquist, 1982; Blomquist and Bagnères, 2010). In social insects, CHCs are directly involved in nestmate recognition, formation and maintenance of social castes, and determination of the health and fecundity of the reproductive caste (Howard and Blomquist, 2005).

The CHC profiles of insects can range from relatively simple mixtures of only a few compounds to complex blends of more than 100 substances (Dani et al., 2001; van Wilgenburg et al., 2010). However, little is known about how insects perceive and process the information that is encoded in the cuticular lipids (Dani et al., 2005; Ozaki et al., 2005; van Wilgenburg et al., 2012; Ozaki and Wada-Katsumata, 2010). It is assumed that insects use only a small subset of the cuticular compounds as semiochemicals (Espelie et al., 1990; Ginzel et al., 2003; Sugeno et al., 2006; Lacey et al., 2008; Silk et al., 2009) with the majority of CHC components being considered to have little or no communicative function (Dani et al., 2001; Châline et al., 2005; van Wilgenburg et al., 2010). The biological activity of methylalkanes and alkenes is directly correlated with their chain lengths and the positions of methyl branch points and double bonds, respectively (Ginzel et al., 2003, 2006; Sugeno et al., 2006; Lacey et al., 2008; van Wilgenburg et al, 2010, 2012). This suggests that methylalkanes and alkenes are better suited for use as signal molecules than straight-chain alkanes because

they possess additional structural features that provide for discrimination using criteria other than chain length alone (Dani et al., 2001; Châline et al., 2005; Espelie et al, 1994). Methylalkanes often occur on the insect cuticle as series of homologs, with methyl branch points at the same position in chains of variable length (van Wilgenburg et al., 2010). It is still unclear whether insects are able to discriminate such homologs or if they "generalize" them. In the latter case, methylalkanes differing only in chain length might convey the same amount methylalkanes differing only in chain length might convey the same amount of information and therefore be used as "synonyms" (Lacey et al., 2008; van Wilgenburg et al., 2010, 2012). This would make CHC profiles functionally far less complex than one would expect from the mere number of compounds (van Wilgenburg et al., 2010; Bos et al., 2012). Further potential information might be encoded in the stereochemistry of methylalkanes (Duff et al., 2001; Ablard et al., 2012; Kühbandner et al., 2012) and the relative proportions in which they occur in the CHC profile of insects (Dani et al., 2001, 2005). However, despite the substantial body of literature on the semiochemical functions of CHCs, many details on the relationships between structural features and bioactivity remain to be elucidated.

Lariophagus distinguendus Förster (Hymenoptera: Pteromalidae) is an idiobiont ectoparasitoid that parasitizes the larvae and pupae of several species of beetles that infest stored products (Steidle and Schöller, 1997; Hansen et al., 2013). Females produce a contact sex pheromone on their cuticles. Males are arrested by this pheromone and respond by performing stereotypical courtship behavior that includes high-frequency wing-fanning (Ruther et al., 2000; Benelli et al., 2013). Interestingly, the pupae of both sexes as well as newly emerged males apparently produce the same pheromone blend as females, but young males deactivate the pheromone within 32 hours after emergence. This deactivation is accompanied by the loss of 3-methylheptacosane (3-MeC27) and some minor CHCs (Steiner et al., 2005, 2007a). The mechanism behind the disappearance of 3-MeC27 from the aging male cuticle is not yet known, but it has been shown that males killed before the pheromone deactivation period retain the attractive hydrocarbon blend indefinitely (Steiner et al., 2005). Reapplication of synthetic 3-MeC27 onto the cuticle of aged males fully reinstates the pheromonal activity, so that they are courted by sexually mature males (Kühbandner et al., 2012). Thus, 3-MeC27 is a key component of the *L. distinguendus* contact sex pheromone. However, experiments using fractionated bioactive lipid extracts revealed that 3-MeC27 only elicits a response when it is presented in combination with a chemical background of the other CHCs and triacylglycerides that also occur on the cuticle of *L. distinguendus* wasps (Kühbandner et al., 2012). The results mentioned above have shown that the disappearance of

a single compound from a bioactive CHC profile can terminate the wing fanning response of *L. distinguendus* males. It is not known, however, whether a bioactive CHC profile can also be disturbed by adding individual compounds as has been shown in the context of nestmate recognition in social insects (van Zweden and D'Ettore, 2010).

In this study, we investigated the structure-bioactivity relationships of methyl-branched CHCs in *L. distinguendus*. In particular, we tested whether the responses of males to 3-MeC27 are specific with respect to chain length, position of the methyl branch, and absolute configuration. In addition, we tested the hypothesis that the responses elicited by bioactive CHC profiles, such as those of females and newly emerged males, can be disrupted by the addition of synthetic methylalkanes and n-alkanes to those cuticular profiles.

4.3. Materials and Methods

Insects

Lariophagus distinguendus wasps were reared on late instar larvae and prepupae of the granary weevil *Sitophilus granarius* (Curculionidae) at 25 °C and 40%-50% relative humidity under a photoperiod of 12 h:12 h light:dark (Steidle and Schöller, 1997). Male wasps used as responders in bioassays were isolated shortly after emergence and kept separately for two days under the described rearing conditions. Two types of dead wasps were used as dummies to study the effects of added synthetic alkanes on the pheromonal activity of the wasps' CHC profiles. The first type of dummies were males that had been isolated for four days and were subsequently freeze-killed (referred to as 4-d-old males). These males no longer elicit pheromonally induced wing-fanning responses from courting males (Steiner et al., 2005) and were used in Experiment 1 (see below). The second type of dummies were males and females which were freeze-killed immediately after emergence (referred to as 0-d-old males/females). These dummies elicit intense wing-fanning behavior in responding males (Steiner et al., 2005) and were utilized in Experiment 2. All dummies were stored at -23 °C, and were defrosted immediately prior to bioassays.

Synthesis of Reference Chemicals

General Methods and Information for Synthesis

All solvents were Optima grade (Fisher Scientific, Pittsburgh, PA, USA). Tetrahydrofuran (THF) was distilled from sodium/benzophenone under argon. ^{1}H- and ^{13}C-NMR spectra were recorded with a Varian INOVA-400 (400 and 100.5 MHz, respectively) spectrometer (Palo Alto, CA, USA), as $CDCl_3$ solutions. ^{1}H-NMR chemical shifts are expressed in ppm relative to residual $CHCl_3$ (7.27 ppm) and ^{13}C-NMR chemical shifts are reported relative to $CDCl_3$ (77.16 ppm). Unless otherwise stated, solvent extracts of reaction mixtures were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Crude products were purified by vacuum flash chromatography or column flash chromatography on silica gel (230-400 mesh; Fisher Scientific). Yields refer to isolated yields of chromatographically pure products. Mass spectra were obtained with a Hewlett-Packard (HP) 6890 GC (Hewlett-Packard, Avondale, PA, USA) interfaced to an HP 5973 mass selective detector, in EI mode (70 eV) with helium as carrier gas. The GC was equipped with a DB17-MS column (25 m × 0.20 mm i.d., 0.33 μm film). Reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under argon.

Synthesis of (*S*)-3-Methylnonacosane [(*S*)-7], (*S*)-3-Methylhentriacontane [(*S*)-8], (*R*)-3-Methylnonacosane [(*R*)-7], and (*R*)-3-Methylhentriacontane [(*R*)-8] (Fig. 1)

(*S*)-2-Methyl-1-butanol **1** (1.21 g, 13.7 mmol, Alfa Aesar, Ward Hill, MA, USA) was dissolved in 15 mL of CH_2Cl_2 and cooled to -10 °C, then pyridine (1.1 mL, 13.7 mmol) and triflic anhydride (2.81 mL, 16.4 mmol) were added sequentially, and the resulting mixture was stirred for 1.5 h at -10 °C. The reaction was then diluted with 45 mL of pentane, filtered through a plug of silica gel, and concentrated to afford (*S*)-2-methylbutyl triflate **2** as a colorless oil in quantitative yield, which was used immediately in the next step without further purification (Wang and Zhang, 2008).

(11-(*tert*-Butyldimethylsilyloxy)undecyl)magnesium bromide (1.5 M, 8.2 mL, 12.3 mmol) was prepared by dropwise addition of 11-(*tert*-butyldimethylsilyloxy)undecyl bromide (5.5 g, 15.02 mmol) to Mg turnings (365 mg, 15 mmol) in 10 mL of dry Et_2O, followed by stirring 2 h at 23 °C. Triflate **2** (2.8 g, 12.7 mmol) was taken up in 40 mL of dry Et_2O and cooled to -30 °C under argon, Li_2CuCl_4 (0.48 M, 1.45 mL, 0.69 mmol) was added dropwise, and the solution was stirred for 15 min. The freshly prepared Grignard reagent was then added dropwise over 30 min and the resulting mixture was stirred at -40 °C for 4 h. The reaction was

then quenched with saturated aqueous NH_4Cl (50 mL) and extracted with hexane. The hexane extract was washed with water and brine, dried and concentrated, and the residue was purified by column chromatography (Et_2O/hexane, 1:19) to afford silyl ether **3** (3.35 g, 78%) as a colorless oil (Cahiez et al., 2000).

A solution of Ph_3PBr_2 (9.62 g, 22.8 mmol) was prepared by treatment of Ph_3P (5.96 g, 22.8 mmol) with Br_2 (1.17 mL, 22.8 mmol) in 100 mL CH_2Cl_2 at 0 °C. After warming to room temperature, silyl ether **3** (3.25 g, 9.1 mmol) in 20 mL dry CH_2Cl_2 was added slowly, the mixture was stirred for 1 h, then diluted with hexane (200 mL) and filtered through a plug of silica gel. After concentration, the crude product was purified by column chromatography (Et_2O/Hex, 1:99) to afford alkyl bromide **4** (2.55 g, 92%) (Aizpurua et al., 1986).

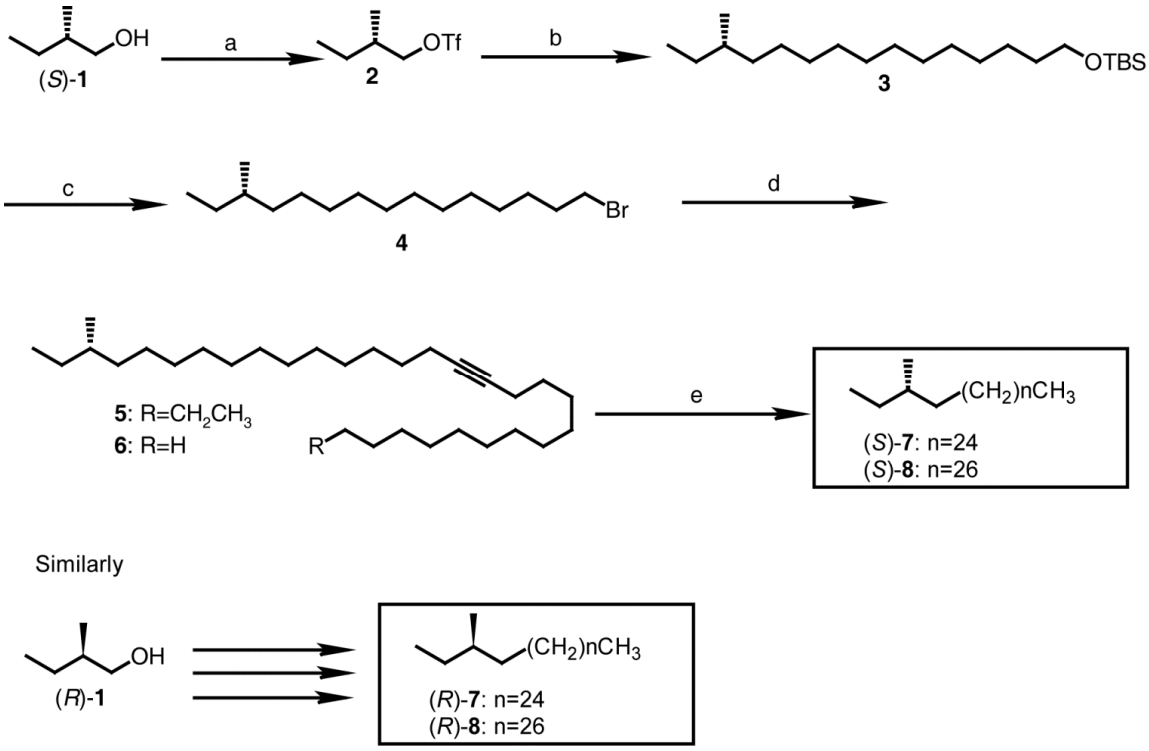

Figure 1. Synthesis of (*S*)-3-methylnonacosane [(*S*)-**7**], (*S*)-3-methylhentriacontane [(*S*)-**8**], (*R*)-3-methylnonacosane [(*R*)-**7**], and (*R*)-3-methylhentriacontane [(*R*)-**8**]. Reagents: (**a**) Tf_2O, pyridine, CH_2Cl_2 (quantitative); (**b**) (11-(*tert*-butyldimethylsilyloxy)undecyl)-magnesium bromide, Li_2CuCl_4, Et_2O (78%); (**c**) Ph_3PBr_2, CH_2Cl_2 (91.5%); (**d**) tetradecynyl lithium for **7**, hexadecynyl lithium for **8**, THF (82% and 85%, respectively); (**e**) 5% Rh/C, H_2, hexane (98%).

1-Tetradecyne (416 mg, 2.14 mmol, Farchan/GFS Chemicals, Powell, OH, USA) was dissolved in 15 mL dry THF in a 3-necked flask under Ar and after cooling to -78 °C, *n*-BuLi (2.2 M in hexanes, 972 μL, 2.14 mmol) was added dropwise over 10 min. The reaction was stirred for 30 min at -78 °C, then warmed to 23 °C. Alkyl bromide **4** (600 mg, 2.04 mmol) and NaI (50 mg, 0.24 mmol) were then added and the mixture was refluxed for 8 h. The mixture was then cooled to 23 °C, quenched with saturated aqueous NH_4Cl, and extracted with hexane. The hexane extract was washed with water and brine, dried, and concentrated. Unreacted 1-tetradecyne was removed by Kugelrohr distillation of the crude product (oven temp. 50 °C, 0.1 mm Hg), affording (*S*)-27-methylnonacos-13-yne (*S*)-**5** (746 mg, 82%) as a colorless oil (Buck and Chong, 2001). The oil was added to a slurry of 5% Rh/C (80 mg) and anhydrous Na_2CO_3 (700 mg, 5.2 mmol) in hexane (10 mL) and stirred for 10 h under a slight positive pressure of H_2. The mixture was filtered through a plug of silica gel and concentrated to afford 763 mg of crude crystalline (*S*)-3-methylnonacosane (Zou and Millar, 2011).

Recrystallization from hexane/acetone (1:5) gave 737 mg of pure (>98%) (*S*)-**7** in 53% overall yield in 5 steps, mp 34 °C, $[\alpha]_D^{23}$ = +3.63° (c = 1.52, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$), ∂_H (ppm): 0.84 (3H, d, *J* = 6.3 Hz), 0.85 (3H, t, *J* = 6.7 Hz), 0.87 (3H, t, *J* = 6.5 Hz), 1.16-1.4 (53 H, broad m). ^{13}C-NMR, ∂c (ppm): 11.62, 14.32, 19.45, 22.91, 25.67, 27.36, 29.58, 29.72, 29.93, 30.25, 31.81, 32.16, 34.62, 36.88. MS (EI, 70 eV, *m*/*z*, relative abundance): 422 (1, M+), 407 (1), 393 (12), 379 (1), 365 (1), 351 (1), 337 (1), 323 (1), 309 (2), 295 (2), 281 (2), 267 (2), 253 (2), 239 (3), 225 (3), 211 (2), 197 (3), 183 (3), 169 (5), 155 (5), 141 (7), 127 (10), 113 (11), 99 (19), 85 (30), 71 (43), 57 (99), 43 (100).

(*S*)-3-Methylhentriacontane [(*S*)-**8**] was prepared in analogous fashion in 51% yield by substitution of hexadecynyllithium for tetradecynyllithium in the 4th reaction. 1-Hexadecyne was obtained from treatment of 1-tetradecyl bromide with lithium acetylide-ethylene diamine complex in DMSO (Sonnet and Heath, 1980). Mp = 36 °C, $[\alpha]_D^{23}$ = +3.43° (c = 1.55, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$) ∂_H (ppm): 0.84 (3H, d, *J* = 6.5 Hz), 0.85 (3H, t, *J* = 6.7 Hz), 0.87 (3H, t, *J* = 6.8 Hz), 1.16-1.4 (57 H, broad m). ^{13}C-NMR, ∂c (ppm): 11.59, 14.32, 19.41, 22.87, 25.48, 27.36, 29.28, 29.58, 29.73, 29.94, 30.25, 31.81, 32.16, 34.62, 36.88. MS (EI, 70 eV, *m*/*z*, relative abundance): 450 (1, M+), 435 (2), 421 (43), 407 (1), 393 (17), 379 (1), 365 (1), 351 (1), 337 (1), 323 (1), 309 (1), 295 (2), 281 (2), 265 (3), 253 (3), 239 (3), 225 (3), 211 (5), 197 (5), 183 (5), 169 (5), 155 (7), 141 (9), 127 (13), 133 (17), 99 (30), 85 (39), 71 (52), 57 (99), 43 (100).

(*R*)-3-methylnonacosane [(*R*)-**7**] (52% overall yield, purity >97%) was prepared in analogous fashion to (*S*)-**7** by substitution of (*R*)-2-methyl-1-butanol for (*S*)-2-methyl-1-butanol in the 1st reaction. (*R*)-2-Methyl-1-butanol was obtained by enzymatic resolution of racemic 2-methyl-1-butanol with Amano *Pseudomonas fluorescens* lipase (Aldrich Chemical Co., Milwaukee, WI, USA) and vinyl acetate in dry CH_2Cl_2 (Barth and Effenberger, 1993). Mp = 33 °C, $[\alpha]_D^{23}$ = -3.53° (c = 1.52, CH_2Cl_2). Its spectroscopic data were equivalent to those of (*S*)-**7**.

(*R*)-3-methylhentriacontane [(*R*)-**8**] (50% overall yield, purity >99%) was prepared in analogous fashion to (*S*)-**8** by substitution of (*R*)-2-methyl-1-butanol for (*S*)-2-methyl-1-butanol in the 1st reaction. Mp = 36 °C, $[\alpha]_D^{23}$ = -3.37° (c = 1.53, CH_2Cl_2). Its spectroscopic data were equivalent to those of (*S*)-**8**.

Synthesis of (*R*)-5-Methylheptacosane [(*R*)-15] and (*S*)-5-Methylheptacosane [(*S*)-15] (Fig. 2)

(*R*)-4-Isopropyloxazolidin-2-one **9** (2.25 g, 17.7 mmol) (Benoit et al., 2008) was dissolved in dry THF (70 mL) and cooled to -78 °C, *n*-BuLi (2.89 M in hexanes, 6.4 mL, 18.5 mmol) was added dropwise over 10 min and the reaction was stirred for 1 h. Hexanoyl chloride (2.82 mL, 19.5 mmol) was then added dropwise and the resulting mixture was stirred at -78 °C for 20 min, then warmed to 0 °C for 1.5 h. The reaction was quenched with 1 M aqueous K_2CO_3 (50 mL) and extracted with hexane. The hexane extract was washed with water and brine, dried and concentrated, and the residue was purified by column chromatography (EtOAc/hexanes, 1:9) to afford oxazolidinone amide **10** (3.91 g, 96%) as a colorless oil (Kanomata et al., 2003).

