Chemical communication in the genus *Leptopilina* (Hymenoptera: Figitidae), a parasitoid of *Drosophila*

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES
DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN
DER UNIVERSITÄT REGENSBURG

vorgelegt von

Ingmar Weiss

aus

Augsburg

im Jahr 2015

Der Promotionsgesuch wurde eingereicht am:
13.02.2015

Die Arbeit wurde angeleitet von:
Dr. Johannes Stökl

Unterschrift:

Ingmar Weiss

List of publications

Chapter 2 has been published as **Weiss, I., Rössler, T., Hofferberth, J., Brummer, M., Ruther, J., and Stökl, J. A nonspecific defensive compound evolves into a competition avoidance cue and a female sex pheromone. *Nature Communications*, 4, 2013. doi: 10.1038/ncomms3767**. This chapter is licensed under the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported license. To view a copy of the license, visit `https://creativecommons.org/licenses/by-nc-sa/3.0/legalcode`.
Author contributions: I.W., J.R., and J.S. designed the study; I.W. (sex pheromone identification), T.R. (competition avoidance agent identification), M.B. (headspace), and J.S. (species specificity) performed the experiments; J.H. synthesized the compounds; I.W., J.H., J.R., and J.S. wrote the manuscript.

Chapter 4 has been published as **Weiss, I., Hofferberth, J., Ruther, J., and Stökl, J. Varying importance of cuticular hydrocarbons and iridoids in the species-specific mate recognition pheromones of three closely related *Leptopilina* species. *Frontiers in Ecology and Evolution*, 3 (19), 2015. doi: 10.3389/fevo.2015.00019**. This chapter has been licensed under the Creative Commons Attribution 4.0 International license. To view a copy of the license, visit `https://creativecommons.org/licenses/by/4.0/legalcode`.
Author contributions: I.W., J.R., and J.S. designed the study; I.W. performed the experiments; J.H. synthesized the compounds; I.W., J.H., J.R., and J.S. wrote the manuscript.

Contents

List of figures

List of tables

Summary

Animal communication systems employ a variety of different modalities, including visual, acoustic, electrical, and chemical stimuli. In insects, the use of chemicals for information transfer, the so-called semiochemicals, is a common phenomenon.

In this doctoral thesis, I investigate the chemical communication of the genus *Leptopilina*, a genus of solitary parasitoids of *Drosophila*. I mainly study the sexual communication of the closely related species *L. heterotoma*, *L. boulardi*, and *L. victoriae*, with particular emphasis on mate attraction, mate recognition, and receptiveness elicitation.

In chapter 2, I identify the female mate attraction pheromone of *L. heterotoma*. The pheromone is composed of several iridoids; its main component is (−)-iridomyrmecin, which is also shown to mediate competition avoidance in host-searching females. Because (−)-iridomyrmecin has previously been identified as defensive compound of *L. heterotoma*, an evolutionary route can be hypothesised, leading from the defensive function to competition avoidance and mate attraction pheromone accompanied by an increase in specificity and complexity of the semiochemical blend.

In chapter 3, I investigate the mating frequency and post-mating attractiveness of *L. heterotoma* females. Previous studies by different authors mentioned that females are monandrous and lose their attractiveness after mating. These reports, however, rely on incidental observations rather than dedicated experiments. I show, through a series of mating trials that *L. heterotoma* females are indeed monandrous, but do not lose their attractiveness. The found monandry implies the need for species-specific mate recognition in *L. heterotoma*, and the post-mating attractiveness is interpreted as a side effect of the parsimonous use of iridoids in *L. heterotoma*.

In chapter 4, I study the species specificity and chemical diversity of the female mate recognition pheromones of *L. heterotoma*, *L. boulardi*, and *L. victoriae*. Mating trials with conspecific and heterospecific females show that mate recognition is highly species specific in the three species. I then continue to investigate the chemical composition of the female mate recognition pheromones. In *L. heterotoma*, mate recognition is mediated by iridoids alone, whereas in *L. victoriae*, cuticular hydrocarbons are of major importance. The picture is yet different in *L. boulardi*: iridoids and cuticular hydrocarbons are equally important in mate recognition. The chemical diversity of the mate recognition pheromone very likely ensures its species specificity.

In chapter 5, I show the species specificity of the putative male antennal aphrodisiac pheromones in *L. heterotoma*, *L. boulardi*, and *L. victoriae*. For this, I devise a setup, in which the odour profile of heterospecific females is manipulated, so that the females are recognized as conspecifics by males. Males readily court heterospecifics in this setup, but can elicit receptiveness only in conspecific females. The identity of the putative aphrodisiac pheromones is not investigated, but the setup can likely be used to test candidate compounds for their behavioural activity.

The results presented in this thesis provide new insights into the evolution of chemical communication in *Leptopilina*. The suggested evolutionary route from defensive compound to mate attraction pheromone in *L. heterotoma* strongly supports the hypothesis that communicative functions can evolve for non-communicative compounds—and that thus the composition of chemical signals may be partially predestined by an inventory of non-communicative compounds. A similar inventory of such non-communicative compounds, however, does not necessarily lead to the same pheromone composition. In the investigated *Leptopilina* species, the female mate recognition pheromones differ greatly between the species. This supports the hypothesis that signals under strong stabilizing selection can evolve through saltational shifts, thus allowing rather drastic changes despite strong stabilisation. Building upon the new insights presented here, the chemical communication of more *Leptopilina* species should now be investigated to further advance our understanding of the evolution of chemical communication.

1. General introduction

Communication in animals

Communication in animals is an intensely researched topic. What exactly defines communication, however, is debatable. According to Scott-Phillips (2008), communication is often defined in terms of either adaptions or information transfer. Scott-Phillips himself defines communication (in terms of adaptions) as

> '[t]he completion of corresponding signals and responses.'

A 'signal' is defined as

> '[a]ny act or structure that (i) affects the behaviour of other organisms; (ii) evolved because of those effects; and (iii) which is effective because the effect (the response) has evolved to be affected by the act or structure'

and a 'response' is defined as

> '[a]ny act or structure that (i) is the effect of some act or structure of another organism; (ii) evolved to be affected by that act or structure; and (iii) which is affected because the other act or structure (the signal) has evolved to affect this act or structure.'

According to Endler (1993), animal communication enables individuals to base decision on the behaviour or physiology of others. This implies, that during communication, information is transferred (via a signal, as defined above) from one individual (the 'sender') to another (the 'receiver'). According to Endler (1993), the evolution of the signal increases communication efficiency and the benefit the sender receives from the receiver's response, and the evolution of the receiving mechanisms increases efficiency and reliability of the signal's perception.

The term 'signal' needs to be very clearly differentiated from the term 'cue'. A signal has evolved to alter the behaviour of other organisms (and is effective because a response evolved in the receiver), and because a signal requires adaptations in both sender and receiver, a benefit for both is implied. Cues, on the other hand, have not evolved to cause a response, and do not necessarily benefit the sender. Scott-Phillips (2008) defines a 'cue' as

> '[a]ny act or structure that (i) affects the behaviour of other organisms; and (ii) which is effective because the effect has evolved to be affected by the act or structure; but which (iii) did not evolve because of those effects.'

Cues are e. g. chemical compounds that are employed by the sender for non-communicative functions, including defensive secretions, cuticular compounds, and hormones, and that are released into the environment. Information transfer only takes place because the receiver 'eavesdrops' on the available cue and infers information from it. If a cue benefits the sender, the cue will likely evolve into a signal optimized for information exchange (Sorensen and Stacey, 1999; Steiger et al., 2011; Weiss et al., 2013; Wyatt, 2014).

Chemical communication in insects

Chemical communication, i. e. communication that employs chemical compounds to transfer information, is considered to be the oldest and most widespread form of communication (Wyatt, 2003). Although insects use a variety of sensory modalities for communication, chemical communication can be found throughout the insect taxa and is therefore intensely investigated. Chemical compounds employed for information transfer are called 'semiochemicals', regardless of being a signal or a cue. Semiochemicals are divided into 'pheromones' that mediate intraspecific information transfer and 'allelochemicals' that mediate interspecific information transfer (Nordlund and Lewis, 1976).

Allelochemicals are further divided into 'allomones' that benefit only the sender, 'kairomones' that benefit only the receiver, and 'synonomes' that benefit both sender and receiver (Nordlund and Lewis, 1976; Wyatt, 2014).

Pheromones can be further divided into primer pheromones that cause a developmental process in the receiver and releaser pheromones that cause a specific behavioural reaction in the receiver (Wyatt, 2014). Additionally, pheromones can be further divided by their behavioural function. This includes, e. g. aggregation, recruitment, alarm, and sex pheromones (Tillman et al., 1999).