A solution of **10** (3.0 g, 12.95 mmol) in dry THF was cooled to -78 °C under Ar and sodium hexamethyldisilazide (NaHMDS, 2.0 M in THF, 7.12 mL, 14.25 mmol) was added dropwise over 15 min. The reaction was stirred at -78 °C for 1 h, then MeI (3.22 mL, 52 mmol) was added dropwise, and the resulting solution was stirred at -78 °C for 2 h. The reaction was quenched with saturated aqueous NH_4Cl (75 mL) and extracted with hexane. The hexane extract was washed sequentially with 1 M HCl, saturated $NaHCO_3$, and brine, then dried and concentrated. The residue was purified by column chromatography to afford ((*R*)-2-methylhexanoyl)oxazolidinone (**11**) (3.01 g, 94%) as a colorless oil (Evans et al., 1982).

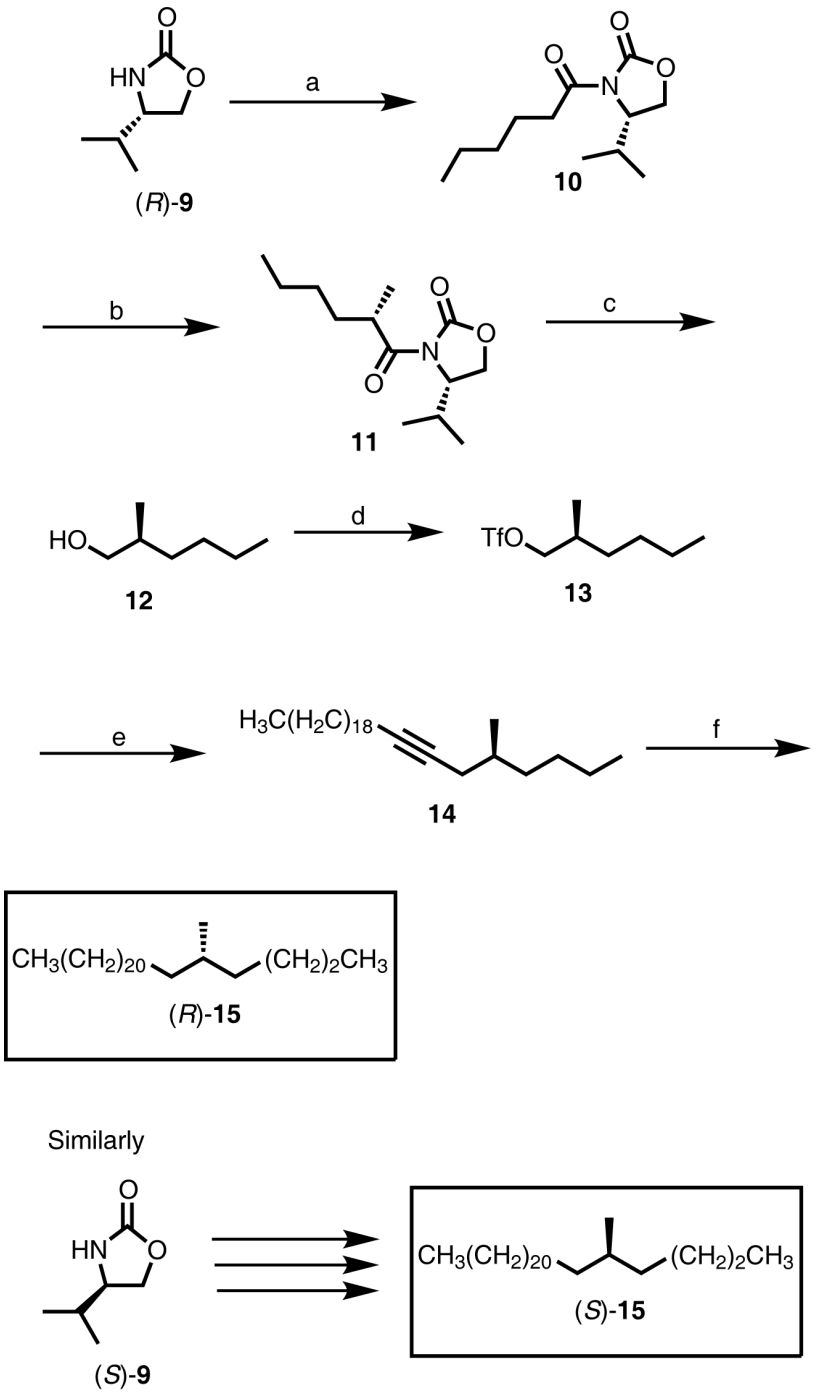

Figure 2. Synthesis of (*R*)- and (*S*)-5-methylheptacosane (**15**). Reagents: (**a**) i. *n*-BuLi, THF; ii. $CH_3(CH_2)_4COCl$ (96%); (**b**) i. NaHMDS, THF; ii. MeI (94%); (**c**) $LiBH_4$, Et_2O (92%); (**d**) Tf_2O, pyridine, CH_2Cl_2 (quantitative for **7**); (**e**) heneicosynyl lithium, THF (76%-80%); (**f**) H_2 (1 atm), 5% Rh/C, hexane (96%-97%).

A solution of ((*R*)-2-methylhexanoyl)oxazolidinone (**11**) (3.0 g, 12.9 mmol) in Et_2O (80 mL) was cooled to 0 °C, and dry MeOH (1.9 mL, 25.3 mmol) was added, followed immediately by $LiBH_4$ (562 mg, 25.8 mmol). The reaction was stirred at 0 °C for 3 h, then quenched with saturated $NaHCO_3$ (60 mL), and extracted with Et_2O. The ether extract was washed with saturated NH_4Cl, water and brine, and after drying and concentration, the residue was purified by column chromatography (EtOAc/hexane 1:5) to afford alcohol **12** (1.41 g, 92%) as a colorless oil (Williams et al., 2003).

Alcohol **12** (500 mg, 4.3 mmol) was treated sequentially with pyridine (346 μL, 4.3 mmol) and triflic anhydride (880 μL, 5.16 mmol) in CH_2Cl_2 (20 mL) at -10 °C for 2 h. The reaction then was diluted with pentane (60 mL) and filtered through a plug of silica gel, rinsing with 3:1 hexane:CH_2Cl_2. Concentration of the filtrate gave alkyl triflate **13** (1.07 g, quantitative) as a colorless oil, which was used immediately without further purification (Wang and Zhang, 2008).

A solution of 1-heneicosyne (1.23 g, 4.2 mmol) (Corey and Fuchs, 1972) in 10 mL of dry THF was cooled to -10 °C, *n*-BuLi (2.89 M in hexanes, 1.46 mL, 4.22 mmol) was added dropwise over 10 min, and the reaction was stirred for 1 h. Alkyl triflate **13** (1.07 g, 4.3 mmol) in 5 mL THF was then added by syringe pump over 30 min, and the reaction was stirred at -10 °C for 5 h. The reaction was quenched with water (20 mL) and extracted with hexane. The hexane extract was washed with brine, dried and concentrated, and the residue was purified by vacuum flash chromatography (hexane) to afford (*R*)-5-methylheptacos-7-yne [(*R*)-**14**] (1.35 g, 80%) (Armstrong-Chong et al., 2004).

(*R*)-5-methylheptacos-7-yne [(*R*)-**14**] (1.35 g, 3.44 mmol) was added to a slurry of 5% Rh/C (135 mg) and anhydrous Na_2CO_3 (1.09 g, 10.3 mmol) in hexane (15 mL) (Zou and Millar, 2011). The reaction was stirred under a slight positive pressure of H_2 for 8 h, then filtered through a plug of silica gel to afford crude (*R*)-5-methylheptacosane. After concentration, the residue was dissolved in boiling acetone (10 mL) and the solution was cooled to -20 °C. Filtration and vacuum drying yielded pure (*R*)-5-methylheptacosane [(*R*)-**15**] (1.29 g, 96%) as white waxy crystals in 64% overall yield in 6 steps. Mp = 32 °C, $[\alpha]_D^{23}$ = -0.77° (c = 1.33, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) ∂_H (ppm): 0.83 (3H, d, *J* = 6.3 Hz), 0.85 (3H, t, *J* = 6.7 Hz), 0.87 (3H, t, *J* = 6.5 Hz), 1.16-1.4 (49 H, broad m). ^{13}C-NMR, ∂c (ppm): 11.52, 14.14, 19.72, 22.61, 23.67, 27.09, 29.38, 29.70, 29.93, 30.35, 31.81, 32.16, 34.62, 36.73. MS (EI, 70 eV, *m*/*z*, relative abundance): 394 (M+, 1), 365 (3), 337 (39), 308 (12), 295 (1), 281 (1), 253 (3), 225 (3), 197 (1), 183 (2), 169 (2), 155 (1), 141 (9), 112 (35), 85 (42), 71 (98), 57 (100), 43 (50).

(*S*)-5-methylheptacosane [(*S*)-**15**] (61% yield, purity >99%) was prepared in analogous fashion by substitution of (*S*)-4-isopropyloxazolidin-2-one [(*S*)-**9**] (Aizpurua et al., 1986) for (*R*)-4-isopropyloxazolidin-2-one [(*R*)-**9**] in the first reaction, mp = 31.5 °C, $[\alpha]_D^{23}$ = +0.73° (c=1.35, CH_2Cl_2). Its spectroscopic data were analogous to those of (*R*)-**15**.

Synthesis of (*R*)- and (*S*)-7-Methylheptacosane

The enantiomers of 7-methylheptacosane were synthesized as previously described (Bello and Millar, 2013).

Synthesis of (*R*)-and (*S*)-3-Methylpentacosane, and (*R*)- and (*S*)-3-Methylheptacosane The enantiomers of 3-methylpentacosane and 3-methylheptacosane were synthesized as previously described (Marukawa et al., 2001).

Bioassays

General Procedures for Bioassays

Bioassays were performed in a round test arena (diameter: 10 mm; height: 3 mm) as described previously (Ruther et al., 2000). Aliquots of 1 µL containing 150 ng of synthetic compounds (treatment) or the pure solvent (dichloromethane, control) were applied evenly to the cuticle of individual dummies with a 5 µL syringe (Hamilton, Bonaduz, Switzerland). After the solvent had evaporated for 2 min, treated dummies were transferred to the test arena and the total duration of wing-fanning of a test male was recorded during the following 5 min using a stereo microscope and The Observer XT 9.0 scientific software (Noldus Information Technology, Wageningen, The Netherlands). Each male was tested twice, first with a control dummy and subsequently with a treated dummy. Test males that did not perform wing-fanning behavior towards the dummy in a given bioassay were additionally exposed to a 0-d-old female dummy as a positive control to make sure that they were responsive. Data from those few males (<1% of all tested males) that did not respond to this positive control were discarded. All experiments were conducted with a sample size of 20 replicates (N = 20). After every replicate, the test arena was thoroughly cleaned with ethanol.

Experiment 1: Structure-Bioactivity Relationship of Methylalkanes for the Restoration of the Pheromone in 4-d-Old Male Dummies

This experiment was performed to determine if other structurally related methylalkanes, differing in chain length or methyl-branch position, could mimic the pheromonal activity of 3-MeC27 when added to the cuticle of 4-d-old male dummies. For this purpose, the following enantiomerically pure methylalkanes (synthesized as described above) were applied at doses of 150 ng each to the cuticle of 4-d-old male dummies: (*R*)- and (*S*)-enantiomers respectively of 3-MeC25, 3-MeC29, 3-MeC31 (correct position of the methyl branch, differing chain length), and 5-MeC27 and 7-MeC27 (correct chain length, differing position of the methyl branch). (*R*)- and (*S*)-3-MeC27 also were tested as positive controls. The dose of 150 ng for all compounds was chosen because it is the approximate amount of 3-MeC27 found on the cuticle of female wasps (Steiner et al., 2005). All compounds tested in this experiment are minor components of the *L. distinguendus* CHC profile (Steiner et al., 2005). The absolute configurations of the natural products are unknown.

Experiment 2: Interruption of Pheromone Activity in 0-d-Old Male and Female Dummies by the Addition of Individual CHCs

The disappearance of 3-MeC27 from the cuticle of aging males results in the deactivation of the contact pheromone response. Therefore we tested whether specific changes to the bioactive CHC profiles of newly emerged male and female dummies, such as the addition of isomers and homologs of 3-MeC27, could inhibit or interrupt the responses of courting males. For this purpose, the following methylalkanes were applied individually at doses of 150 ng to 0-d-old male or female dummies: (*R*)- and (*S*)-enantiomers of 3-MeC29, 3-MeC31, 5-MeC27, and 7-MeC27. Additionally, we tested whether the addition of straight chain alkanes (150 ng n-C27, n-C29, or n-C31) or an excess of the key component 3-MeC27 (150 ng of the (*R*)- or (*S*)-enantiomer) added to the cuticle of otherwise attractive 0-d-old male dummies, affected the wing-fanning behavior of test males. All n-alkanes tested in this experiment are minor components of the *L. distinguendus* CHC profile (Steiner et al., 2005).

Statistical Analysis

Data did not meet the assumptions for parametric statistical analysis. Therefore, non-parametric Wilcoxon signed rank tests were used for the comparison of the duration of wing-fanning exhibited by responding males towards different treatments (addition of a given synthetic alkane) and the corresponding solvent controls. For statistical calculations, the software R version 2.15.1 (R Core Team R, 2012) was used.

4.4 Results

Experiment 1: Structure-Bioactivity Relationship of Methylalkanes for the Restoration of the Pheromone in 4-d-Old Male Dummies

The addition of either (*R*)- or (*S*)-3-MeC27 to unattractive 4-d-old male dummies restored the wing-fanning behavioral responses elicited from test males, with responding males wing-fanning for significantly longer periods in the presence of pheromone-treated dummies than in the presence of solvent-treated controls (Figure 3). None of the other compounds when applied at doses of 150 ng to 4-d-old male dummies affected the wing-fanning behavior of test males (Figure 3). Thus, males specifically detected and responded to the key component 3-MeC27, but did not distinguish between the enantiomers when they were applied to 4-d-old dummies.

Experiment 2: Interruption of Pheromone Activity in 0-d-Old Male and Female Dummies by the Addition of Individual CHCs

The application of 150 ng of any of the tested straight chain or methyl-branched alkanes other than (*R*)- or (*S*)-3-MeC27 onto bioactive, wing-fanning inducing 0-d-old male dummies resulted in a significant decrease in the duration of wing-fanning in test males (Figure 4a). Similar results were found for the methylalkanes when added to 0-d-old female dummies, except for (*S*)-7-MeC27, for which the decrease in wing-fanning duration was not statistically significant when compared to the solvent control (Figure 4b).

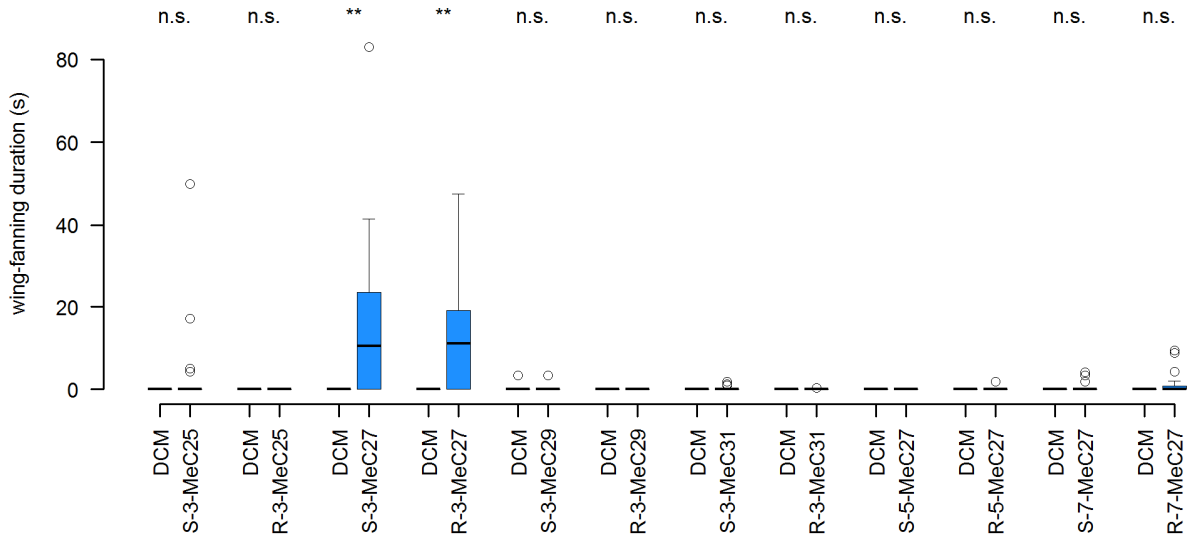

Figure 3. Restoration of pheromone activity in 4-d-old male dummies (Experiment 1). Wing-fanning duration during a 5-min observation period performed by *L. distinguendus* responder males towards 4-d-old male dummies treated with dichloromethane (DCM, control = white) and with 150 ng of different methyl-branched alkanes in dichloromethane, respectively (blue). Box-and-whisker plots show median (horizontal line), 25-75 percent quartiles (box), maximum/minimum range (whiskers) and outliers (>1.5× above box height). Asterisks indicate significant differences between a methylalkane treatment and the corresponding DCM control ($p > 0.05$ = non-significant (n.s.), $p < 0.01$ = **, Wilcoxon signed rank test; N = 20).

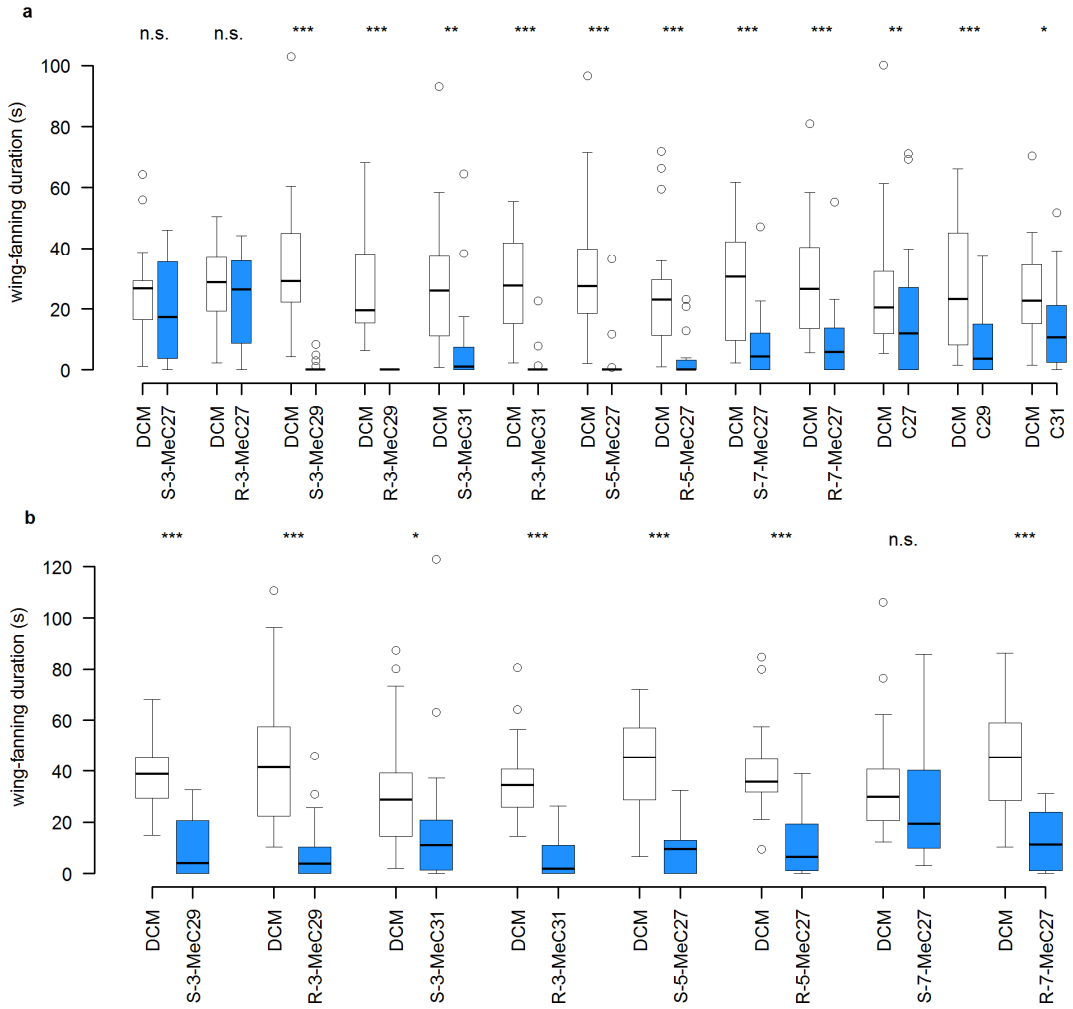

Figure 4. Interruption of pheromone bioactivity in 0-d-old male and female dummies (Experiment 2). Wing-fanning duration during a 5-min observation period performed by *L. distinguendus* responder males towards 0-d-old (**a**) male and (**b**) female dummies treated either with dichloromethane (DCM, control = white) or 150 ng of methyl-branched and straight-chain alkanes, respectively (blue). Box-and-whisker plots show median (horizontal line), 25-75 percent quartiles (box), maximum/minimum range (whiskers) and outliers (>1.5× above or below box height). Asterisks indicate significant differences ($p > 0.05$ = non-significant (n.s.), $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***) between alkane treatment and the corresponding DCM control (Wilcoxon signed rank test; N = 20).