To date, thousands of pheromones have been identified in a number of different taxa, including mammals, reptiles, and insects (El-Sayed, 2014). Pheromones fulfill a number of different roles, including the elicitation of alarm behaviour (e. g. in bees), the guiding of nestmates to a food source (e. g. in ants), the marking of a territory (e. g. in canines), and the attraction of mates (e. g. in moths). In fact, the very first pheromone to be identified was the sex pheromone of the silkworm, *Bombyx mori* (Butenandt et al., 1959). A pheromone may consist of only one single compound or a mixture of several compounds, the number of components reaching over a dozen in some pheromones (Wyatt, 2014). Pheromones can be practically applied in pest management, e. g. as attractants in traps to monitor pest populations or in techniques to limit reproduction in pest species, such as 'mating disruption'.

Parasitoids

A parasitoid can be defined (Eggleton and Gaston, 1990) as

> 'an organism which develops on or in another single ("host") organism, extracts nourishment from it, and kills it as a direct or indirect result of that development.'

The above definition does not restrict the term parasitoid to insect taxa, as is often the case (Eggleton and Gaston, 1990). Parasitoid lifecycles are commonly found in insects, but Hymenoptera show an especially high proportion of parasitoid species (Eggleton and Belshaw, 1992). In fact, 70 % of the about 95 000 Hymenoptera species are parasitoids and make up about 80 % of all parasitoids (Eggleton and Belshaw, 1992). Hosts of parasitoids are usually other insects, but numerous species of other Arthropoda have been found to be hosts as well (Eggleton and Belshaw, 1993). Despite the large number of parasitic Hymenoptera, only a small number of their sex pheromones have been identified to date (Ruther, 2013).

Sexual communication in parasitoids

In insects, sexual communication, i. e. communication that ultimately occurs for the purpose of mating, heavily relies on sex pheromones (Godfray, 1994; Quicke, 1997). Sex pheromones fulfill a variety of functions. They mediate mate attraction, mate recognition, mate assessment, and receptiveness elicitation. Mate attraction pheromones are released by one sex and enable the other sex to locate potential mating partners over some distance, they are usually effective at long range. At close range, mate recognition pheromones enable individuals to identify other individuals as conspecific members of the opposite sex, i. e. as a suitable mate. Mate recognition pheromones usually elicit stereotyped courtship behaviour in one or both sexes. Courtship is typically required to elicit readiness to mate in the female sex. During courtship, individuals can assess the quality of the other individual as a mate. This is especially important for females, as they usually have a greater interest than males to only copulate with a suitable male of high quality, so they will produce high quality offspring (Andersson, 1994). Once a female has accepted a male as mate, the female will indicate its receptiveness and copulation will eventually occur. A species may possess different pheromones for the functions mentioned above, but a single pheromone can also fullfil more than one function. Furthermore, sexual communication may consist of not only pheromones, but can include signals from other sensory modalities (Andersson, 1994).

The genus *Leptopilina* and its ecology

Wasps of the genus *Leptopilina* Förster, 1862 are solitary parasitoids of Drosophilid flies. The genus *Leptopilina* is currently comprised of 32 species (Nordlander, 1980; Quinlan, 1988; Nordlander and Grijpma, 1991; Allemand et al., 2002; Novkovic et al., 2011; Forshage et al., 2013; Wachi et al., 2015), some of which have been classified into 3 species groups within the genus: the *longipes* group, the *heterotoma* group, and the *boulardi* group (Nordlander, 1980). Members of the *heterotoma* group are mainly found in the Oriental region, whereas species from the *boulardi* group occur mainly in Africa. Species from the *longipes* group appear in Europe, South America, and the Carribean. Although several morphological and genetical phylogenies have been described (van Alphen et al., 1991; Schilthuizen et al., 1998; Allemand et al., 2002; Novkovic et al., 2011), these phylogenies have a low resolution and only low statistical support. Also, none of the published phylogenies includes all *Leptopilina* species, but only varying subsets of the known species.

Females of *Leptopilina* lay their eggs into early instar larvae of *Drosophila*. As most parasitoids, *Leptopilina* species tend do parasitize larvae of only one or very few *Drosophila* species. The tight interaction of *Leptopilina* and *Drosophila* has made the genus *Leptopilina* a model organism to study host-parasite interactions. Most studies, however, focused on the immune defense of *Drosophila* against the *Leptopilina* larvae (Fleury et al., 2009). The wasps' ecology has so far been studied with respect to host searching behaviour, foraging strategies, and patch time allocation (Fleury et al., 2009; Kaiser et al., 2009). However, only little is known about mate finding, mate recognition, and courtship behaviour of *Leptopilina*, three levels of sexual communication that often involve sex pheromones in parasitic Hymenop-

tera (Ruther, 2013).

Drosophila species, the host species of *Leptopilina*, are usually no pests. Thus, a potential role of *Leptopilina* in pest control had not been investigated until recently. With the emergence of *Drosophila suzukii* in Europe and North America (Hauser, 2011; Calabria et al., 2012), however, the situation has changed. In contrast to most other *Drosophila* species, *D. suzukii* females possess a serrated ovipositor (Cini et al., 2012). Thus, *D. suzukii* females can lay eggs into undamaged fruit, thereby damaging the fruit and eventually ruining crops, while most other *Drosophila* species can lay their eggs only into already damaged or overripe fruits or mushrooms (Cini et al., 2012). *Drosophila suzukii* is the only known *Drosophila* pest species, and it is rapidly emerging in Europe and North America (Hauser, 2011; Calabria et al., 2012). This has lead to first initial investigations into the potential role of parasitoids, including *Leptopilina*, as antagonists against *D. suzukii* (Chabert et al., 2012). To establish *Leptopilina* as a control agent of *D. suzukii*, however, in-depth knowledge of the ecology, including the chemical ecology, of *Leptopilina* is required. This is particularly true for ecological aspects of reproduction, such as mate attraction, mate recognition, and courtship behaviour, but also for host-finding and competition avoidance mechanisms. The mechanisms for host-finding and competition avoidance in *Leptopilina* can be exploited to direct parasitoids towards specific areas in the field. Detailed knowledge of the mating behaviour allows efficient rearing of the parasitoids, e. g. aphrodisiac pheromones can be used to increase receptiveness in females and thereby increase the number of offspring per generation. Thus, thorough knowledge of the chemical ecology of *Leptopilina* is required to efficiently rear and deploy *Leptopilina* as a potential control agent of *D. suzukii*.

Leptopilina heterotoma (Thomson, 1862) and *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillaut, 1979) females have been found to orientate towards odours specific to *Drosophila* (Wiskerke et al., 1993; Hedlund et al., 1996). More specifically, host-searching females eavesdrop on the aggregation pheromone of their host species to find hosts for oviposition.

Competition avoidance in host-searching *Leptopilina* females has been investigated by Janssen et al. (1995a,b). They found that, in the field, host-searching *L. heterotoma* females avoided host patches that were already exploited by *Leptopilina clavipes* females. Subsequently, Janssen et al. could show in laboratory experiments that this avoidance is odour-mediated and that the avoidance behaviour is not only interspecific, but also intraspecific. The identity of the avoidance-inducing odour, however, has not been investigated so far.

Fauvergue et al. (1999) found that males of both *L. heterotoma* and *L. boulardi* were attracted towards female-baited traps in orchards. In wind tunnel experiments, Fauvergue et al. (1999) could demonstrate that a volatile sex pheromone is responsible for the attraction of males towards females. The composition of the sex pheromone, however, has not been investigated so far.

The mating behaviour of *L. heterotoma* has been described by several authors (Jenni, 1951; van den Assem, 1968; Isidoro et al., 1999). Males typically display wing-fanning, a high-frequency vibration of the wings, when they recognize a female. After their recognition, males approach the female and then touch the female with the antennae. Afterwards, the female is mounted. Once the female has been mounted, it usually folds back its antennae, so the male's head comes to lie between the female antennae. Males then show so-called antennal stroking (or paddling), moving their antennae in a circular pattern, thereby bringing their own proximal antennomeres into contact with the female's distal antennomeres in a rhythmical way. The female may then accept the male as mate. If the male is accepted, the female will lower her antennae and open her genital orifice (and extrude the ovipositor, personal observation). The male then moves backwards and eventually copulation occurs. Wing fanning is typically maintained during all stages of courtship and stops only when copulation occurs or courtship is abandoned. I found that the mating behaviour of *L. boulardi* and *Leptopilina victoriae* Nordlander, 1980 is very similar to that of *L. heterotoma*. The role of pheromones in elicitation of both male courtship and female receptiveness has not been investigated so far, although a male antennal aphrodisiac pheromone has been proposed (Isidoro et al., 1999).