4.5 Discussion

The results of the present study in combination with previous work on *L. distinguendus* (Steiner et al., 2005; Ruther and Steiner, 2008; Kühbandner et al., 2012) shed new light on the role of CHCs as contact sex pheromones by showing that the CHC profile is perceived as a whole by males of this species. This is in contrast to the CHC-based contact sex pheromones of some other insects, in which individual methylalkanes elicit behavioral responses (Carlson et al., 1978, 1998b; Sugeno et al., 2006; Lacey et al., 2008; Rutledge et al., 2009 Silk et al., 2009; Spikes et al., 2010). In *L. distinguendus*, both the removal as well as the addition of individual components to bioactive CHC profiles disrupted the behavioral response of the receiver. Under natural conditions, conspecific males stop responding to aging males as the major contact sex pheromone component, 3-MeC27, disappears from their cuticle. The evolution of this process has presumably been driven by the fitness costs imposed on young males by the courtship activities of conspecific males (Ruther and Steiner, 2008) and has been the prerequisite for the sex-specific conveyance of information. The deactivation of the pheromone in older males was shown to be reversible experimentally by the addition of synthetic 3-MeC27. Furthermore, the response was very specific to 3-MeC27 because when equal amounts of structurally related methylalkanes with differing chain lengths or methyl branch positions were applied to dummies, they did not restore the wing-fanning response. Thus, *L. distinguendus* males respond very specifically to 3-MeC27 and can discriminate variations in chain length of two carbons, and variations in methyl branch position of two or more positions. These results suggest that a missing key component in the CHC profile cannot be replaced by a structurally related analogue, and emphasizes the critical role of 3-MeC27 in the *L. distinguendus* contact sex pheromone. In contrast, the leaf beetle *Gastrophsa atrocyanea* has been shown to tolerate slight variations in the chain lengths and methyl branch points of methylalkanes in its contact pheromone without loss of bioactivity (Sugeno et al., 2006). Similarly, in the longhorned beetle *Neoclytus acuminatus acuminatus*, three methylalkanes (7-MeC25, 7-MeC27 and 9-MeC27) differing in chain length or position of the methyl branch have been identified as the female's contact sex pheromone. Each compound was active alone, but a combination of all three was required to elicit the full behavioral response from males (Lacey et al., 2008).

The designation of 3-MeC27 as a key component of the contact sex pheromone of *L. distinguendus* is corroborated by the fact that treatment of attractive 0-d-old male dummies with an unnaturally high dose of synthetic 3-MeC27 resulted in no significant change in the wing-fanning responses elicited from courting males. In contrast, the application of any of the

other synthetic methylalkanes onto 0-d-old male dummies resulted in a significant decrease in the wing-fanning response. The same was true when 0-d-old female dummies were treated with synthetic methylalkanes other than 3-MeC27, with the exception of those treated with (*S*)-7-MeC27, which had no significant effect on the bioactivity of 0-d-old female dummies as compared to the solvent treated controls ($p = 0.08$). The reason for this anomaly is unclear, particularly as both enantiomers of 7-MeC27 significantly disrupted responses from males when applied to male 0-d-old dummies. All compounds interrupting the pheromone response in *L. distinguendus* males when added to bioactive CHC profiles are minor components of the natural CHC profile of this species (Steiner et al., 2005). This suggests that it was not the appearance of a novel foreign compound but a shift in the ratios of familiar compounds what caused the loss of pheromone activity.

Disturbance of CHC profiles by the addition of synthetic compounds also has been demonstrated in the context of nestmate recognition in social insects. Addition of specific alkanes to the CHC profile of individual workers increased aggressive behavior by nestmates in some species (reviewed by van Zweden and d'Ettore, 2010). Some studies provided evidence that methyl-branched alkanes might be more important in this respect than straight chain alkanes (Dani et al., 2001, 2005; Châline et al., 2005; Guerrieri et al., 2009; van Wilgenburg et al., 2012). The results of the present study show that the addition of both straight chain and methyl-branched alkanes can disturb bioactive CHC profiles in the context of sexual communication (Figure 4a). In the context of nestmate recognition, it should be much easier to render a nestmate unacceptable by experimental manipulation of its CHC profile than the reverse, *i.e.*, rendering a non-nestmate acceptable. That is, if a nestmate recognizes a non-nestmate as foreign by perceiving the species-specific CHCs in ratios differing from the known colony blend, only the exact correction of the imbalance should render it acceptable. In contrast, many different compounds added to the cuticle of a nestmate could make it unacceptable. Transferring these considerations to the present study might explain why *L. distinguendus* responded only to 3-MeC27 in the pheromone restoration experiment (Experiment 1), whereas in the pheromone interruption experiment (Experiment 2) many different compounds disrupted the pheromonal response equally well.

The striking parallels between the role of CHCs in nestmate recognition of social insects and sexual communication in *L. distinguendus* suggest that there might also be analogies in the sensory organs used to detect these compounds. It has been suggested that single CHCs used as sex pheromone components are perceived by gustatory sensilla. However, conventional gustatory sensilla have been predicted to be unsuitable for the perception of complex CHC

profiles because they typically are innervated by only a small number of receptor neurons (Ozaki and Wada-Katsumata, 2010). This idea was corroborated by the identification of a specialized olfactory sensillum type in the ant *Camponutus japonicus* which is innervated by about 130 olfactory receptor neurons and capable of discriminating complex CHC profiles originating from nestmates and non-nestmates, respectively (Ozaki et al., 2005; Ozaki and Wada-Katsumata, 2010). Given the results of the present study suggesting that *L. distinguendus* wasps, like the ants, perceive CHC profiles as a whole, it will be interesting to determine whether similar specialized sensilla also are present on the antennae of this species.

Apart from 7-MeC27 tested on 0-d-old female dummies, *L. distinguendus* males did not discriminate between the enantiomers of synthetic methylalkanes. However, in a previous study (Kühbandner et al., 2012), males preferred (*S*)-3-MeC27 over (*R*)-3-MeC27 when presented in a different context, *i.e.*, when applied to filter paper together with a chemical background of the other CHCs and triacylglycerides. This preference was not seen in the present study when the enantiomers were tested with three-dimensional 4-d-old male dummies (Kühbandner et al., 2012). These results suggest that chemically-based sex recognition in *L. distinguendus* is supported by visual and/or tactile stimuli, as previously shown for the pteromalid wasps *Nasonia vitripennis* (Steiner et al., 2006) and *Dibrachys cavus* (Ruther et al., 2011). Thus, the absolute configuration of 3-MeC27 occurring on the cuticle of *L. distinguendus* wasps could not be concluded unambiguously from the behavior of males as has been done, for instance, for the enantiomers of 5-MeC27 with the egg parasitoid *Oenocyrtus kuvanae* (Encyrtidae) (Ablard et al., 2012). Thus, the chirality of 3-MeC27 in *L. distinguendus* remains to be established by analytical methods. However, before this can be accomplished, methods need to be developed for resolving the enantiomers of methylalkanes or of determining their absolute configuration on microgram to nanogram scale (Hefetz et al., 2010; Millar, 2010).

4.6. Conclusions

Subsets of CHCs have evolved to have secondary functions as contact sex pheromones in several insect taxa (Howard and Blomquist, 2005). However, details of the structure-activity relationships of CHCs and the contribution of individual components to the bioactivity of complex CHC profiles have been elucidated in very few species. It appears that the mechanisms for the perception of CHCs in the context of sexual communication differ

significantly among taxa. In Coleoptera and Diptera, for instance, individual key components may elicit behavioral responses when presented alone (Carlson et al., 1978, 1998b; Ginzel et al., 2003, 2006; Sugeno t al., 2006; Lacey et al., 2008; Silk et al., 2009). In contrast, for *L. distinguendus*, CHC profiles are perceived as a whole and the key compound 3-MeC27 needs to be present with a chemical background of other cuticular lipids to elicit behavioral responses in males. Furthermore, the response elicited by an attractive individual could be disturbed by distortion of the CHC profile by enhancement of individual compounds. Given that purified CHC fractions also have been shown to elicit behavioral responses in other parasitic wasp species (Ruther, 2013), further studies are needed to understand the role of individual CHCs in these species, and to investigate whether the perception mechanism found in *L. distinguendus* is common in parasitic wasps.

4.7 Acknowledgments

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4.8 Conflicts of Interest

The authors declare no conflict of interest.

Personal contribution:

I established the bioassays of this paper and carried out the biological experiments assisted by Sergej Sperling and Theresa Hammerl. I analyzed the data from the bioassays, created the biological figures and wrote the text of this paper without the chemical synthesis part as a first version.

Chapter 5

Solid phase micro-extraction (SPME) with *in situ* transesterification: An easy method for the analysis of non-volatile cuticular triacylglycerides

5.1 Abstract

Triacylglycerides (TAGs) have been found in large amounts in the internal tissues of insects but their occurrence on the insect cuticle has been widely ignored. Most studies investigating cuticular lipids of insects involve solvent extraction, which runs the risk of the extraction of lipids from internal tissues. Here, we present a new method (**SPME-FAME-GC-MS**) that allows the solvent-free analysis of insect cuticular TAGs. With this method, TAGs are sampled by rubbing a solid phase micro-extraction (**SPME**) fibre over the insect cuticle. The sampled TAGs are transesterified *in situ* with trimethyl sulfonium hydroxide into more volatile fatty acid methyl esters (**FAME**), which can be analysed by standard gas chromatography coupled with mass spectrometry (**GC-MS**). Two types of control experiments have been performed: (1) to rule out contamination of the GC-MS system with FAMEs originating from former analyses and (2) to exclude the analysis of free fatty acids, instead of TAGs, on the insect cuticle. Only the combination of SPME-FAME-GC-MS with the two types of controls actually shows whether TAGs occur on the insect cuticle. Six insect species from four insect orders have been analysed with SPME-FAME-GC-MS and with common whole body solvent extraction and subsequent GC-MS analysis after transesterification into FAMEs (FAME-GC-MS). Both methods have been evaluated, with SPME-FAME-GC-MS probably being a more suitable method for analysing cuticular TAGs than solvent extraction, the former method involving no risk of extracting lipids from internal tissues. The most abundant TAG fatty acids are: tetradecanoic acid, (Z)-hexadec-9-enoic acid, hexadecanoic acid, (9Z,12Z)-octadeca-9,12-dienoic acid, (Z)-octadec-9-enoic acid and octadecanoic acid. They can be detected on the cuticle of all species analysed with SPME-FAME-GC-MS. Thus, TAGs are far more common cuticular compounds than usually thought.

5.2 Introduction

Insects often live in rough environments under changing conditions. To protect themselves from desiccation and pathogens, they cover their cuticle with a thin layer of lipids (Gibbs and Rajpurohit, 2010). These cuticular lipids often have a second function in the inter- and intra-specific communication of many insects. The non-polar cuticular hydrocarbons (CHCs) are the focus of most studies and comprise straight-chain alkanes, alkenes and methylalkanes. CHCs are abundant and are relatively easy to isolate and identify (Gibbs and Rajpurohit, 2010). Insects use them to recognize the species and gender of other individuals in order to find potential mating partners or to defeat opponents (Singer, 1998; Howard and Blomquist, 2005). In social insects, CHCs are also employed to recognize nestmates, caste and kinship (Howard and Blomquist, 1982, 2005; Singer, 1998). More polar compounds found on the insect cuticle are alcohols, aldehydes, ketones and wax-esters (Buckner, 2010). A mostly unnoticed, but not unimportant group of cuticular compounds are the triacylglycerides (TAG). TAGs can, for example, play a role in the sexual communication of insects. Thus, in *Drosophila mojavensis* and *D. arizonae*, cuticular TAGs located in the males anogenital region are transferred to the female during mating (Yew et al., 2011a). Further, TAGs have recently been shown to be an elementary compound of the female courtship pheromone of the pteromalid wasp species *Lariophagus distinguendus* (Kühbandner et al., 2012). TAGs are chemically characterized by their fatty acid chain length, the number and position of their double bonds and the position of the fatty acids on their glycerol backbone (Cvačka et al., 2006c; Kofroňová et al., 2009). TAGs occur in large amounts as lipid droplets in the adipocytes of the insect fat body (Downer and Matthews, 1976; Arrese and Soulages, 2010), which is a storage site for energy and an important metabolic tissue that converts TAGs into precursors of pheromones (Arrese and Soulages, 2010). The TAGs are either taken up in the diet or synthesized de novo (Arrese and Soulages, 2010). Whereas TAGs occur in large amounts and as the major lipid class in the internal tissues of insects, they are not a major component of the cuticular lipids of most insects (Buckner, 1993). Additionally, TAGs might be often overlooked, because they are not detectable without transesterification into more volatile fatty acid methyl esters (FAMEs) by standard gas chromatography coupled with mass spectrometry (GC-MS), because of their high melting points (Cvačka et al., 2006a; Cvačka et al., 2006b; Millar, 2010). Nevertheless, they have been found in whole-body extracts of insect species from several orders, for instance, the big stonefly *Pteronarcys californica* (Armold et al., 1969), the cigarette beetle *Lasioderma serricorne* (Baker et al., 1979), the pea aphid

Acyrthosiphon pisum (Brey et al., 1985), the grasshoppers *Melanoplus bivittatus, Melanoplus femurrubrum* and *Melanoplus dawsoni* (Jackson, 1981) and the kissing bug *Triatoma infestans* (Juárez et al., 1984). Most of those studies share one problem; they rely on whole-body solvent based extracts that are usually analysed by gas chromatography (GC) or high performance liquid chromatography (HPLC) techniques (Cvačka et al., 2006a). Solvent extraction however involves the risk of extracting lipids from internal tissues such as the fat body (Hadley, 1980; Jackson, 1981; Jackson et al., 1981; Lockey, 1985; Buckner, 1993). Therefore, whether the TAGs reported in such studies are really present on the cuticle or whethr they originate from internal tissues remains unclear.

One method to verify the origin of TAGs is the extraction of exuviae or puparia, as performed with the grasshopper *Melanoplus bivittatus* (Jackson, 1981) and the Australian sheep blowfly *Lucilia cuprina* (Goodrich, 1970). However, the method is impractical for most species, because of its dependence on obtaining the cast skin or puparia for analysis. This restricts the analysis to distinct times shortly after the emergence or moulting of the insect. A solvent-free method has recently been reported by Yew et al. (2009, 2011a, 2011b). They have demonstrated the occurrence of TAGs on the cuticle of several *Drosophila* species by using direct ultraviolet laser desorption/ionization orthogonal time-of-flight mass spectrometry (UV-LDI-o-TOF MS). This method, unlike matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), does not require a matrix and has the advantage that insects can be used without prior extraction. However, UV-LDI-o-TOF MS is perhaps more invasive than has been assumed (Everaerts et al., 2010) and an absolute quantification of compounds is not possible with MALDI-MS and UV-LDI-o-TOF MS, because the elemental composition of a compound influences ionization and detection success. For example, saturated hydrocarbons cannot be analysed with those methods (Cvačka and Svatoš, 2003; Yew et al., 2009). Additionally UV-LDI-o-TOF MS is usually lethal for the analysed insect (Everaerts et al., 2010), is expensive and is thus not standard in most laboratories.

A practical alternative might be to carry out wipe-sampling by the solid phase micro-extraction (SPME) technique in combination with GC-MS (SPME-GC-MS). This method works without solvent and therefore does not include the risk of extracting internal lipids. Several studies have compared the effectiveness of SPME wipe-sampling with solvent extraction for CHCs and found that both methods yield comparable results, although some cuticular compounds associated with mate recognition are detected in higher relative amounts by SPME wipe-sampling than by solvent extraction (Ginzel et al., 2006; Lacey et al., 2008;

Spikes et al., 2010). These findings have led to the hypothesis that cuticular compounds that are accessible to the male antennae are more representatively detected by SPME wipe-sampling than are those after solvent extraction (Ginzel et al., 2006; Lacey et al., 2008; Spikes et al., 2010). Another advantage of SPME is that living insects can be used allowing the repeated measurement of cuticular compounds and thus the tracking of temporal changes of cuticular lipid profiles (Peeters et al., 1999; Lacey et al., 2008; Ouyang et al. 2011). A strong limitation of SPME-GC-MS wipe-sampling has been, until recently, that some compounds such as TAGs cannot be analysed, because of their high melting points. We have developed **SPME-FAME-GC-MS** as a new method that makes high molecular weight compounds such as TAGs also detectable with GC-MS. With this method, TAGs can be sampled by rubbing a solid phase micro-extraction (**SPME**) fibre over the insect cuticle. The sampled TAGs are transesterified *in situ* with trimethyl sulfonium hydroxide into more volatile fatty acid methyl esters (**FAME**) (Butte, 1983; Kühbandner et al., 2012), which can be analysed with standard **GC-MS**. Two types of control experiments have been performed (1) to rule out contamination of the GC-MS system with FAMEs originating from former analyses and (2) to exclude the occurrence of free fatty acids (FFAs), instead of TAGs, being analysed on the insect cuticle. Only the combination of SPME-FAME-GC-MS with the two types of controls actually shows whether TAGs occur on the insect cuticle. With this method, TAGs were detected on the cuticle of the parasitic wasp species *Lariophagus distinguendus* (Kühbandner et al., 2012). *L. distinguendus* is the first species in which a communicative function of TAGs has been shown to date. The cuticular TAGs of *L. distinguendus* are essential components of the female contact sex pheromone in this species (Kühbandner et al., 2012). However, SPME-FAME-GC-MS has until now only been used to identify cuticular TAGs in one insect species (Kühbandner et al., 2012) and, thus, further analysis with other species from several insect orders are necessary to establish this new method.

In this study, the effectiveness of SPME-FAME-GC-MS as a solvent-free extraction method for cuticular TAGs will be compared with common whole body solvent extraction. Therefore, TAGs from females of six insect species have been extracted with SPME-FAME-GC-MS and common whole body solvent extraction with dichloromethane (FAME-GC-MS). The results of both extraction techniques have been compared and evaluated.