Thesis outline

In this work, the sexual communication and competition avoidance behaviour of the species *L. heterotoma*, *L. boulardi*, and *L. victoriae* is investigated. Both *L. heterotoma* and *L. boulardi* have a cosmopolitan distribution (Nordlander, 1980) while *L. victoriae* is restricted to Africa (Nordlander, 1980) and Asia (Novkovic et al., 2011).

In chapter 2, the composition of the female mate attraction pheromone in *L. heterotoma* is investigated. Additionally, the competition avoidance odour is identified and the evolutionary pathway that might have lead to the composition of the pheromone is discussed.

In chapter 3, the mating frequency of *L. heterotoma* females and the loss of attractiveness of mated females that has been postulated in previous studies are studied.

In chapter 4, the species specificity of the mate recognition pheromones of *L. heterotoma*, *L. boulardi*, and *L. victoriae* is investigated. Furthermore, a high chemical diversity of the mate recognition pheromones is demonstrated.

In chapter 5, the species specificity of the putative male antennal aphrodisiac pheromone of *L. heterotoma*, *L. boulardi*, and *L. victoriae* is shown.

2. The evolution of mate attraction and competition avoidance in *Leptopilina heterotoma*

This chapter has been published as **Weiss, I., Rössler, T., Hofferberth, J., Brummer, M., Ruther, J., and Stökl, J. A nonspecific defensive compound evolves into a competition avoidance cue and a female sex pheromone. *Nature Communications*, 4, 2013. doi: 10.1038/ncomms3767**.

Author contributions: I.W., J.R., and J.S. designed the study; I.W. (sex pheromone identification), T.R. (competition avoidance agent identification), M.B. (headspace), and J.S. (species specificity) performed the experiments; J.H. synthesized the compounds; I.W., J.H., J.R., and J.S. wrote the manuscript.

The evolution of chemical communication and the origin of pheromones are among the most challenging issues in chemical ecology. Current theory predicts that chemical communication can arise from compounds primarily evolved for non-communicative purposes but experimental evidence showing a gradual evolution of non-informative compounds into cues and true signals is scarce. Here we report that females of the parasitic wasp *Leptopilina heterotoma* use the defensive compound (−)-iridomyrmecin as a semiochemical cue to avoid interference with con- and heterospecific competitors and as the main component of a species-specific sex pheromone. Although competition avoidance is mediated by (−)-iridomyrmecin alone, several structurally related minor compounds are necessary for reliable mate attraction and recognition. Our findings provide insights into the evolution of insect pheromones by demonstrating that the increasing specificity of chemical information is accompanied by an increasing complexity of the chemical messengers involved and the evolution of the chemosensory adaptations for their exploitation.

Introduction

Chemical senses are the oldest and most widespread in nature and chemical communication was likely the original mechanism of information transfer between individuals (Wyatt, 2003). One major advantage of chemical communication is the enormous diversity of potential signals. To date, several thousand chemical pheromone components (chemical signals used for intraspecific communication) are known to science (El-Sayed, 2009–2013), and using these compounds in combination results in an astounding collection of possible chemical signals.

What is the origin of a chemical signal and what qualifies a certain compound to be used as a pheromone? Even considering all of the physiological and physical limitations, such as the availability of chemical precursors and biosynthetic pathways or functional signal constraints such as volatility, hundreds or thousands of different chemical compounds would be equally well suited as messengers. Thus, other factors must have a role in the evolution of such signals.

Current evolutionary theory predicts that pheromones may evolve from compounds already in use for non-communicative functions (Steiger et al., 2011; Bradbury and Vehrencamp, 2011; Wyatt, 2010). For example, hormones, defensive secretions, or cuticular compounds can be the basis for pheromone evolution. If these compounds, or their metabolites, are released by one individual and perceived by another individual, they might provide information about the condition of the sender. This condition can be the simple presence of the sender or, in the case of hormones, a physiological state (Sorensen and Stacey,

1999). A compound perceived in this way serves only as a cue: it supplies information without being selected for this function. If the sender benefits from the receiver's response, chemical ritualization may alter the status of the cue into a true signal by it being selected for information transfer (Steiger et al., 2011; Tinbergen, 1952).

The use of a chemical substance for two or more purposes is well established and referred to in the literature as 'semiochemical parsimony' (Blum, 1996). Classic examples of primarily non-communicative compounds becoming informative in intraspecific interactions are cuticular hydrocarbons (CHCs). Found on the cuticle of almost every insect, CHCs function primarily as a desiccation barrier (Gibbs, 1998). But in addition, CHCs serve as semiochemical cues (for example, nestmate recognition in social insects) or have even evolved into signals (Howard and Blomquist, 2005), and in some cases, the selective forces driving this process have been identified (Steiner et al., 2005; Ruther and Steiner, 2008; Kühbandner et al., 2012). Defence compounds are also candidate chemicals that may be employed for intraspecific information transfer. To fulfil their function, they are typically produced and released in relatively large quantities and thus are often easily detectable. Consequently, defensive compounds have been found to function as alarm pheromones (for example, (Löfqvist, 1976)) or even more specifically as sex pheromones (Ruther et al., 2001; Geiselhardt et al., 2008; Boppré, 1986). However, most studies investigating multi-functional defence compounds are limited to the description of the multiple functions of the compounds but do not address the ecological framework that might have favoured their evolution. In particular, it is unclear how an increase in specificity and information reliability is achieved when defence compounds evolve into chemical signals, because the same defence chemicals are often used by several species (Laurent et al., 2005). One way the specificity and reliability of the information could increase is via species-specific alterations of the chemical messengers. Such alterations could include the presence and relative proportion of other compounds in the blend. However, to the best of our knowledge, experimental evidence for this type of signal evolution is missing.

Leptopilina heterotoma is a solitary larval parasitoid of Drosphilid flies, including *Drosophila melanogaster* (Jenni, 1951; Hedlund et al., 1996). Owing to the model status of its host, *Leptopilina* has been intensively studied during the past years (reviewed in Fleury et al. 2009). Recently we have shown that females and males of *L. heterotoma* produce iridomyrmecins in a cephalic gland and that the wasps use it as a repellent against insect predators (Stökl et al., 2012). Furthermore, it is known, that females of *L. heterotoma* are able to distinguish between host patches with and without conspecifics, based on olfactory cues only (Janssen et al., 1995a). Iridomyrmecin is the major volatile compound produced by *L. heterotoma* and therefore it is a likely candidate to serve as a competition avoidance cue for female *L. heterotoma*. Males and females of *L. heterotoma* use different stereoisomers of iridomyrmecin for defence. While females produce (−)-iridomyrmecin and, in much smaller quantities (+)-isoiridomyrmecin (Stökl et al., 2012), males produce only (+)-isoiridomyrmecin. This sex-specific difference and the observation that males are attracted to virgin females by olfactory signals (Fauvergue et al., 1999) suggest that (−)-iridomyrmecin, in addition to its defensive function, might also be the female sex pheromone in *L. heterotoma*.

In this study, we present a case of threefold semiochemical parsimony in *L. heterotoma* and provide evidence for the evolution of a nonspecific defensive compound into an agent mediating inter- and intraspecific competition avoidance and a species-specific female sex pheromone. This increased specificity of information is accompanied by a diversification of the chemical messengers and behavioural adaptations.

Results

Chemical analysis. To identify the chemical cues and pheromone compounds mediating competition avoidance and mate finding, we extracted the wasps in dichloromethane (DCM). The extract contains iridoids and CHCs (fig. 2.1a). We subsequently fractionated the extract into CHCs and iridoids by solid phase extraction or size-exclusion chromatography (SEC). The iridoid fraction contained (−)-iridomyrmecin as major component (fig. 2.1b, 'p5' and (+)-isoiridomyrmecin 'p6' as a minor component. In addition, three further iridoids ('p1', 'p2', 'p7') were present in small amounts.

Mass spectra of p1 and p2 were very similar to a published mass spectrum of iridodial (fig. A.1a–c, Ohmura et al. 2009) suggesting that p1 and p2 are stereoisomers of iridodial. As synthetic references for iridodial were not available, we produced them by catalytic hydrogenation of (epi-)/chrysomelidial (fig. A.4) extracted from *Phaedon cochleariae* larvae. Two iridoidials formed by hydrogenation of (epi-)/chrysomelidial showed mass spectra identical to those of p1 and p2, respectively (fig. A.1d, e). Additionally, these iridodials co-eluted with p1 and p2 on a non-polar GC-column (fig. A.2). One of the (epi-)/chrysomelidial-derived iridodials co-eluted with p2 also on the Beta DEX 225 column (fig. A.3). We conclude from these results that p1 and p2 are irido-

dials. We suggest that p2 is identical to one of the (epi-)/chrysomelidial-derived iridodials, whereas p1 is likely the enantiomer of one of the other iridodials formed by hydrogenation of (epi-)/chrysomelidial.