5.3 Materials and Methods

Insects

As described in Steidle and Schöller (1997) *Lariophagus distinguendus* (LD) wasps were reared on grain kernels infested with late instar larvae and prepupae of the granary weevil *Sitophilus granarius* (Curculionidae) at 28 % relative humidity and a light/dark cycle of 12:12 h. Female individuals were isolated shortly after emergence, frozen and stored at -23 °C.

Nasonia vitripennis (NV) wasps were reared on puparia of the green bottle fly *Lucilia caesar* as described elsewhere (Steiner et al., 2006) at 28 % relative humidity. Wasp pupae were isolated from the host puparia and kept separately in micro-centrifuge vials under the same conditions. Female wasps were killed by freezing at -23 °C two days after emergence.

Drosophila melanogaster (DM) was reared on a standard fly diet under conditions described by Stökl et al. (2012). Female individuals were isolated shortly after emergence, frozen and stored at -23 °C.

Asobara tabida (AT) wasps were reared on *D. melanogaster* hosts (rearing of the hosts and conditions as described above). For each new charge, 30 flies of each sex were placed together with standard fly diet (see above) into a container for 48 h. After the flies had laid eggs, the adult individuals were removed and exchanged against 10 mated *A. tabida* females. Parasitized fly pupae were isolated and kept separately in micro-centrifuge vials. Two days after emergence, they were frozen and stored at -23 °C.

Nicrophorus vespilloides (NI) females used for TAG analysis were the F2-generation offspring of beetles collected from carrion-baited pitfall traps in a deciduous forest near Freiburg in southwestern Germany (48°00’ N, 07°51’ E) (Steiger et al., 2008). The F1- and F2-generation beetles had been reared by fostering parents of laboratory cultures and were therefore free of nematodes or mites that are typically found on field-caught beetles. Beetles were maintained under conditions according to the protocol of Eggert et al. (1998). Adult females were frozen and stored at -23 °C.

Cockroaches of the species *Periplaneta americana* (PA) were obtained from laboratory populations kept at the University of Regensburg. They were reared under conditions described by Herzner et al. (2013). Adult females were frozen and stored at -23 °C.

SPME-FAME-GC-MS

To analyse putative TAGs occurring on the cuticle of insects, the SPME technique was used combined with *in situ* transesterification of TAGs with trimethyl sulfonium hydroxide (TMSH) (Butte, 1983). This agent converts TAGs into more volatile fatty acid methyl esters (FAMEs) thereby allowing their analysis by GC-MS. A 24-gauge polydimethylsiloxane-coated SPME fibre (PDMS, Supelco, Bellefonte, PA, USA) was conditioned for 90 min in the injector of a gas chromatograph (GC) at 280 °C. Afterwards the SPME fibre was soaked with TMSH solution (Sigma-Aldrich, Steinheim, Germany) for 30 s and subsequent desorbed in the injector of a GC-MS (Fisons GC 8000 series, Fisons MD 800) for one minute. The corresponding GC run was recorded in order to exclude the presence of FAMEs and served as a negative control (SPME control 1). If the fibre and the GC-MS system were free of FAMEs, the conditioned SPME fibre was rubbed for a total time of five minutes over the cuticle of the following number of female insects: 5 LD, 5 NV, 5 AT, 5 DM, 1 NI or 1 PA. Afterwards, the SPME fibre was soaked with TMSH solution for 30 seconds and subsequently desorbed in the injection port of a GC-MS system. The FAMEs were analysed by GC-MS. To exclude that FFAs occurring on the insect cuticle were analysed instead of TAGs, analogous experiments were performed but without soaking the SPME fibre with TMSH and served as another negative control (SPME control 2). The parameters of the corresponding GC-MS analyses are given below.

Preparation of solvent-based extracts (FAME-GCMS)

Twenty-five female insects of one species (LD, NV and DM) per analysis were extracted in 60 µl dichloromethane (DCM) for 30 s. Ten female insects per analysis were used for AT. Because of their larger size, only one female of NI was extracted in 1 ml DCM for 30 s and one female of PA with 2 ml DCM for 30 s. All extracts were filtered through clean cotton wool to remove small particles originating from the animals or their rearing substrates. Afterwards, 100 µl fresh DCM were used to rinse the remaining compounds off the cotton wool. The DCM was completely evaporated under a stream of nitrogen and the sample was resolved in 20 µl DCM. The extracts were fractionated by size exclusion high performance liquid chromatography (SE-HPLC) to separate the TAGs from cuticular hydrocarbons (CHC) and other compounds (see SE-HPLC).

SE-HPLC

Size exclusion high performance liquid chromatography (SE-HPLC) was used to separate the TAGs from the other compounds within the DCM samples. A LC-20AD HPLC pump (Shimadzu Europe, Duisburg, Germany) pumped DCM (GC grade, Sigma-Aldrich, Steinheim, Germany) at a pressure of 1.8-2.0 MPa resulting in a flow rate of 1 ml/min through a 300 x 7.5 mm PLgel SE-HPLC column (particle size 5 µm, pore size 100 Å, Agilent Technologies Deutschland, Waldbronn, Germany). The extracts (20 µl) were injected into a Rheodyne model 7125 HPLC injector equipped with a 20 µl sample loop (Rheodyne, Cotati, CA, USA). The fraction containing the TAGs was collected into clean GC vials for between 6.05 and 6.35 min.

Addition of internal standard and transesterification into FAMEs

The isolated TAGs were supplemented with an internal standard (heptadecanoic acid) and transesterified by means of methanol and acetyl chloride into more volatile FAMEs for subsequent GC-MS analysis. The internal standard (heptadecanoic acid) had to be added after the fractionation with SE-HPLC, since the TAGs would otherwise have been separated from the internal standard because of their different molecular size. Each TAG fraction from the SE-HPLC purification was supplemented with 60 µl heptadecanoic acid solution in DCM (20 ng/µl). For AT extracts, only 30 µl heptadecanoic acid solution in DCM (20 ng/µl) was added. Each sample was transesterified into FAMEs according to the protocol of Blaul and Ruther (2011). The solvent was evaporated under a constant stream of nitrogen and, subsequently, 100 µl methanol and 10 µl acetyl chloride solution (10% in methanol) were added. The samples were kept for 1 h at 60 °C. Next, 200 µl sodium bicarbonate solution (5 % in methanol) and 200 µl pentane were added and shaked for 1 min. The upper pentane phase containing the FAMEs was transferred into a clean GC vial. Afterwards, the solvent was evaporated under a constant stream of nitrogen and 20 µl pentane was added.

GC-MS analysis

The SPME samples and 1 µl aliquots of the solvent-based extracts were analysed by GC-MS (split less mode) on a Fisons GC-MS (Fisons GC 8000 series, Fisons MD 800) equipped with a RH-5ms column (30 m, 360/370°C, ID 0.32 mm, film thickness 0.25 µm, CZT, Kriftel, Germany). Helium (4.6) was used as the carrier gas with an inlet pressure of 15 kPa. Samples

were ionized by electron impact ionization (EI) at 70 eV. The mass spectrometer scanned a mass range (m/z) from 35 to 600 (scan time 0.2 s, inter scan delay 0.05 s). The oven program for the SPME-FAME-GC-MS analysis started at 50 °C for 4 min and increased at a rate of 3 °C/min up to 280 °C and was held at this temperature for 45 min. The oven program for the solvent-based extracts was analogous but the maximum temperature was held only for 15 min. The co-injection of straight-chain alkanes allowed the estimation of linear retention indices (LRI) of FAMEs (van den Dool and Kratz, 1963). FAMEs were identified by a comparison of their mass spectrum, their retention time or LRI with those of synthetic reference chemicals (37 component FAME mix, Supelco, Bellefonte, PA, USA).

Data analysis

For both methods (SPME-FAME-GC-MS and solvent extraction), three replications per species were performed (N=3). Relative fatty acid amounts per individuum were calculated by dividing the area of a distinct fatty acid peak by the total area of all fatty acid peaks. Absolute fatty acid amounts could only be calculated for the solvent extracts from the added amount of internal standard (heptadecanoic acid). Absolute amounts are given in nanograms and were calculated as a mean of three samples (N=3).

5.4 Results

SPME control 1 and 2

The fatty acids analysed by SPME-FAME-GC-MS originated from TAGs located on the insect cuticle, because no contamination with TAGs was found in the GC-MS system for the analysis of *Lariophagus distinguendus* (LD) and *Periplaneta americana* (PA) (Fig. S1 and S6; SPME control 1). For the analysis of *Nasonia vitripennis* (NV), *Drosophila melanogaster* (DM), *Asobara tabida* (AT) and *Nicrophorus vespilloides* (NI), only minor contamination of the GC-MS system with TAGs was found (Fig. S2 – S5; SPME control 1). FFAs could not be detected on the cuticle of any analysed insect (SPME control 2; Fig. S1 – S6; rubbing the SPME fibre over the insect cuticle without soaking it in TMSH for transesterification).

Comparison of SPME-FAME-GC-MS with solvent extraction (FAME-GC-MS)

A comparison of the two methods (SPME-FAME-GC-MS and solvent extraction) revealed differences in the relative amounts of fatty acids in some samples (table 1 - 2, Fig. 1). A 5 % to 10 % higher relative amount of (Z)-tetradec-9-enoic acid (DM), tetradecanoic acid (DM), hexadecanoic acid (NV, PA) and octadecanoic acid (NV, DM, PA) was detected by SPME-FAME-GC-MS compared with solvent-based extracts. The relative amounts of (Z)-hexadec-9-enoic acid (PA), hexadecanoic acid (AT, NI), (9Z,12Z)-octadeca-9,12-dienoic acid (LD) and octadecanoic acid (NI) analysed by SPME-FAME-GC-MS were even more than 10 % higher than in solvent-based extracts. On the other hand, (Z)-heptadec-10-enoic acid (NI), (9Z,12Z)-octadeca-9,12-dienoic acid (PA) and icosanoic acid (NI) were detected at a 5 % to 10 % lower relative amount by SPME-FAME-GC-MS compared with solvent extraction. The relative amount of (Z)-octadec-9-enoic acid analysed by solvent extraction was more than 10 % higher in all species when compared with the SPME-FAME-GC-MS samples. (Z)-octadec-9-enoic acid was also the fatty acid with the highest ratio by far in all solvent-based extracts, whereas in SPME-FAME-GC-MS, hexadecanoic acid was detected at a higher (NV,DM,AT,NI) or comparable ratio (LD,PA) to (Z)-octadec-9-enoic acid (table 1-2, Fig.1).

A comparison of representative SPME-FAME-GC-MS chromatograms of each insect species scaled at the same intensity level revealed that the total amount of cuticular TAG fatty acids was much higher in LD than in all other species analysed (Fig. 2).

Major compounds

Six fatty acids contributing together between 80 % and 100 % to the total fatty acid profile (depending on species and extraction method) were present in all species: tetradecanoic acid, (Z)-hexadec-9-enoic acid, hexadecanoic acid, (9Z,12Z)-octadeca-9,12-dienoic acid, (Z)-octadec-9-enoic acid and octadecanoic acid (table 1 - 3, Fig. 1), with the exception of NV solvent-based extracts in which tetradecanoic acid could not be detected. These six fatty acids can be considered as major components of cuticular TAGs of the species analysed in this study.

Minor compounds

Most of the following fatty acids occurred at a percentage of rarely over 3 % and were absent in all species and samples (table 1 and 2, Fig.1). Therefore, they were classified as minor compounds. Most of them were exclusively found in solvent-based extracts (FAME-GC-MS) of PA (PA, table 2): unknown fatty acid 3, pentadecanoic acid, unknown fatty acid 4, unknown fatty acid 5, unknown fatty acid 6, (Z)-heptadec-10-enoic acid, (6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid, unknown fatty acid 7, unknown fatty acid 8, (11Z,14Z,17Z)-eicosa-11,14,17-trienoic acid, unknown fatty acid 9, (11Z,14Z)-eicosa-11,14-dienoic acid, (Z)-eicos-11-enoic acid and tetracosanoic acid.

Tables

Table 1. Mean relative amounts of fatty acids per individuum (±SE, N=3) analysed by SPME-FAME-GC-MS. *Lariophagus distinguendus* = LD; *Nasonia vitripennis* = NV; *Drosophila melanogaster* = DM; *Asobara tabida* = AT; *Nicrophorus vespilloides* = NI; *Periplaneta americana* = PA; Molecular weight = MW.

Fatty Acids	Formula	MW	LD	NV	DM	AT	NI	PA
dodecanoic acid	C12:0	200	0.1±0.06	0.7±0.1	3.1±0.3	0	1.0±0.5	0
unknown fatty acid 1			0	0	4.6±2.2	0	0	0
(Z)-tetradec-9-enoic acid	C14:1	226	0	0	6.1±1.2	0	0	0
tetradecanoic acid	C14:0	228	1.1±0.1	2.4±0.1	16.6±1.0	4.3±2.3	3.8±1.7	1.0±0.5
Pentadecenoic Acid	C15:1	240	0	0	0	0	0.3±0.2	0
pentadecanoic acid	C15:0	242	0.2±0.1	0.9±0.1	1.5±0.3	0	0.4±0.3	0
C16:1?	C16:1	254	0.4±0.03	1.6±0.4	0.2±0.2	0	1.1±0.5	0.0
(Z)-hexadec-9-enoic acid	C16:1	254	1.6±0.04	2.8±0.07	11.4±0.6	5.0±2.8	2.0±1.0	20.9±11.8
hexadecanoic acid	C16:0	256	27.0±3.1	29.9±1.8	26.9±2.2	60.5±16.2	35.3±3.9	23.6±3.0
(Z)-heptadec-10-enoic acid	C17:1	268	0	0	0	0	0.8±0.6	0
(9Z,12Z)-octadeca-9,12-dienoic acid	C18:2	280	27.5±3.7	13.5±2.3	3.8±0.3	4.1±1.7	11.2±1.3	15.3±3.2
(Z)-octadec-9-enoic acid	C18:1	282	31.9±3.7	28.2±0.9	15.7±1.5	14.4±5.9	25.5±2.5	25.8±5.4
(E)-octadec-9-enoic acid	C18:1	282	0.7±0.1	1.8±0.07	0	0	0.5±0.4	0
octadecanoic acid	C18:0	284	8.7±1.5	16.3±2.0	8.0±1.0	11.7±5.4	17.3±4.1	13.5±3.4
icosanoic acid	C20:0	312	0.8±0.1	1.1±0.2	2.1±0.2	0	0.4±0.3	0
docosanoic acid	C22:0	340	0	0.7±0.3	0	0	0.5±0.4	0

Table 2. Mean relative amounts of fatty acids per individuum (±SE, N=3) analysed by solvent extract GC-MS analyses. *Lariophagus distinguendus* = LD; *Nasonia vitripennis* = NV; *Drosophila melanogaster* = DM; *Asobara tabida* = AT; *Nicrophorus vespilloides* = NI; *Periplaneta americana* = PA; Molecular weight = MW.

Fatty Acids	Formula	MW	LD	NV	DM	AT	NI	PA
dodecanoic acid	C12:0	200	0	0	0.4±0.3	0	0	0
unknown fatty acid 1	-	-	0	0	0.3±0.2	0	0	0
(Z)-tetradec-9-enoic acid	C14:1	226	0	0	0.5±0.2	0	0	0
unknown fatty acid 2	-	-	0	0	0.1±0.07	0	0	0
tetradecanoic acid	C14:0	228	1.1±0.04	0	6.9±0.6	1.4±1.2	1.0±0.3	0.5±0.02
unknown fatty acid 3	-	-	0	0	0	0	0	0.1±0.03
pentadecanoic acid	C15:0	242	0	0	0	0	0	0.1±0.05
C16:1?	C16:1	254	0.5±0.1	1.1±0.5	0.3±0.1	0	1.0±0.3	0.3±0.1
(Z)-hexadec-9-enoic acid	C16:1	254	2.3±0.3	6.0±1.7	16.4±1.8	9.0±1.2	2.8±0.7	0.8±0.1
hexadecanoic acid	C16:0	256	24.0±	24.4±3.6	23.0±0.9	30.3±1.7	9.4±1.8	16.4±0.8
unknown fatty acid 4	-	-	0	0	0	0	0	0
unknown fatty acid 5	-	-	0	0	0	0	0	0
unknown fatty acid 6	-	-	0	0	0	0	0	0
(Z)-heptadec-10-enoic acid	C17:1	268	0	0	0	0	7.8±3.6	0
(6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	C18:3	278	0	0	0	0	0	0.1±0.04
(9Z,12Z)-octadeca-9,12-dienoic acid	C18:2	280	16.5±1.3	17.6±2.4	7.0±1.4	4.3±1.8	15.7±2.3	24.3±0.4
(Z)-octadec-9-enoic acid	C18:1	282	44.5±0.6	40.6±3.0	42.5±1.2	36.8±4.7	49.4±4.3	45.4±1.8
(E)-octadec-9-enoic acid	C18:1	282	0	3.2±2.2	0	0	0.03±0.02	1.3±0.2
octadecanoic acid	C18:0	284	10.6±0.3	7.1±2.1	2.6±0.8	14.8±1.1	2.9±0.1	7.7±1.1
nonadecanoic acid	C19:0	298	0	0	0	0	0.2±0.2	0.5±0.1
unknown fatty acid 7	-	-	0	0	0	0	0	0.6±0.3
unknown fatty acid 8	-	-	0	0	0	0	0	0.2±0.1
(11Z,14Z,17Z)-eicosa-11,14,17-trienoic acid	C20:3	-	0	0	0	0	0	0
unknown fatty acid 9	-	-	0	0	0	0	0	0
(11Z,14Z)-eicosa-11,14-dienoic acid	C20:2	308	0	0	0	0	0	0.5±0.1
(Z)-eicos-11-enoic acid	C20:1	310	0	0	0	0	0	0.9±0.1
icosanoic acid	C20:0	312	0.5±0.01	0	0.1±0.04	0	5.9±2.3	0.2±0.1
docosanoic acid	C22:0	340	0	0	0	3.3±1.9	3.9±1.7	0
tetracosanoic acid	C24:0	368	0	0	0	0	0	0

Table 3. Mean amounts of fatty acids per individuum in ng (±SE, N=3) analysed by solvent extract GC-MS analyses and calculated from the amount of heptadecanoic acid, which was added as an internal standard. *Lariophagus distinguendus* = LD; *Nasonia vitripennis* = NV; *Drosophila melanogaster* = DM; *Asobara tabida* = AT; *Nicrophorus vespilloides* = NI; *Periplaneta americana* = PA; Molecular weight = MW.