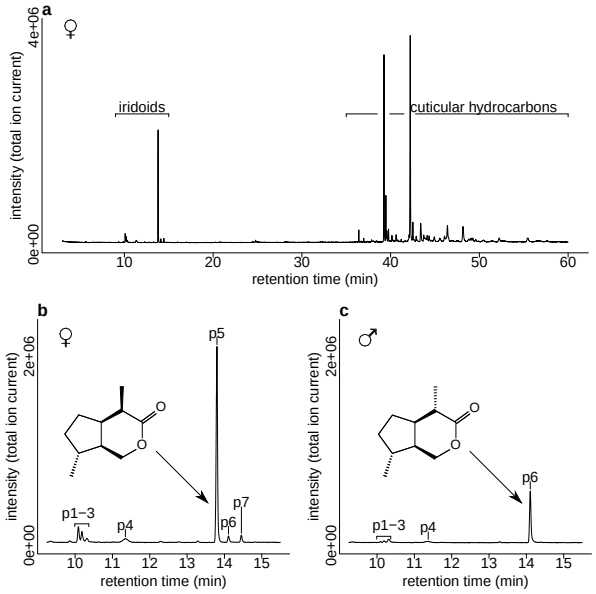

Figure 2.1.: Chemical compounds produced by *L. heterotoma*. Total ion current chromatograms of (**a**) *L. heterotoma* female extract and the iridoid fraction of (**b**) female and (**c**) male extract. Compound p5 is (−)-iridomyrmecin and p6 is (+)-isoiridomyrmecin. The inserts in (**b**) and (**c**) show their structure and absolute configuration. Compounds p1 and p2 were tentatively identified as iridodials; p7 was tentatively identified as a third iridomyrmecin. P3 and p4 were behaviourally inactive and thus not identified.

Peak p7 showed a mass spectrum similar to (−)-iridomyrmecin (fig. A.5a, b) suggesting that p7 is another stereoisomer of iridomyrmecin. Mass spectra of trans-fused stereoisomers of iridomyrmecin show different mass spectra (m/z 95 is always smaller than m/z 81, fig. A.5c, Hilgraf et al. 2012) and we thus excluded the presence of these compounds. From the eight possible cis-fused stereoisomers of iridomyrmecin we excluded (+)- and (−)-iridomyrmecin and (+)- and (−)-isoiridomyrmecin because of differing retention times compared with authentic standards. Thus, four possible cis-fused iridomyrmecin stereoisomers remained as possible structures for p7. Although, those were not available as pure synthetic references, p7 co-eluted with a minor impurity in the synthetic sample of (−)-iridomyrmecin on both the non-polar BPX-5 (fig. A.6) and the Gamma DEX 120 column (fig. A.7). This impurity shows the same mass spectrum as p7 (fig. A.5a, d). We therefore conclude that p7 is the same compound as the impurity in the synthetic sample. The way we synthesized (−)-iridomyrmecin suggests that this impurity, and therefore p7, is either (4R,4aS,7R,7aR)- or (4S,4aS,7R,7aR)-iridomyrmecin (J and K in fig. A.13). The line of argumentation for this conclusion is provided in detail below.

Two further minor compounds in the iridoid fraction ('p3','p4') were found to be behaviourally inactive (see below) and were thus not structurally elucidated. Unlike (−)-iridomyrmecin and (+)-isoiridomyrmecin the minor compounds in p1, p2 and p7 were not available as synthetic standards. To test their contribution to the biological activity, we isolated them from the iridoid fraction by preparative gas chromatography. The two iridodial (p1 and p2) could not be further seperated and were thus tested together in the bioassays. The CHC fraction was behaviourally inactive and thus not analysed any further.

We used female *L. boulardi* and extracts thereof to test the species-specificity of the chemical communication in *L. heterotoma*. *Leptopilina boulardi* occurs sympatrically with *L. heterotoma* and parasitizes the same host species (Hedlund et al., 1996; Fauvergue et al., 1999). Our chemical analysis revealed that the females of *L. boulardi* produce the same iridoid compounds as *L. heterotoma* females, but in different ratios (figs. A.8–A.11).