Fatty Acids	Formula	MW	LD	NV	DM	AT	NI	PA
dodecanoic acid	C12:0	200	0	0	0.5±0.4	0	0	0.5±0.4
unknown fatty acid 1	-	-	0	0	1.0±0.5	0	0	0.6±0.5
(Z)-tetradec-9-enoic acid	C14:1	226	0	0	1.5±0.8	0	0	0
unknown fatty acid 2	-	-	0	0	0.5±0.4	0	0	0
tetradecanoic acid	C14:0	228	1.2±0.2	0	18.3±9.3	0.6±0.5	47.1±28.6	11.5±3.2
unknown fatty acid 3	-	-	0	0	0	0	0	1.1±0.5
pentadecanoic acid	C15:0	242	0	0	0	0	0	2.1±0.9
C16:1?	C16:1	254	0.6±0.04	0.4±0.2	1.1±0.7	0	42.9±25.3	6.5±3.0
(Z)-hexadec-9-enoic acid	C16:1	254	2.5±0.3	2.3±1.4	43.2±23.9	2.1±1.1	115.6±65.1	20.6±9.2
hexadecanoic acid	C16:0	256	27.4±4.5	5.8±2.3	57.0±28.5	6.3±2.8	1209.0±930.7	371.7±125.8
unknown fatty acid 4	-	-	0	0	0	0	0	0.6±0.5
unknown fatty acid 5	-	-	0	0	0	0	0	1.2±1.0
unknown fatty acid 6	-	-	0	0	0	0	0	1.8±1.5
(Z)-heptadec-10-enoic acid	C17:1	268	0	0	0	0	74.7±33.4	0
(6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	C18:3	278	0	0	0	0	0	0.7±0.5
(9Z,12Z)-octadeca-9,12-dienoic acid	C18:2	280	19.3±3.9	5.5±2.6	19.2±9.7	1.0±0.5	1877.8±1431.5	547.5±174.9
(Z)-octadec-9-enoic acid	C18:1	282	51.4±9.4	10.6±4.0	102.1±48.9	6.3±1.7	5284.2±3965.5	1049.8±363.8
(E)-octadec-9-enoic acid	C18:1	282	0.0	1.7±1.3	0.0		6.7±5.5	24.2±3.0
octadecanoic acid	C18:0	284	12.4±2.5	1.4±0.5	5.0±1.6	3.1±1.3	244.4±176.0	156.6±33.5
nonadecanoic acid	C19:0	298	0	0	0	0	2.3±1.9	7.8±0.5
unknown fatty acid 7	-	-	0	0	0	0	0	9.5±4.2
unknown fatty acid 8	-	-	0	0	0	0	0	3.0±1.5
(11Z,14Z,17Z)-eicosa-11,14,17-trienoic acid	C20:3	-	0	0	0	0	0	1.7±1.4
unknown fatty acid 9	-	-	0	0	0	0	0	1.7±1.3
(11Z,14Z)-eicosa-11,14-dienoic acid	C20:2	308	0	0	0	0	0	10.7±2.7
(Z)-eicos-11-enoic acid	C20:1	310	0	0	0	0	0	18.1±3.0
icosanoic acid	C20:0	312	0.6±0.1	0	0.3±0.2	0	85.9±6.2	4.1±1.9
docosanoic acid	C22:0	340	0	0	0	0.5±0.2	50.2±7.9	0
tetracosanoic acid	C24:0	368	0	0	0	0	0	1.4±1.2

Figures

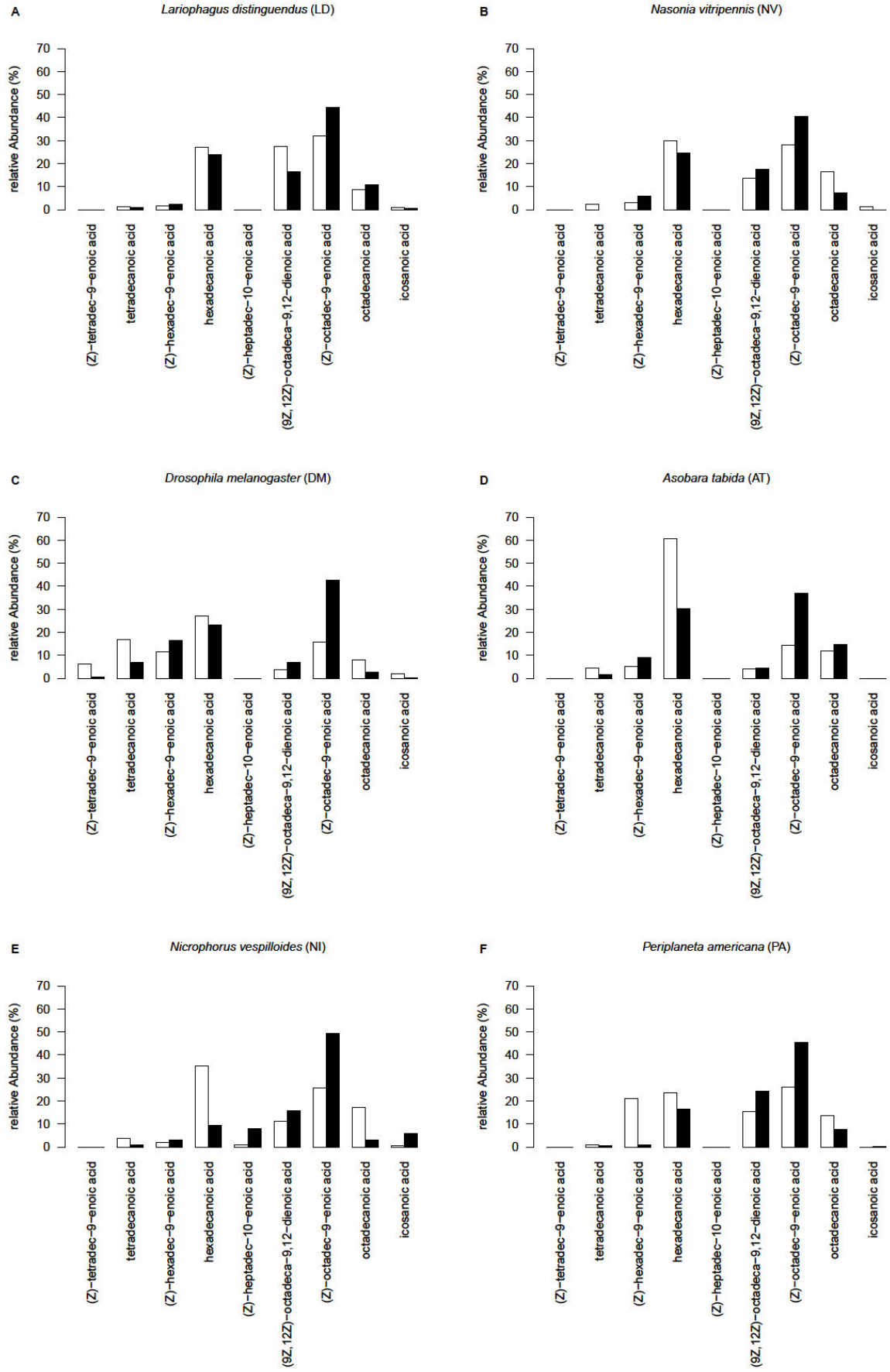
A
Lariophagus distinguendus (LD)
B
Nasonia vitripennis (NV)
C
Drosophila melanogaster (DM)
D
Asobara tabida (AT)
E
Nicrophorus vespilloides (NI)
F
Periplaneta americana (PA)
relative Abundance (%)
0
10
20
30
40
50
60
70
(Z)−tetradec−9−enoic acid
tetradecanoic acid
(Z)−hexadec−9−enoic acid
hexadecanoic acid
(Z)−heptadec−10−enoic acid
(9Z,12Z)−octadeca−9,12−dienoic acid
(Z)−octadec−9−enoic acid
octadecanoic acid
icosanoic acid

Fig. 1. Comparison of SPME-FAME-GC-MS with solvent-based FAME-GC-MS analyses for the detection of fatty acids of: **A** *Lariophagus distinguendus* (LD), **B** *Nasonia vitripennis* (NV), **C** *Drosophila melanogaster* (DM), **D** *Asobara tabida* (AT), **E** *Nicrophorus vespilloides* (NI), **F** *Periplaneta americana* (PA). White bars represent fatty acids detected on the insect cuticle by using SPME-FAME-GC-MS (table 1). Black bars show fatty acids detected by solvent-based FAME-GC-MS analysis (table 2). Fatty acid ratios are a mean of three samples. Only fatty acids that contribute to the total amount of fatty acids by > 5 % are shown.

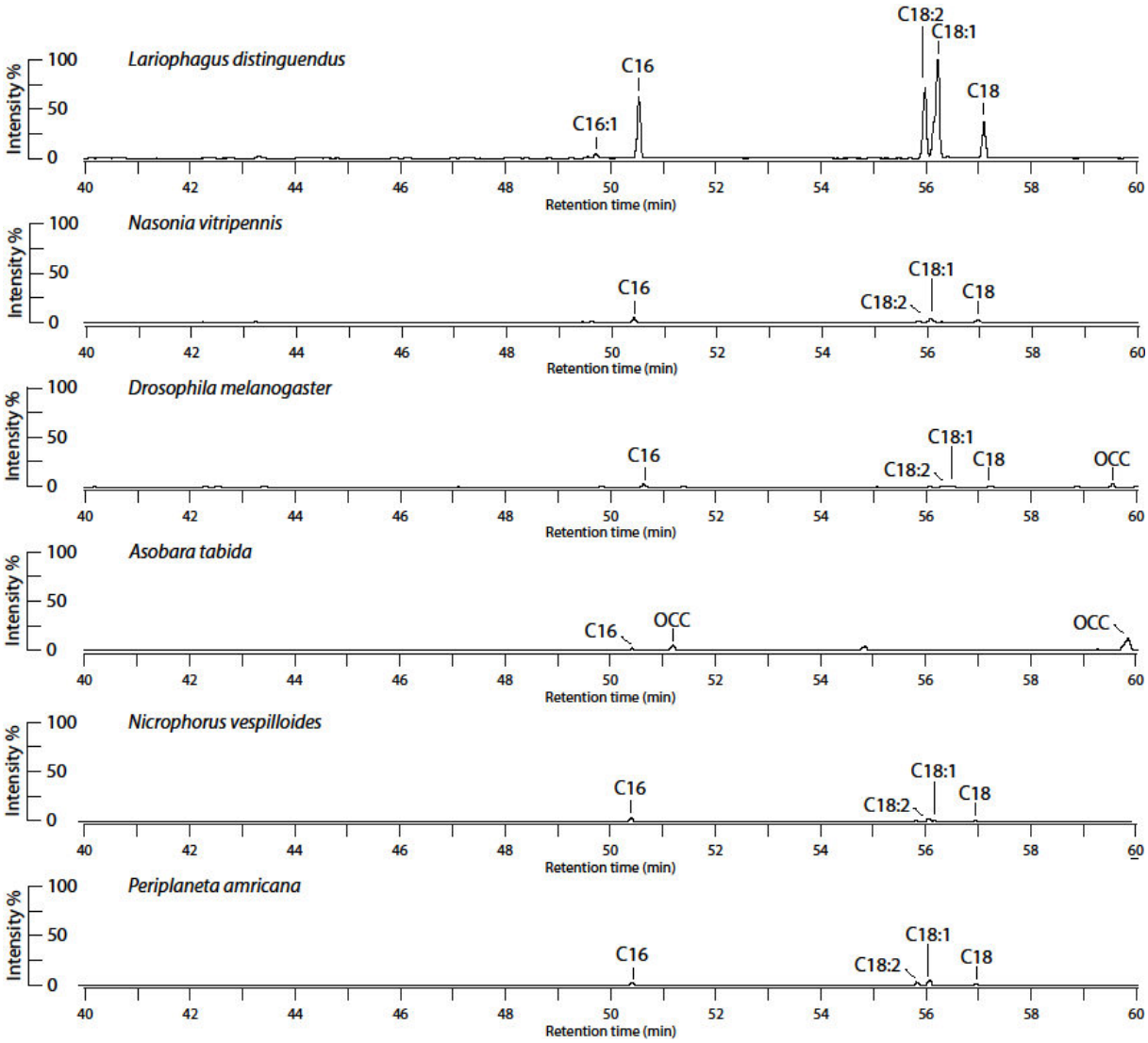

Fig.2. Comparison of the total amount of fatty acids that occur on the cuticle of several insect species as analysed by SPME-FAME-GC-MS. The following fatty acids were detected: C16 = hexadecanoic acid, C16:1 = (Z)-hexadec-9-enoic acid, C18 = octadecanoic acid, C18:1 = (Z)-octadec-9-enoic acid, C18:2 = (9Z,12Z)-octadeca-9,12-dienoic acid, OCC = other cuticular compounds). Chromatograms are scaled at the same intensity level and the retention time is shown below.

5.5 Discussion

In this study, we have shown that the fatty acids analysed by SPME-FAME-GC-MS actually originated from TAGs located on the insect cuticle and not from contamination of the GC-MS system (SPME control 1) or from free-fatty acids on the insect cuticle (SPME control 2). Further, TAG fatty acids have been detected on the cuticle of all analysed insect species; this indicates that TAGs are far more common cuticle compounds than previously thought, because of the low number of studies in which they have been mentioned. Interestingly, the total amount of TAG fatty acids is much higher in *Lariophagus distinguendus* compared with all other species; *L. distinguendus* is to date the only species in which a communicative function of TAGs has been reported (Kühbandner et al., 2012). Extraction of TAGs from the insect cuticle with SPME-FAME-GC-MS has turned out to be a practicable alternative to solvent extraction (FAME-GC-MS) without the risk of extracting TAGs from internal tissues. SPME-FAME-GC-MS is easy to use and does not require expensive equipment. It can thus be performed in any laboratory equipped with a GC-MS system.

The following major TAG fatty acids have been found on the cuticle of nearly all analysed insects: tetradecanoic acid, (Z)-hexadec-9-enoic acid, hexadecanoic acid, (9Z,12Z)-octadeca-9,12-dienoic acid and (Z)-octadec-9-enoic acid and octadecanoic acid. Together, they contributed 80% to 100% of the total fatty acid profile. They have been detected with both extraction methods (SPME-FAME-GC-MS and solvent extraction) and have also been reported in previous studies (Armold et al., 1969; Baker et al., 1979; Juárez et al., 1984). Jackson et al. (1981) have identified tetradecanoic acid, (Z)-hexadec-9-enoic acid, hexadecanoic acid, (9Z,12Z)-octadeca-9,12-dienoic acid and (Z)-octadec-9-enoic acid as the major acylglycerol fatty acids of *Drosophila melanogaster*. Their findings have been verified by the results of this study. The comparison between TAG fatty acids detected with the two extraction methods has revealed some qualitative and quantitative differences, which can be explained by the various origins of the fatty acids. Whereas the major compounds as described above are detected with both methods (SPME-FAME-GC-MS and solvent extraction), minor compounds are more frequently found with solvent extraction. Most of them have been detected exclusively in solvent-based extracts of *Periplaneta americana*. SPME-FAME-GC-MS has found, in comparison with solvent extraction, particularly high amounts of hexadecanoic acid and octadecaonic acid, whereas solvent extraction has delivered higher relative amounts of (Z)-hexadec-9-enoic acid. The TAG fatty acid with the highest relative abundance in solvent-based extracts is (Z)-hexadec-9-enoic acid, whereas in

SPME-FAME-GC-MS, hexadecanoic acid has been found at a higher or comparable ratio to (Z)-hexadec-9-enoic acid. Other studies that have analysed cuticular TAGs by solvent extraction have also reported (Z)-hexadec-9-enoic acid as the TAG fatty acid with the highest relative abundance (Hadley and Jackson, 1977; Baker et al., 1979; Jackson et al., 1981; Juárez et al., 1984). These differences between TAG fatty acids detected with the two extraction methods might be explained by the difference in the composition of TAGs located on the cuticle or in internal tissues. The fact that solvent extraction involves the risk of the extraction of fatty acids from internal tissues such as the fat body has been discussed by several authors (Hadley, 1980; Jackson et al., 1981; Lockey, 1985; Buckner, 1993). The TAG composition of the cuticle itself might also be inhomogeneous and layers might differ in their TAG profile. Everaerts et al. (2010) have found no qualitative, but quantitative differences when they compare CHCs of *D. melanogaster* sampled by SPME and solvent extraction. Using SPME, they have detected larger amounts of unsaturated CHCs and lower levels of straight-chain and methyl-branched alkanes than with solvent extraction. They suggest that SPME detects mainly CHCs located on the outer layers of the cuticle; these layers might deviate not only from the internal tissues of the insect, but also from the deeper layers of the cuticle that are accessible only by solvent extraction (Everaerts et al., 2010). Several authors have suggested that SPME is the method of choice for detecting those compounds, which are actually available for the sensory organs of other individuals (Ginzel et al., 2006; Lacey et al., 2008; Spikes et al., 2010 Everaerts et al., 2010). The same might be true for cuticular TAGs. A remarkable result of this study is that no FFAs have been detected with the two methods. Other studies of cuticular lipids by using solvent extraction have reported the occurrence of FFAs, in addition to triacylglycerides and other polar compounds (Armold et al., 1969; Goodrich, 1970; Downer and Matthews, 1976; Hadley and Jackson, 1977; Jackson et al., 1981; Juárez et al., 1984; Brey et al., 1985). Furthermore, the composition of FFAs and TAGs is often similar (Baker et al., 1979; Lockey, 1985). The results from our study suggest that the FFAs detected in the studies mentioned above were extracted from deeper tissues of the insects and are not accessible to the SPME fibre and presumably not for the antennae of other insects. This is supported by the findings of Yew et al. (2011a), who have analysed the cuticle of *Drosophila arizonae* and *D. mojavensis* by using solvent-free UV-LDI-o-TOF-MS. They do not report the occurrence of FFAs on the cuticle of one of the analysed *Drosophila* species. Disadvantages of SPME-FAME-GC-MS are that currently no quantitative analyses are possible and that the orientation of the fatty acids on the glycerol backbone is lost during the transesterification into FAMEs. We cannot rule out completely that the fatty acids analysed in

this study originate from other compounds than TAGs, which include bound fatty acids. However, this is unlikely, because Aleš Svatoš has analysed *L. distinguendus* extracts by using ultra-performance liquid chromatography coupled with MS (UPLC-MS) analysis and has not found any other fatty-acid-containing compounds than TAGs (unpublished data). In future studies, SPME-FAME-GC-MS could be used in combination with UV-LDI-o-TOF-MS (Yew et al., 2011a, 2011b) for the analysis of cuticular TAG in order to overcome the disadvantages of the two methods. UV-LDI-o-TOF-MS is also unlikely to extract TAGs from internal tissues and has the advantage that the fatty acid composition of TAGs can be analysed. SPME-FAME-GC-MS on the other hand is less costly and non-lethal for the analysed insects (Peeters et al., 1999; Lacey et al., 2008; Ouyang et al. 2011), can analyze saturated CHCs (Everaerts et al., 2010) and is perhaps less destructive (Everaerts et al., 2010). Further studies with UV-LDI-o-TOF-MS and SPME-FAME-GC-MS in combination might reveal the existence of cuticular TAGs in many more species than are known today. A communicative function of TAGs is probably also not restricted to *L. distinguendus*. Therefore bioassays addressing the biological function of cuticle TAGs in other species are necessary.

5.6 Funding

This work was supported by a doctoral scholarship granted by the Universität Bayern e.V. to SK.

5.7 Acknowledgements

The authors thank Sarah Hayer and Florian Königseder for their assistance with the GC-MS analysis. *Nicrophorus vespilloides* beetles were kind gifts of Sandra Steiger.

5.8 Supplemental material

Fig.S1.

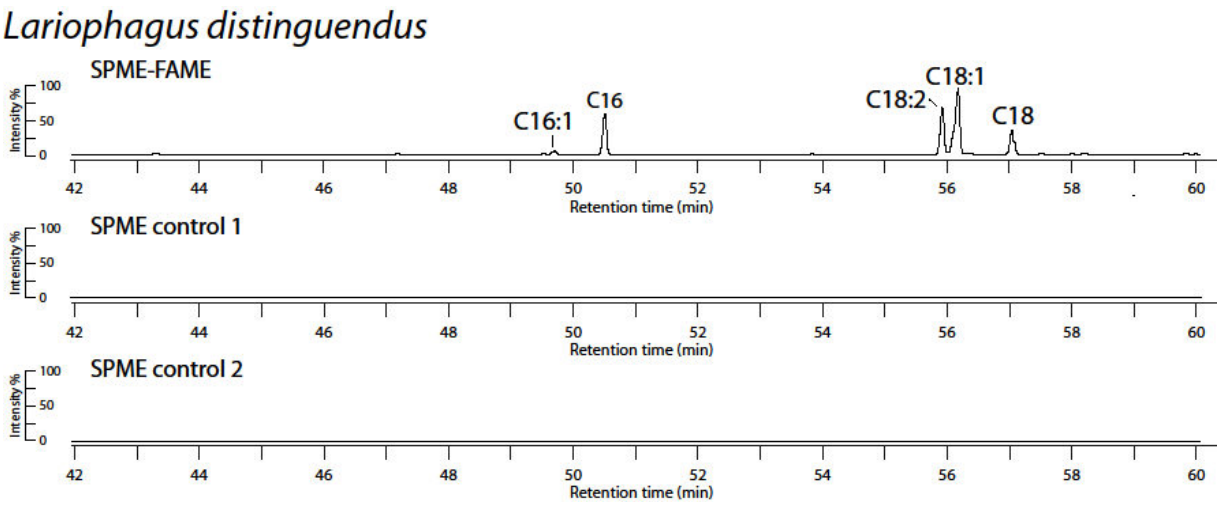

Fig.S2.

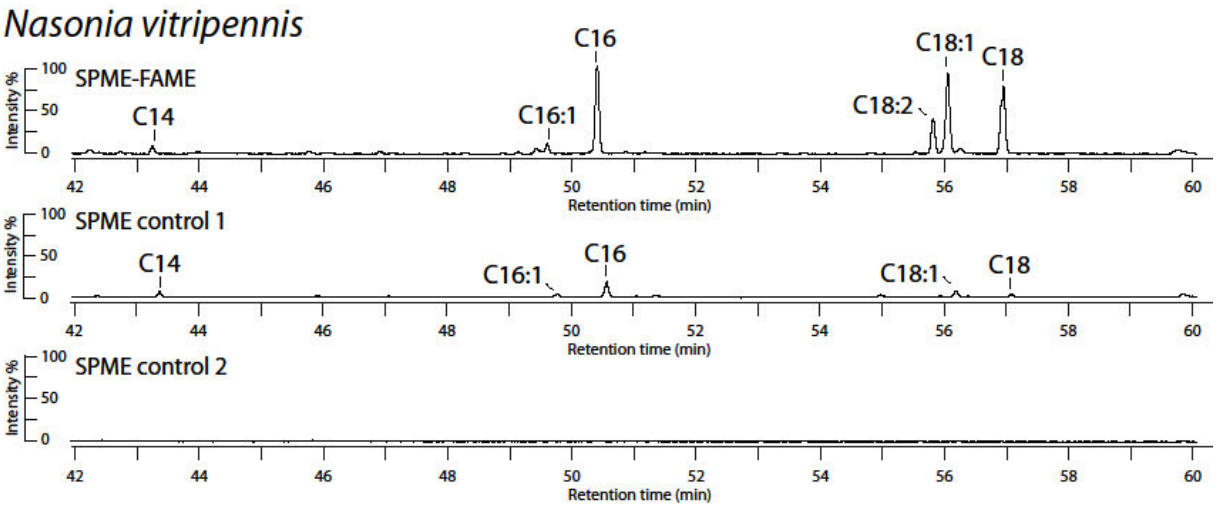

Fig.S3.

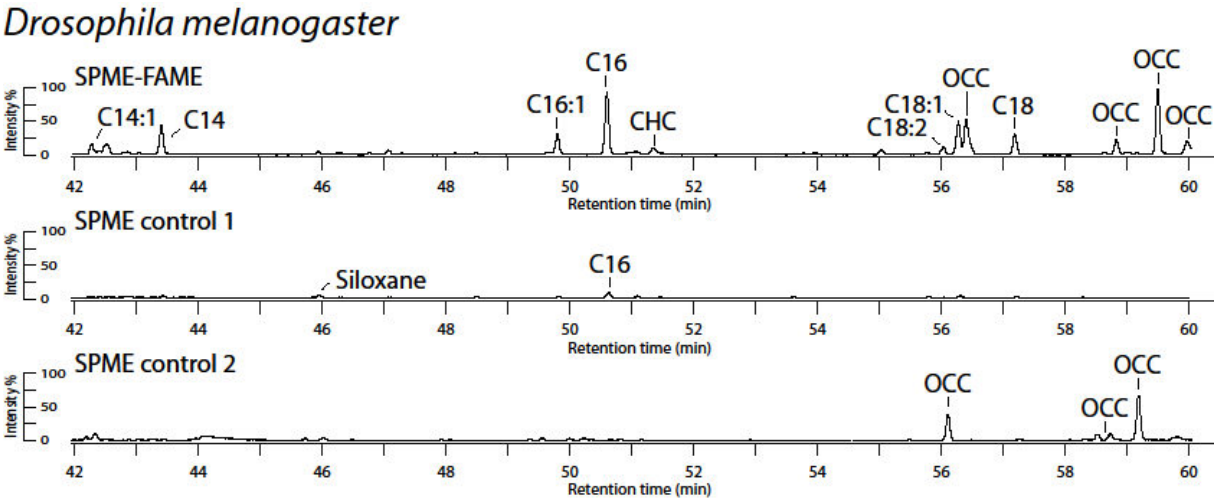
Drosophila melanogaster
SPME-FAME
C14:1
C14
C16:1
C16
CHC
C18:1
C18:2
OCC
C18
OCC
OCC
OCC
SPME control 1
Siloxane
C16
SPME control 2
OCC
OCC
OCC
Intensity %
100
50
0
42
44
46
48
50
52
54
56
58
60
Retention time (min)

Fig.S4.

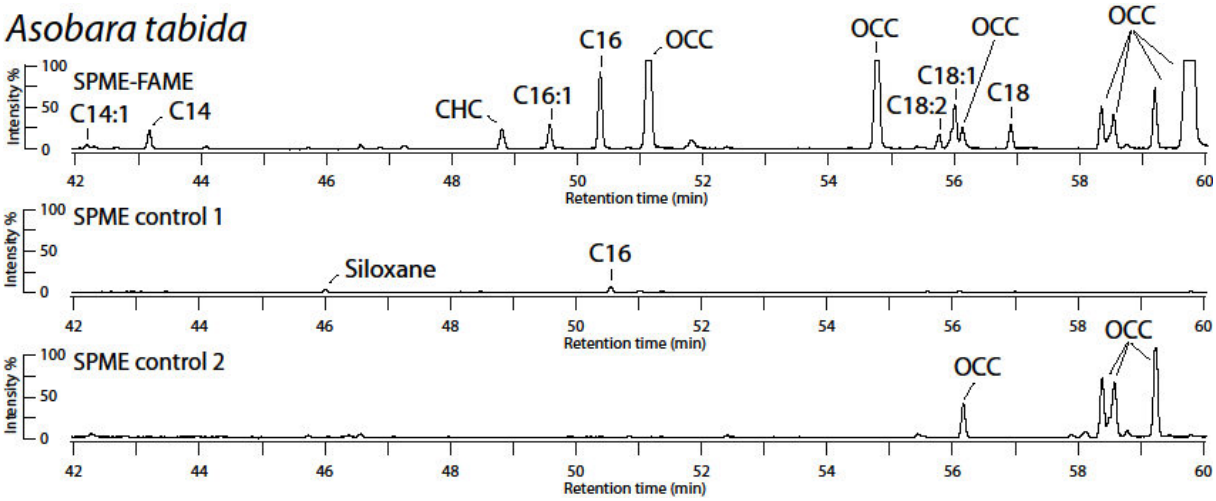
Asobara tabida
SPME-FAME
C14:1
C14
CHC
C16:1
C16
OCC
OCC
OCC
C18:1
C18:2
C18
OCC
SPME control 1
Siloxane
C16
SPME control 2
OCC
OCC
Intensity %
100
50
0
42
44
46
48
50
52
54
56
58
60
Retention time (min)

Fig.S5.

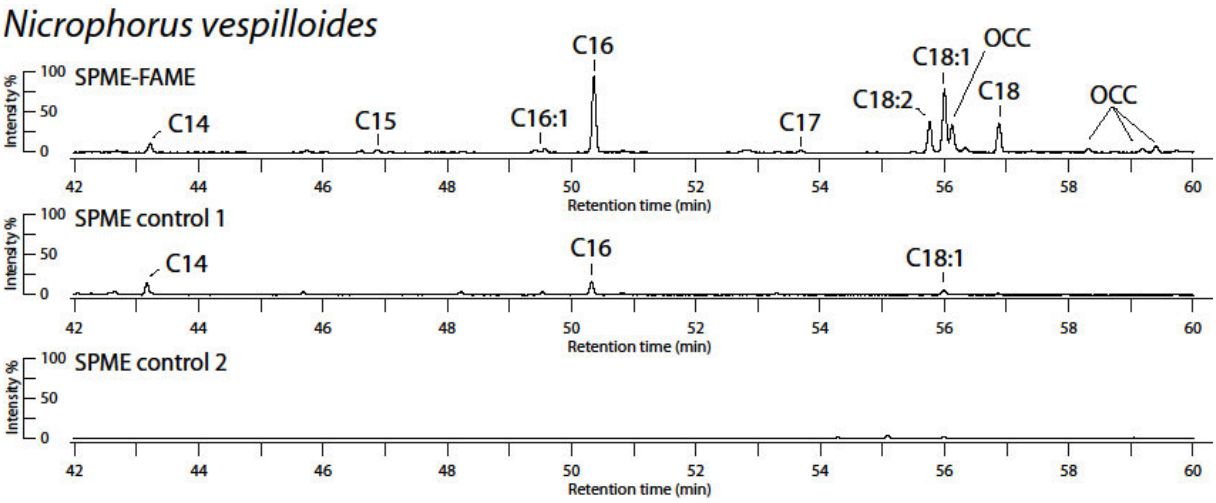
Nicrophorus vespilloides
SPME-FAME
C14
C15
C16:1
C16
C17
C18:2
C18:1
OCC
C18
OCC
SPME control 1
C14
C16
C18:1
SPME control 2
Intensity %
Retention time (min)

Fig.S6.

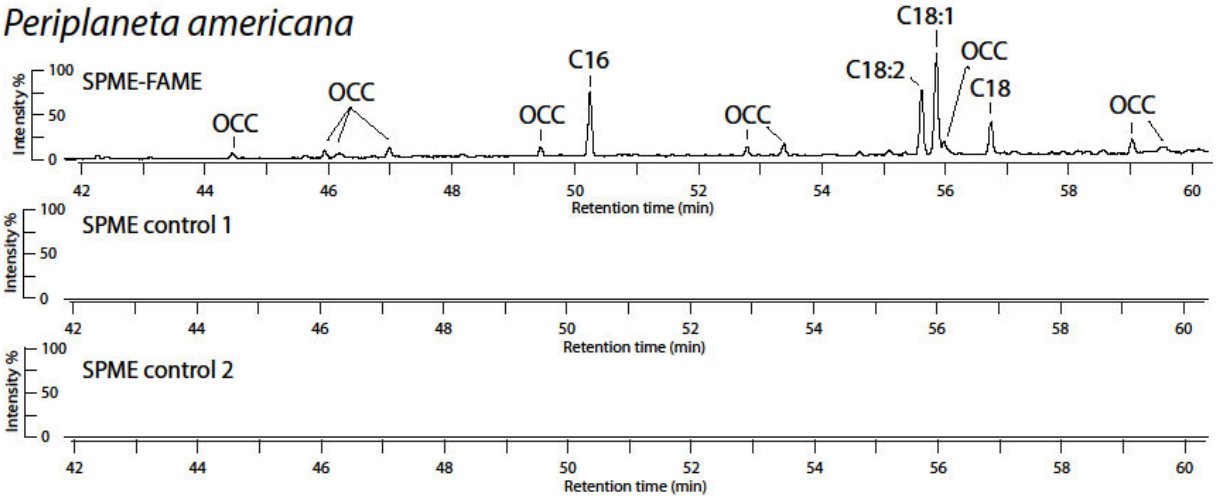
Periplaneta americana
SPME-FAME
OCC
OCC
OCC
C16
OCC
C18:2
C18:1
OCC
C18
OCC
SPME control 1
SPME control 2
Intensity %
Retention time (min)

Fig.S1-S6. SPME-FAME-GC-MS analyses of fatty acids on the cuticle of several insect species. A conditioned SPME fibre was rubbed over the cuticle of female individuals. Subsequently, TAGs were transesterified *in situ* to FAMEs by using TMSH. Finally, the SPME fibre was desorbed for 1 minute in the GC-MS injector. As a control for the absence of TAG contamination of the GC-MS, the fibre was soaked in TMSH without being rubbed over the insect cuticle (SPME control 1). To exclude that the occurrence of free fatty acids on the insect cuticle caused the peaks, a SPME fibre was rubbed over the female insects, but without the fibre being soaked into TMSH (SPME control 2). The following fatty acids were detected: C14 = tetradecanoic acid, C14:1 = (Z)-tetradec-9-enoic acid, C15 = pentadecanoic acid, C16 = hexadecanoic acid, C16:1 = (Z)-hexadec-9-enoic acid, C17 = heptadecanoic acid, C18 = octadecanoic acid, C18:1 = (Z)-octadec-9-enoic acid, C18:2 = (9Z,12Z)-octadeca-9,12-dienoic acid, OCC = other cuticular compounds. Chromatograms within one figure (SPME-FAME, SPME control 1 and SPME control 2) are scaled at the same intensity level and the retention time is shown below.

Personal contribution:

I established the experiments of this paper and carried out the experimental work partly assisted by Sarah Hayer and Florian Königseder. I analyzed the data, created the figures and wrote the text of this paper.

Chapter 6

General Discussion

During the course of this doctoral thesis, new intriguing insights into the chemical communication system of the pteromalid wasp species *Lariophagus distinguendus* were gained. Furthermore, a new and easy-to-use method for the solvent-free analysis of cuticular triacylglycerides (TAG) was developed. I have shown that 3-MeC27 is the key component of *L. distinguendus* female contact sex pheromone but it is only effective in combination with other cuticular components as a chemical background. This chemical background comprises not only cuticular hydrocarbons (CHC), but also more polar TAGs (**chapter 3**). *L. distinguendus* males responded highly specifically to the key component 3-MeC27. An enantioselective response only occurred when synthetic 3-MeC27 was applied together with the remaining cuticular CHCs and TAGs onto filter paper, but not when 3-MeC27 was applied onto ageing male dummies. The attractiveness of ageing males that lacked 3-MeC27 could only be restored by the addition of 3-MeC27, but not with structurally related components that slightly deviated in chain length or position of the methyl branch. The addition of n-alkanes and methylalkanes differing from 3-MeC27 in chain length or methyl-branch position onto the cuticle of behaviourally active male and female wasp dummies disrupted the pheromone bioactivity (**chapter 4**). These findings suggest that *L. distinguendus* males perceive the CHC profile of conspecific females and young males as a whole.

Another important finding was that the CHC profile of *L. distinguendus* was host-dependent. CHC profiles from wasps reared on different host species were significantly distinguishable from each other, whereas wasps from spatially isolated strains reared on the same host had similar CHC profiles. Wasps reared on *Stegobium paniceum* were significantly distinguishable from all strains reared on other hosts. Further, a host shift from *Sitophilus granarius* to *Stegobium paniceum* was shown to lead to distinguishable CHC profiles of female wasps within one generation (**chapter 2**). The results of these experiments suggest that such host shifts can lead, via the modification of their CHC profiles, to the reproductive isolation of host races in parasitic wasps.

The occurrence of more polar TAGs on the insect cuticle has been widely ignored, whereas TAGs are well known as components of the internal tissues of insects. In order to investigate the occurrence of TAGs on the cuticle of *L. distinguendus* wasps and also in other species, SPME-FAME-GC-MS has been developed as a part of this thesis (**chapter 3 and 5**). Conventionally used solvent extraction techniques run the risk of the extraction of lipids from internal tissues. The solid phase microextraction (SPME) technique does not run this risk but has until recently been restricted to non-polar cuticular compounds such as CHCs. Now, more polar TAGs can be sampled with the SPME fibre and *in situ* transesterified by using trimethylsulphonium hydroxide into more volatile fatty acid methyl esters (FAME) that can be analysed with standard gas chromatography coupled with mass spectrometry (GC-MS). In a direct comparison with solvent extraction to investigate TAGs from six insect species from four orders, SPME-FAME-GC-MS has turned out to be a convenient method for analysing cuticular TAGs (**chapter 5**). TAGs can be found on the cuticle of all investigated insect species and thus cuticular TAGs might be far more common cuticular components than usually thought.

Is the contact sex pheromone of *L. distinguendus* a cue or a signal?

In *L. distinguendus*, both genders produce, during the pupal stage, a species-specific contact sex pheromone (Ruther et al., 2000; Steiner et al., 2005). This pheromone consists of 3-MeC27, CHCs and TAGs (**chapter 3**) and elicits courtship behaviour in the males. The males actively decompose the pheromones key component 3-MeC27 and other minor components from their cuticular lipid profile within 32 hours after emergence and thus render the pheromone unattractive (Steiner et al., 2005; 2007a).

The cuticular lipids in *L. distinguendus* are assumed to have originally had a non-communicative function, such as the prevention of desiccation, and the males are thought to have gained, in a first step, the ability to use the CHC profile to locate conspecifics (Steiner et al., 2005; Ruther and Steiner, 2008). This improved their ability to localize grains containing females about to emerge and thus enhanced their mating chances with virgin females. In a second step, the males might have evolved the active decomposition of 3-MeC27 and other minor components from their cuticle. The introduction of this step was necessary, because the

possession of a species specific contact pheromone on the cuticle is detrimental for the young males, as it reduces their competitive ability with other males (Ruther and Steiner, 2008). Thus, the *L. distinguendus* males have adapted, in a two-step process, a cuticular lipid profile first into a species-specific cue and finally into a sex-specific cue. However, the females appear not to have been actively involved in this process.

According to the definition of Smith and Harper (2003), signals are acts or structures that alter the behaviour of other organisms and both the signal and the receivers' response have been evolved for this purpose. The emitter and the receiver of a signal can have different interests, but it is important for the stability of a signal that both of them benefit from it on the whole. However, in the case of *L. distinguendus*, the pheromone evolved because of adaptations of the male, whereas adaptations of the females in order to make the pheromone more specific could not be shown until now (Steiner et al., 2005; Ruther and Steiner, 2008). Therefore, the question is raised as to whether the contact sex pheromone of *L. distinguendus* is more a cue than a signal. Signals evolve from released compounds such as hormones, cuticular lipids or defensive secretions without a communicative function (Sorensen and Stacey, 1999; Wyatt, 2003; Weiss et al., 2013)). If other organisms evolve the ability to perceive such compounds and respond to it, then components without a communicative function can turn into a cue. This is also called the 'eavesdropping' or 'spying' stage and is only beneficial for the receiver of the cue. For the evolution of a cue into a signal, the emitter has to benefit from the receivers' response (Sorensen and Stacey, 1999; Wyatt, 2003; Weiss et al., 2013). The contact sex pheromone of *L. distinguendus* is presumably in an evolutionary stage between a cue and a signal. The males have developed mechanisms to perceive and adapt the species-specific cuticular lipid profile and to benefit from it. However, the females either benefit from the cue to a lesser extent than the males or the time that has been elapsed since the males adapted the cue has been too short for the females to evolve adaptions in order to make the pheromone more specific.

Importance of methyl branched alkanes in the contact sex pheromone of *L. distinguendus*

3-MeC27 is the key component of *L. distinguendus* contact sex pheromone (**chapter 3**). However, 3-MeC27 has not only been found to occur on the cuticle of *L. distinguendus*, but also on the cuticle of some other pteromalid wasp species, namely *Pteromalus cerealellae* (Howard, 2001), *Roptrocerus xylophagorum* (Sullivan, 2002) and *Dibrachys cavus* (Ruther et al., 2011), and of some cerambycid beetles, namely *Xylotrechus colonus* (Ginzel et al., 2003), *Megacyllene caryae* (Ginzel et al., 2006), *Neoclytus acuminatus* (Lacey et al., 2008) and *Mallodon dasystomus* (Spikes et al., 2010). However, most species produce 3-MeC27 only in small amounts and a behavioural function of 3-MeC27 has been shown in none of these cases so far.

The two genders of the pteromalid wasp species *Dibrachys cavus* are unlike *L. distinguendus* non-attractive to males shortly after their emergence. After emergence they have relatively high amounts of 3-MeC27 and other monomethylalkanes on their cuticle, whereas 3-MeC29 and 3-MeC31 occur only in traces (Ruther et al., 2011). However, within the first two days after emergence, the amount of 3-MeC29 and 3-MeC31 increases in females and, during the same period of time, the females become sexually attractive to males. Therefore, 3-MeC29 and 3-MeC31 are thought to have a contact sex pheromone function in this species. Interestingly, 3-MeC27 and other monomethylalkanes largely decrease on the cuticle of both *D. cavus* genders, whereas the relative amounts of 3-MeC29 and 3-MeC31 increase only in females (Ruther et al., 2011). Whether the disappearance of 3-MeC27 and other monomethylalkanes from the cuticle of *D. cavus* is an active process like in *L. distinguendus* and necessary for the functionality of the female contact sex pheromone would be interesting to know. Moreover, it will be interesting to investigate whether the lipid carrier protein lipophorin is involved in the disappearance of 3-MeC27, as has been discussed for *L. distinguendus* (Steiner et al., 2005, 2007a).

Monomethylalkanes have also been found on the cuticle of a number of other insects species (Howard, 2001; Sullivan, 2002; Ginzel et al., 2003; Sugeno et al., 2006; Lacey et al., 2008; Guedot et al., 2009; Silk et al., 2009; Darrouzet et al., 2010; Holman et al., 2010; Spikes et al., 2010; Ruther et al., 2011). In some insect of these, single monomethylalkanes, mainly produced by females, have a contact sex pheromone function in males. The contact sex pheromone of *Gastrophysa atrocyanea* (Chrysomelidae), for example, is a blend of several

monomethylalkanes. However, each of the following single components alone has a contact pheromone function when tested in higher amounts in bioassays (Sugeno et al. 2006): 9-MeC27, 11-MeC27, 9-MeC29 and 11-MeC29. In the emerald ash borer *Agrilus planipennis* (Buprestidae) 9-MeC25 and 3-MeC23 are considered to have a contact sex pheromone function (Lelito et al., 2009; Silk et al. 2009) and *Cacopsylla pyricola* (Hemiptera: Psyllidae) females produce 13-MeC27, which elicits courtship behaviour in males (Guedot et al. 2009). In other insect species, mixtures of two or more monomethylalkanes are necessary to elicit courtship behaviour. For example, 2-MeC26 and 2-MeC28 each elicit courtship behaviour in *Mallodon dasystomus* (Cerambycidae) alone but only the combination of the two components elicits the full behavioural response (Spikes et al., 2010). Moreover, in *Xylotrechus colonus* (Cerambycidae), three components n-C25, 9-MeC25 and 3-MeC25 in combination are necessary to elicit the full behavioural response, whereas the single compounds of the contact sex pheromone are almost behaviourally inactive (Ginzel et al., 2003). In *Neoclytus acuminatus* (Cerambycidae), 7-MeC27 is the major component of the contact sex pheromones being synergised by 9-MeC27 and 7-MeC25 (Lacey 2008).

Taken together, these examples demonstrate that monomethylalkanes such as 3-MeC27 mediate both species- and sex-specificity of CHC profiles and play key roles, either alone or in combination with other components, as insect contact sex pheromones.

A possible explanation for methylalkanes being particularly suitable as recognition cues comes from studies suggesting that social insects can perceive methylalkanes and alkenes better than straight-chain alkanes. This is because methylalkanes and alkenes not only have variable chain lengths, but also differ in their position of the methyl branch or double bond (Dani et al., 2001; Châline et al., 2005; van Wilgenburg et al., 2012). Thus, they possess additional discrimination features compared with straight chain alkanes. Further, some social insects have a homologous series of methylalkanes on their cuticle. Social insects often discriminate between methylalkanes with the same chain length and different branching patterns but not always between methylalkanes that have their methyl branch at the same position but of variable chain-lengths (van Wilgenburg et al., 2010, 2012; Bos et al., 2012). The insects either fail to discriminate those homologous methylalkanes that only differ in their chain length or they "generalize" them like the "synonyms" in human language, because they bear the same amount of information (van Wilgenburg et al., 2010; Bos et al., 2012). Generalisation is thought to make insect cuticular lipid profiles less complex. This seems not to be the case in *L. distinguendus*. The males of this species neither seem to have difficulties

in discriminating straight-chain alkanes nor do they generalize homologue methylalkanes. The response of *L. distinguendus* males towards 3-MeC27 is highly specific and it cannot be replaced by structurally related components (**chapter 4**). Males are at least able to discriminate variations in chain length of two carbons and variations in methyl branch position of two or more positions. Thus, further research is necessary to determine why 3-MeC27 became the key component of *L. distinguendus* contact sex pheromone and why it is perceived with such a high specificity.

Stereochemistry of methyl-branched hydrocarbons

Most methyl-branched hydrocarbons have, according to their number of methyl-branches, one or several stereogenic centres and therefore several stereoisomers of one molecule can exist. Whereas methyl-branched hydrocarbons with one methyl branch can occur as two different enantiomers, hydrocarbons with two, three or four methyl branches have 4, 8 and 16 stereoisomeres, respectively (Millar, 2010). The key component of *L. distinguendus* contact sex pheromone, 3-MeC27, has one chiral centre at the third C-atom and, therefore, the two enantiomers (*S*)-3-MeC27 and (*R*)-3-MeC27 exist (**chapter 3**). If only one stereoisomer occurs naturally, then several possibilities can be envisaged (Eliyahu et al., 2004; Mori, 2007; Ablard et al., 2012): the naturally occurring stereoisomer is bioactive, whereas the other stereoisomers are either also active or neutral or inhibit the response to the active stereoisomer. If several stereoisomers occur naturally, either all of them might be separately active, several stereoisomers might be necessary for bioactivity, or one stereoisomer might be more active than the others. Moreover, one stereoisomer might elicit bioactivity in males, whereas the other has this function in females or both in a different species. Within this work, it has been shown that both stereoisomers of 3-MeC27 alone were behaviourally active, if they were perceived by the males together with a chemical background consisting of long chain CHCs and TAGs. Further, *L. distinguendus* males turned out to respond enantiospecifically to synthetic (*S*)-3-MeC27 and to prefer it over (*R*)-3-MeC27 when applied onto filter paper together with a chemical background consisting of long chain CHCs and TAGs. (**chapter 3**). Thus *L. distinguendus* males are able to discriminate between the two enantiomers of 3-MeC27 and either one or both of the enantiomers occur naturally on the wasp cuticle. The preference of the wasps for (*S*)-3-MeC27 over (*R*)-3-MeC27 does not

necessarily mean that (*S*)-3-MeC27 is the naturally occurring enantiomer. For example, (3*S*,11*S*)-dimethylnonacosan-2-one is the naturally occurring stereoisomer of the female-produced contact sex pheromone of the German cockroach *Blatella germanica*, despite it being the least bioactive than all other stereoisomers (Mori et al., 1981; Eliyahu et al., 2004). The enantiospecific response to (*S*)-3-MeC27 disappears when synthetic (*S*)-3-MeC27 and (*R*)-3-MeC27 are applied onto 4-day-old wasp dummies instead of filter paper (**chapter 3**). The dummies are three-dimensional objects and thus are a visual and tactile stimuli, as involved in the sex recognition of other parasitic wasps such as *Nasonia vitripennis* (Steiner et al., 2006) and *Dibrachys cavus* (Ruther et al., 2011). Therefore, visual or tactile stimuli probably also play a role in the sex recognition of *L. distinguendus* males.

The way that insects distinguish the enantiomers of monomethylalkanes is largely unclear but Mori (2011) has proposed a pheromone-binding protein (PBP) that binds the alkyl groups of monomethylalkanes by forming stereochemically specific complexes with each stereoisomer. This is possible, because the monomethylalkanes are 'divided' by the branching point into two alkyl groups of different chain length that fit into two different hydrophobic clefts of the PBP. Although some insects have been shown to discriminate between the enantiomers of monomethylalkanes (Duff et al., 2001; Ablard et al. 2012), we do not know which enantiomers occur on the insect cuticle and if they act behaviourally differently. This is attributable to the problem that the discrimination between the enantiomers of methyl-branched hydrocarbons with state of the art analytical techniques is almost impossible, because chiral stationary phases are not able to separate the enantiomers of long chain methyl branched hydrocarbons. Those hydrocarbons usually have small optical rotations and most insects produce them only in small amounts (Hefetz et al., 2010; Millar, 2010). Alternatively, synthetic enantiomerically pure reference components of monomethylalkanes can be synthesized and used in bioassays in order to identify the bioactive enantiomer (Millar, 2010; Mori, 2010; Ablard et al., 2012). However, the most bioactive enantiomer is not necessarily the natural occurring one as already mentioned. Many studies on CHCs have only identified cuticular components and have not used enantiomerically pure synthetic reference substances in order to establish the absolute configuration of bioactive methyl branched hydrocarbons and their role for the pheromones functionality (Millar, 2010). One reason for this might be that enantiomeric pure methyl branched hydrocarbons are often not commercially available.

L. distinguendus CHC profile and its role in the contact sex pheromone

The findings of this doctoral thesis shed new light on the structure of *L. distinguendus* cuticular CHCs with contact sex pheromone function. The CHC profile of this species is characterized by the occurrence of components with a chain-length between 25 and 37 carbon atoms, including methylalkanes with one to four methyl groups, n-alkanes and alkenes with one double-bond (Steiner et al., 2005, 2007a). The most abundant components of the *L. distinguendus* CHC profile are 3,7,11,15-TetraMeC33, 11,21-DiMeC33 and 13,17-DiMeC35 (Steiner et al., 2007a). Both genders of *L. distinguendus* produce the same CHCs during the pupal stage but the males, in contrast to the females, actively decompose 3-MeC27 and other minor components within 32 hours after emergence (Steiner et al., 2005).

3-MeC27 turned out to be the key component of *L. distinguendus* contact sex pheromone but it is not attractive alone. 3-MeC27 has to be perceived by the males together with the remaining CHCs and TAGs as a chemical background (**chapter 3**).

The observation that a key component of a pheromone has to be perceived together with a chemical background of other components has previously been made in other species (Mumm and Hilker 2005, Schroeder and Hilker 2008). As a response to oviposition of the herbivorous sawfly *Diprion pini*, the Scots pine (*Pinus sylvestris*) attracts the egg parasitoid *Chrysonotomyia ruforum* by increasing the level of the sesquiterpene (*E*)-β-farnesene. However, (*E*)-β-farnesene attracts the egg parasitoid only when it is offered in the correct concentration and in combination with volatiles from pine twigs that are free of eggs as a background (Mumm and Hilker, 2005). A background of odorants can have various effects on the perception of the resource indicating odour (RIO): an irrelevant background odour has no effect on the RIO, the background odour can either mask or enhance the response to the (RIO) and the background odour alone might have an effect or not (Schröder and Hilker, 2008). The background odour can be comprised of single components or be a complex blend. *L. distinguendus* background odour is a complex blend of components from several substance classes and has alone no behavioural effect. It is only behaviourally effective in combination with the key component 3-MeC27.

The CHC profile of *L. distinguendus* is a multicomponent mix but which components are part of the chemical background that is involved in the species contact sex pheromone is unclear. Some studies have demonstrated that single components (Sugeno et al. 2006, Guedot et al. 2009, Silk et al. 2009) or binary and ternary mixtures of components (Ginzel et al. 2003,

Lacey et al. 2008, Spikes et al. 2010) are behaviourally active. Other studies have shown that multicomponent blends comprising of one or several structural or substance classes are behaviourally active as in the ants *Linepithema humile* and *Aphaenogaster cockerelli* (Greene and Gordon, 2007) or the *Drosophila* larval parasitoid *Asobara tabida* (Stökl et al., 2014). The female courtship pheromone of *A. tabida* is a multi-component blend consisting of methyl 6-methylsalicylate (M6M), fatty alcohol acetates (FAAs) and CHCs. It does not contain a key component but combinations of two from three substance classes elicit wing-fanning in males. This means that components from various substance classes are necessary to elicit courtship behaviour (Stökl et al., 2014). In the ants *Linepithema humile* and *Aphaenogaster cockerelli*, only a blend consisting of several structural classes (straight chain alkanes, methylalkanes and alkenes) elicited aggressive behaviour (Greene and Gordon, 2007). In the case of *L. distinguendus* components from several structural classes such as methylalkanes and n-alkanes and substance classes CHCs and TAGs are also necessary to elicit a behavioural response in males (**chapter 3**). A distinct complexity of the contact sex pheromones background is possibly necessary in order to maintain the species specificity of the pheromone. On the contrary, several other studies assume that insects use probably not all of their CHCs for communication with others, but only a small part of it (Dani et al., 2001; van Wilgenburg et al., 2010; Châline et al., 2005).

Steiner et al. (2005) have described some other minor CHC components that disappear together with 3-MeC27 from the cuticle of ageing males: C27, 3-MeC27, 3,7-DiMeC27, 13-MeC29 + 11-MeC29 + 9-MeC29, 3-MeC29, 3,7-DiMeC29 and C31:1(9). In another study Steiner et al. (2007a) have analysed whole body extracts of *L. distinguendus* by GC-MS and applied a principal component analysis (PCA) and a partial least squares-discriminant analysis (PLS-DA) to the data. They have found that the monomethylalkanes 3-MeC27, 3,7-DiMeC27, 4,8-DiMeC28, 3-MeC29, 3,7-DiMeC29 and 13-MeC29 and the monoenes C27:1(9) and C29:1(7) have the highest discriminating power between behaviourally active and inactive extracts. All of these components except 13-MeC29 and C29:1(7) disappear from the cuticle of ageing *L. distinguendus* males. Those components that disappear from the cuticle of ageing *L. distinguendus* males together with 3-MeC27 seem not to be necessary for the functionality of the pheromone, because behaviourally non-active 4-day-old male dummies can be rendered attractive again by the sole addition of synthetic 3-MeC27 (**chapter 3** and **chapter 4**). Therefore, whether those components that disappear from the males' cuticle should be seen as behaviourally active components of *L. distinguendus* contact sex pheromone is questionable. The question as to which components from the *L. distinguendus* CHC profile are part of the

species contact sex pheromone therefore remains open until further investigations have been carried out in order to disentangle the contribution of single components and various substance classes for pheromone functionality.

Probably not only the qualitative composition of *L. distinguendus* CHCs matters, but also the relative amount of components seems to be important for the pheromonal activity, because the augmentation of single components naturally occurring on the insects' cuticle render behaviourally active *L. distinguendus* dummies unattractive (**chapter 4**). This is supported by the finding that a host shift alters the CHC more quantitatively than qualitatively (**chapter 2**). This implies that the relative proportions of at least some components are perceived. This finding is in accordance with a study by Dani et al. (2001), which has shown that the augmentation of single methylalkanes and alkenes naturally occurring on the cuticle of the paper wasp *Polistes dominulus* elicits aggressive behaviour among nestmates.

Influence of nutrition on *L. distinguendus* cuticular hydrocarbon profile

The diet that an insect incorporates has been shown to affect its CHC profiles (Howard, 1998; Liang and Silverman, 2000; Rundle et al., 2005; Fedina et al. 2012; Geiselhardt et al. 2012) and, in some cases, also the insects' mating preferences (Rundle et al., 2005; Sharon et al., 2010).

One important result of this doctoral thesis is that the host species onto which *L. distinguendus* was reared had a significant influence on the wasps CHC profiles. CHC profiles of female wasps were distinguishable already one generation after a host-shift from *Sitophilus granarius* to *Stegobium paniceum.* These differences between the CHC profiles were quantitative rather than qualitative (**chapter 2**). Thus, the relative amounts of single components from the wasps CHC profile seem to be important. A comparison of the CHC profiles between the wasps and their hosts has brought no indication for a direct incorporation of CHCs from hosts larvae into the wasps own CHC profile. Rather, the host species might have differed from each other in the composition of CHC precursors such as fatty acids or amino acids that are needed by the wasps for CHC synthesis (**chapter 2**). Further, the components that mainly contribute to the differences between the CHC profiles of wasps reared on different hosts (3,7,11,15-TetraMeC33, 11,21-DiMeC33 and 13,17-DiMeC35) are

among the major CHCs of *L. distinguendus* (Steiner et al., 2007a). These components belong to the CHC background that has to be perceived together with the more polar TAGs and the pheromones key component 3-MeC27 by the *L. distinguendus* males in order to elicit wing-fanning (**chapter 3**). Another finding of this thesis is that 3-MeC27 is perceived highly specifically and that the augmentation of other structurally related components renders behaviourally active wasp dummies inactive (**chapter 4**). This makes it likely that the relative amounts of CHC components other than 3-MeC27 from the *L. distinguendus* CHC profile might also be perceived highly specifically, and that changes of their ratios as they occur during a host shift might in the long-term end up in the reproductive isolation of host races via the alteration of the CHC profiles of the wasps. However, a causal link that a host-shift actually affects the mate recognition behaviour in *L. distinguendus* still has to be shown. A contribution of CHCs to the reproductive isolation of closely related species is, for example, assumed in some sympatric species of cerambycid beetles whose contact sex pheromones are species-specific (Lacey, 2008). A study with *Drosophila melanogaster* and *D. serrata* populations reared on different substrates has exhibited diverging CHC profiles that lead to mating preferences of the females (Rundle et al., 2005). The findings of the study by Rundle et al. are in accordance with the by-product model of speciation, which predicts that speciation can occur via the evolution of premating isolation as a by-product of divergent selection adapting populations to their different environments (Schluter, 2001). Such a mating preference can subsequently be amplified by sexual selection and finally end in speciation (Schluter, 2001).

Further, Sharon et al. (2010) showed that *Drosophila melanogaster* flies from the same population exhibited a mating preference that was stable over more than thirty generations, even if the flies were reared for only one generation on a different medium. The authors assumed that microbiota, which are associated with the rearing substrates, elicited this phenomenon, because the mating preference could be reversed by antibiotic treatment. Additional experiments with *Lactobacillus plantarum* supported this finding; the results imply that symbiotic bacteria can alter the CHC amounts with sex pheromone function in *D. melanogaster* and thus lead to such a mating preference. An interesting scenario would be if, in the case of *L. distinguendus*, symbiotic bacteria from the beetle hosts are also taken up by the wasps and contribute to the altered CHC profiles. Therefore, experiments should be carried out in which antibiotics are fed to the beetle hosts prior to parasitisation by the *L. distinguendus* females. However, one difficulty might be that the antibiotics have negative effects on the health of the wasps and their beetle hosts.

The authors of the *D. melanogaster* study assume that such a mating preference is supported by further environmental factors such as the geographical separation of host species and that the combined effects might lead to the speciation of the populations (Sharon et al., 2010). Possible scenarios for the reproductive isolation of *L. distinguendus* wasps through a host shift combined with geographical separation could be that the wasps spread themselves and find a different host species, that wasps are transferred as "stowaways" from one grain storage elevator to another, which is contaminated with a different beetle host or that wasps are released as a biological pest control on a different host.

A study by Drès and Mellet (2002) implies that the scenarios of speciation by host shift might not necessarily need a strong geographical separation. The authors propose a model for the gradually sympatric speciation of herbivorous insects. The insects within this model feed on several host-species and might have to adapt to their hosts in order to overcome the hosts defence mechanisms and thus form diverging subpopulations (host-races) that exchange genes at a rate of more than 1 % per generation. Such host-races coexist and gradually evolve into different species (Drès and Mellet, 2002). The finding that a host shift can lead within one generation to significantly different CHC profiles (**chapter 2**) supports a similar scenario for the sympatric formation of reproductively isolated host races in *L. distinguendus*.

Geiselhardt et al. (2012) have recently described a scenario whereby behavioural isolation can occur without prior environmental adaptation. The authors reared the mustard leaf beetle *Phaedon cochleariae* (Chrysomelidae) on two different host plant species that often occur sympatrically. They found that rearing the beetles on different host plants leads within two weeks to quantitatively different CHC phenotypes and that the males preferred mating with females that fed on the same host plant as the males did. The authors assume that the different CHC phenotypes are a by-product of random host plant choice that might be lost in the next generation by random host plant choice or that might be maintained if divergent preferences evolve.

Triacylglycerides as a part of *L. distinguendus* cuticular lipid profile

Until recently it was questionable, whether triacylglycerides (TAGs) are really part of the cuticular lipids of insects. The main reasons were that TAGs have relatively high melting points and thus are not analysable by standard gas chromatography coupled with mass spectrometry (GC-MS) without transesterification into more volatile components (Cvačka et al., 2006a; Cvačka et al., 2006b; Millar, 2010). Further, most studies have found cuticular TAGs by solvent extraction and so it is doubtful whether the TAGs found in such studies really originated from the cuticle and not from internal tissues (Jackson, 1981; Lockey, 1985; Buckner, 1993). Verification that TAGs are indeed part of the cuticular lipid profile of insects has come only recently in a study of Yew et al. (2011a) who used ultraviolet laser desorption/ionization orthogonal time-of-flight mass spectrometry (UV-LDI-o-TOF MS) in order to detect TAGs on the cuticle of two *Drosophila* species.

Within this doctoral thesis, in addition to the CHCs, more polar triacylglycerides (TAGs) have turned out to be essential components of the chemical background of *L. distinguendus* interacting with 3-MeC27. This is to my knowledge the first study that has found a behavioural function of cuticular TAGs. Meanwhile, a study by Chin et al. (2014) found that the males of some desert-adapted *Drosophila* species produce sex-specific TAGs and transfer them during copulation from their ejaculatory bulb to the anogenital region of the females, where the TAGs act synergistically as a pheromone that inhibits courtship from other males.

Further, solid phase micro-extraction with *in situ* transesterification of TAGs into more volatile fatty acid methyl esters (SPME-FAME-GC-MS) has been developed during this work as a new method for the detection of more polar cuticular lipids such as TAGs (**chapter 3 and chapter 5**). This new method makes TAGs detectable by standard GC-MS with reasonable effort and allows the repeated measurement of cuticular TAGs, because it is not lethal for the insects. In a comparative approach with solvent extraction, SPME-FAME-GC-MS has been shown to be a suitable method for the analysis of cuticular TAGs without the risk of extracting TAGs from internal tissues. But, SPME-FAME-GC-MS constitutes only an indirect evidence for the occurrence of cuticular TAGs. Therefore, it cannot be completely ruled out that the analysed fatty acids originate from other components than TAGs like mono- and diglycerides or phospholipids, which contain bound fatty acids. However, this seems unlikely because ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) analysis of *L. distinguendus* extracts performed by Aleš Svatoš (unpublished data) did not find any other fatty-acid-containing compounds than TAGs.

SPME-FAME-GC-MS delivers currently no quantitative results about cuticular TAGs and no information about the orientation of the fatty acids on the glycerol backbone. It may therefore make sense to use SPME-FAME-GC-MS in combination with other mass spectrometry based techniques in order to benefit from the advantages of both techniques. Nevertheless, by using SPME-FAME-GC-MS, TAGs could be found on the cuticle of six insect species from four orders making it likely that the occurrence of cuticular TAGs has been widely underestimated and that they might have a behavioural function in further species other than solely *L. distinguendus*. An interesting candidate for the further application of SPME-FAME-GC-MS would be *Gastrophysa atrocyanea*, because a study that used solvent extractions has come to the result that the crude extract apparently contains other, more polar components not found in the hexane fraction (Sugeno et al., 2006). These polar components seem to be a part of the species contact sex pheromone, because the male response to the crude extract is higher than that to the hexane fraction.

The finding that TAGs are more common cuticular components in a large variety of insect species might also have consequences for our understanding of the barrier function of cuticular lipids against desiccation. Models that predict the properties of cuticular lipids as a barrier against desiccation, as in the "lipid melting model", are based mainly on CHCs (Gibbs, 1995, 1998; Gibbs and Rajpuorhit, 2010). The lipid melting model predicts that the melting of cuticular lipids leads to an increased cuticular permeability above a so-called transition temperature (Gibbs, 1998). Cuticular lipids with low melting points are frequently found on the cuticle of many insects, despite insects in general being assumed to be capable of synthesizing high melting lipids that would widely prevent water loss (Gibbs, 2002). The benefits of having low melting lipids on the cuticle, such as a better dispersal of lipids on the cuticle or advantages in the communicative function of the lipids, seems to outweigh the costs of an increased water loss. A worthwhile goal for future research would be to investigate whether and how the occurrence of cuticular TAGs affects the water-proofing abilities of insect cuticular lipids and their transition temperature.

The finding of an unclear correlation between the melting temperature of the cuticular lipids and the water loss rate of the organism, especially in insect species that have high contents of cuticular alkenes, led to the development of the lipid phase separation hypothesis (Gibbs, 2002). This hypothesis assumes that the composition and properties of the cuticular lipids vary on the cuticle from region to region (Gibbs and Crowe, 1991; Gibbs, 2002). This variation is explained by a lipid phase separation that occurs between melted alkenes and

crystallized n-alkanes. Further, the lipid phase separation hypothesis is assumed to have consequences not only for the water proofing abilities of insect cuticle, but also for the chemical communication between insects. Gibbs (2002) assumes that contact pheromones are better accessible to the chemoreceptors of other insects and thus can be better perceived by them, when they build islands that are surrounded by a liquid lipid environment, than if they are embedded in a solid phase. As a consequence of this model, the ratio between saturated and branched lipids is dependent on the surface temperature and, thus, the temperature might have an influence on the cuticular permeability and the behaviour of the insect.

Therefore, the question rises as to whether or not the TAGs are perceived themselves or if they rather enhance the accessibility of cuticular CHCs for the chemoreceptors of other insects, as assumed in the lipid phase separation hypothesis. The TAGs would then serve as a kind of matrix making the CHCs with contact pheromone function more volatile or better accessible for the perception by other insects. At least some of the TAGs found on the cuticle of *L. distinguendus* should be liquid at physiological temperatures and thus could mediate the perception of solid CHC components.

Further, many studies that deal with cuticular lipids as a protective barrier against desiccation and pathogen attack rely on experiments that involve solvent extraction in order to analyse the cuticular lipids (Gibbs and Crowe, 1991; Gibbs and Rajpurohit, 2010). Therefore, the cuticular lipid composition assumed in those studies and models might not reflect the real conditions on the cuticle, because lipids from internal tissues might have been co-extracted (Gibbs and Crowe, 1991). SPME-FAME-GC-MS might also be a suitable method for establishing whether the assumptions made about the cuticular lipid composition in such studies are indeed correct.

Another interesting aspect considering TAGs in general is that those with the same acyl chains on the glycerol backbone are symmetric and thus achiral, whereas TAGs with different acyl chains are chiral (Mori, 2012). Whether the chirality of cuticular TAGs has any behavioural effect in insects is unclear but is possible, because naturally occurring TAGs are assumed to be synthesized stereoselectively. Recently, Mori (2012) has reported the stereo-selective synthesis of TAGs that subsequently have been used by Chin et al. (2014) in bioassays with *Drosophila* flies. First results indicate that only the (*R*) configuration of a TAG containing oleic acid was active, while the (*S*) configuration was inactive.

Perception of *Lariophagus distinguendus* contact sex pheromone

This work has shown that the contact sex pheromone of *L. distinguendus* is a multicomponent mix consisting of 3-MeC27, other CHCs and more polar TAGs (**chapter 3**). However, what does this mean for the perception of *L. distinguendus* contact sex pheromone by conspecific males? The males of *L. distinguendus* are clearly able to perceive components from several substance classes such as n-alkanes, methylalkanes and triacylglycerides. Further, the males of this species can at least perceive monomethylalkanes highly specifically and can detect differences of two carbon atoms, both in chain length and in the position of the methyl branch (**chapter 3 and chapter 4**). Additionally, the wasps are also able to discriminate between the enantiomers of monomethylalkanes. Insects may achieve this as already mentioned by the use of pheromone-binding proteins (PBP) that bind monomethylalkanes stereochemically and specifically (Mori, 2011). If *L. distinguendus* males possess such PBPs, this could explain the enantioselective response towards 3-MeC27 and possibly also the ability of *L. distinguendus* to detect small differences between 3-MeC27 and structurally related components deviating in chain length and in the position of the methyl branch.

However, the contact sex pheromone of *L. distinguendus* consists of a large variety of components and the males are unlikely to possess a specific PBP for each component. This objection can possibly be resolved by the following model. A specific response to pheromone molecules is thought to be achieved, if the odorant-binding proteins (OBP) and odorant receptors (OR) build two layers of a filter (Leal, 2005). OBPs solubilize hydrophobic pheromone molecules and transport them across a barrier formed by aqueous lymph fluid that fills the inside of olfactory sensilla. The OBPs are thought to transport only a subset of the components over the aqueous barrier to the OR and not all of those components do indeed bind to the OR. The OR on the other hand might be activated by multiple components. Thus, both the OBP and the OR themselves do not have to respond specifically towards distinct pheromone molecules, because specificity is achieved by the filter system that OBP and OR build together. This model is supported by the finding that the numbers of OBPs and ORs are usually much smaller than the number of molecules that an insect can perceive (Leal, 2005).

The response of *L. distinguendus* males to 3-MeC27 is highly specific and 3-MeC27 cannot be replaced by structurally related components (**chapter 4**). The augmentation of naturally occurring monomethylalkanes and n-alkanes on the cuticle of behaviourally active dummies leads to a disruption of their pheromone function, which implies that the wasps perceive not

only the qualitative composition of the species cuticular lipid profiles, but also the relative amounts of those components. This, in combination with the finding that the pheromone is a multicomponent blend consisting of a key component that has to be perceived together with a chemical background of other components, implies that the pheromone might be perceived as a whole. This might occur via a multiporous sensillum as recently discovered by Ozaki et al. (2005) on the antennae of the Japanese carpenter ant *Camponotus japonicus*. This sensillum responds only to non-nestmate CHCs, seems to perceive those CHC profiles as a whole and, for a gustatory sensillum, it is innervated by an unusually high number of receptor neurons. Therefore, it is assumed to be an olfactory sensillum. Until recently, CHCs with contact pheromone function have been thought to be perceived by gustatory sensilla rather than by olfactory sensilla, because such CHCs have relative low volatility. However, gustatory sensilla exhibit only a small number of receptor neurons for fundamental tastes and thus they might be less suitable for the detection of multicomponent pheromones than olfactory sensilla that contain many more receptor neurons (Ozaki et al., 2005; Ozaki and Wada-Katsumata, 2010).

How do the *L. distinguendus* males decide whether the cuticular lipid profile of an encountered insect meets the demands of species and sex specificity? Insects are thought to make such decisions by comparing the cuticular lipid profiles of conspecifics with so-called 'templates'. According to the definition of Sherman et al. (1997), templates are "internal representations of the characteristics of desirable or undesirable recipients. Recognition occurs when phenotypes of recipients match these templates closely enough. Generalized templates are favoured when appropriate responses to all undesirable (or desirable) recipients are the same, despite variation in their exact cues." Sex-recognition templates are either genetically coded or they are learned from parents or social mates or by self-inspection (Sherman et al., 1997). In the case of *L. distinguendus* learning from parents or social mates can be ruled out, because the wasps are haplodiploid (van den Assem, 1970). This means that the males of this species are haploid and therefore have a mother but no father. Further, *L. distinguendus* females usually lay only one egg per grain kernel and leave the oviposition site afterwards (Hase, 1924; Steidle, 2000; Ruther and Steiner, 2008). Learning by self-inspection would be possible, because *L. distinguendus* males produce the species contact sex pheromone by themselves during development prior to emergence, probably in order to distract previously emerged conspecific males from grain kernels that contain unmated females (Steiner et al., 2005, 2007a). Thus, the template formation in *L. distinguendus* is either innate or learned by self-inspection. If template formation in *L. distinguendus* is

achieved by learning, it has to occur early in life, because freshly emerged males are immediately fertile and have the same mating chances as older males (Steiner et al., 2005). From some species, we know that templates are learned ('imprinted') early in life, during a short sensitive period (Sherman et al., 1997). Further support for this comes from findings that the environment of a parasitoid during development (the host and its nutrition) might affect the behaviour of the parasitoid as an adult and that the sensory system of the parasitoid matures within the end of the pupal stage (Vet and Groenewold, 1990).

Studies dealing with nestmate recognition in social insects imply that the decision as to whether an encountering individual is treated as a nestmate or a non-nestmate is made at the level of the antennae or the antennal lobe (Ozaki et al., 2005; Leonhardt et al., 2007; van Zweden and d'Ettorre, 2010). If *L. distinguendus* males possess such a multiporous sensillum that perceives the species contact sex pheromone as a whole, similar to the one described by Ozaki et al. (2005), we can reasonably assume that the decision is also made at the level of the antennae and does not need a feedback from the brain.

Most studies on template formation and matching refer to social insects. Social insect workers are believed to recognize nestmates by comparing mainly the quantitative dissimilarities between the cuticular lipid profiles of an encountered insect with a template that represents those cuticular lipids that are specific for their own colony (label) (van Zweden and d'Ettorre, 2010). Thus, several nestmate recognition models exist for social insects (Crozier and Dix, 1979; Getz, 1982). Sherman et al. (1997) have introduced two models: the desirable-present model (D-present) and the undesirable-absent model (U-absent). The D-present model claims that individuals are accepted as nestmates if they possess desirable cues that are shared by nearly all nestmates, whereas the U-absent model claims that individuals are accepted as nestmates if they lack undesirable cues that are possessed by undesirable individuals. According to the U-present model, the insect workers are habituated to their nestmates' labels and thus are able to filter them out and respond only to non-nestmates. Both models are different in their restrictiveness, because some individuals with undesirable cues are accepted in the D-present model resulting in acceptance errors, whereas the U-absent model is more prone to rejection errors (Couvillon and Ratnieks, 2008; van Zweden and d'Ettorre, 2010). Possibly, the highly specific response of *L. distinguendus* males towards the species contact sex pheromone can also be explained by a recognition model that is based on the presence or absence of distinct components or a combination of both. *L. distinguendus* males could also use a D-present model, although monomethylalkanes structurally related to 3-MeC27 simply

compete with 3-MeC27 at the PBP or the OR, so that the perceived relative amount of 3-MeC27 falls below a distinct recognition threshold relative to the other components of the pheromone and, thus, the bioactivity of the contact sex pheromone is lost.

Whether the acceptance threshold is restrictive depends not only on the fitness consequences of accepting or rejecting desirable or undesirable recipients, but also on the relative rates of encountering recipients (Sherman et al., 1997). In species and sex recognition, a more static template and a more fixed threshold should be expected compared with kin recognition in social insects, because gender and sex recognition cues are stable over time and dissimilarity distributions of cues have a minimal overlap (Liebert and Starks, 2004). In social insects, template formation seems to be based on an experience-derived memory. The cuticular lipids that are specific for the colony (label) are not fixed, can be based on cues that are heritable or acquired from the environment (nestmates, nest material, substances secreted by the queen) and might change over time (Lahav et al., 2001; van Zweden and d'Ettorre, 2010). Therefore, the neural template that represents an experience-derived memory has to be adapted continuously by using the cuticular lipid profiles of the nestmates as a reference in order to reflect the actual label. In contrast, the *L. distinguendus* template seems to be formed prior to emergence and is unlikely to be changed afterwards (Steiner et al., 2005; Sherman et al., 1997). This makes it likely that the template for contact sex recognition in *L. distinguendus* is based on long-term memory.

Conclusion

Insects are known to use cuticular lipids not only as a barrier against water loss, but also for communicative purposes. However, many studies examining the significance of cuticular lipid components for pheromone function conclude bioactivity from the sole occurrence of the components on the insects' cuticle. In contrast, this doctoral research involves behavioural bioassays with synthetic reference components in addition to chemical analysis. The results of this work contribute to the understanding of the role that cuticular lipids having contact sex pheromone function might play in insects and the way that they are perceived. A contact pheromone function of monomethylalkanes has previously been shown in some other insect species but the role of 3-MeC27 in *L. distinguendus* contact sex pheromone is intriguing. The

response towards the key component 3-MeC27 and most likely also to the chemical background is highly specific and fine-tuned. Not only the qualitative composition matters, but also the relative ratios of single components seem to be important. This has led to the assumption that the wasps probably perceive the pheromone as a whole possibly with the help of a multiporous sensillum, as has been shown for the Japanese carpenter ant *Camponotus japonicus*.

The cuticular lipid profile with contact sex pheromone function is produced by both sexes of *L. distinguendus* prior to emergence but only the active decomposition of 3-MeC27 from the cuticle of ageing males turns the species cuticular lipid profile with species recognition pheromone function into a sex pheromone. This underlines that the contact sex pheromone of this species is more than the mere accumulation of a couple of components that elicit behavioural activity in the conspecific males. It is the result of an evolutionary process that is still ongoing. *L. distinguendus* cuticular lipids gained their contact sex pheromone function, because of the conspecific males finding the means of actively being able to decompose 3-MeC27. This presumably turned a species-specific cue into a sex-specific one. In ordinary terms, *L. distinguendus* males mastered the art of omission.

The dietary intake of an insect has been shown to have an influence on its CHC profile. For parasitoids, the host usually represents the exclusive source of nutrition during development. Therefore, a host shift as it has been shown within this thesis might have strong implications for the CHC profiles of the parasitoids, most likely because different host species deliver a diverse subset of components that are used by the parasitoids as CHC precursors, such as fatty acids and amino acids. In *L. distinguendus*, a host shift has been shown to cause the differentiation of CHC profiles within one generation. This could be the starting point for the formation of host races and might lead to reproductive isolation that, in the long-term, might end in sympatric speciation.

The insect cuticle is covered not only by CHCs that are the focus of cuticular lipid research, but also by more polar lipids containing oxygenated functional groups such as triacylglycerides (TAG). The role of TAGs is maybe widely underestimated and, where the occurrence of TAGs has been reported, uncertainty exists with regard to whether the TAGs really originated from the insects cuticle and not from internal tissues following the use of solvent extraction techniques. The development of SPME-FAME-GC-MS, a new method, during this work enables the solvent-free and repeated sampling and analysis of cuticular TAGs. This allows the analysis of TAGs from the cuticle of living insects without killing

them and thus any changes in insect TAG profiles over time can be tracked. TAGs have been shown to be essential components of the *L. distinguendus* cuticular lipid profile and to occur on the cuticle of five other insect species from four orders. To my knowledge, this is the first time that a behavioural activity of TAGs in insects has been shown. These results make it likely that TAGs are far more common components of the cuticular lipid profile of many insect species than has previously been appreciated. If TAGs are indeed a widely underestimated substance class of insect cuticular lipids, further examples should demonstrate the behavioural activity of TAGs in other species.

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