Detailed argumentation for the identity of p7. Because of the way the authentic standards of (−)-iridomyrmecin and (+)-isoiridomyrmecin were prepared, certain minor impurities are likely to contaminate the authentic standards.

Authentic standards of (−)-iridomyrmecin and (+)-isoiridomyrmecin were prepared using a highly diastereoselective synthesis. The stereochemistry in the final products is governed by the stereochemistry of the single stereocenter in the starting material. The parlay of stereochemistry from the C7 stereocenter in citronellal to the bridgehead positions (C4a and C7a) occurs during formation of the five-membered ring. This reaction is thought to occur by a concerted hetero-Diels-Alder reaction because only products with a cis ring junction have ever been observed. With a stereocenter at C7, there are two distinct transition states for the Diels-Alder reaction that have different energies (fig. A.12). The formation of the trans cis product (A) is presumably favourable both kinetically because of the lower energy transition state and thermodynamically because there is less steric compression compared with the alternative cis cis product (B). As first described by Schreiber et al. (1986), the ratio of A to B strongly favours A under both kinetic and thermodynamic conditions. Specifically, he found that the ratio of A to B at short reaction times to be about 4:1. If the re-

action is allowed to continue for long reaction times, the ratio of A to B increases to >20:1. We have used this method to generate the iridoid carbon framework extensively and have never isolated B and because it forms in such small proportions with respect to A, we have not seen evidence of it in our spectral analysis. Chances are high that the cis cis diasteromer B is present in our product mixtures albeit in very small relative amounts. In contrast, there is little chance that the cis trans or trans cis isomers (fig. A.12, in the box) will form because of the stereospecificity of the concerted Diels-Alder reaction.

The stereochemistry of the stereocenters at C4a, C7 and C7a do not change during the remainder of the synthesis of iridomyrmecin and isoiridomyrmecin. However, the stereocenter at C4 is generated with low selectivity later in the synthesis (fig. A.13). During the hydration of C (and D), the C4 and C3 stereocenters are formed in all possible orientations. Using silica gel chromatography, the hydrated products E and G can be separated. Because we do not detect the alternate products derived initially from B (via D), F and H, we cannot determine whether either F and/or H is/are present as impurities in E or G. So when E and G are separately oxidized to form (−)-iridomyrmecin and (+)-isoiridomyrmecin, we cannot say if J and/or K is/are present as very minor impurities.

In summary, although the authentic standards of (−)-iridomyrmecin and (+)-isoiridomyrmecin are very pure (>99%), it is possible that two side products, J and K, formed during their synthesis. Although it is possible that one or both of these side products are present, it is very unlikely that the corresponding cis trans or trans cis isomers (derived from the intermediates in the box in fig. A.12) are present.

Because a very minor component (p7) of the natural blend rich in (−)-iridomyrmecin co-elutes with a very minor impurity in the authentic standard of (−)-iridomyrmecin and has an identical mass spectrum, it is likely the identity of the minor component (p7) is either J or K.

When the authentic standard of (−)-iridomyrmecin is injected with the natural compounds, the minor impurity in the authentic standard co-elutes with p7 on both non-chiral (fig. A.6) and chiral columns (fig. A.7). This is consistent with the idea that p7 forms as a side product during the biosynthesis of the (−)-iridomyrmecin in the insect produced blend. One would not expect the insect to produce the enantiomer of p7 (called here ent-p7). This enantiomer (ent-p7) would be indistinguishable from p7 on the non-chiral GC-column but not on a chiral phase GC-column. The enantiomers of J and K, ent-J and ent-K, would be expected as minor impurities in the preparation of (+)-iridomyrmecin. Therefore, one would anticipate that the minor impurity in (+)-iridomyrmecin will co-elute with p7 on the non-chiral column and not co-elute on the chiral phase column. This expected result is borne out by experiment further supporting that p7 is J and/or K.

All lines of evidence support that p7 is J and/or K. Our understanding of the synthesis of (−)-iridomyrmecin implicates J and K as likely impurities in the authentic standard. Without the isolation and full characterization of the impurity, we cannot prove that J and/or K are the structures we propose but it is highly likely based on the chemistry.

Competition avoidance cue. Having the volatiles produced by *L. heterotoma* at hand, we used a y-tube olfactometer to test the hypothesis that the iridoids, particularly (−)-iridomyrmecin , mediate competition avoidance in host-searching females of *L. heterotoma*. In a first basic experiment, we demonstrated that mated female *L. heterotoma* prefer host patch odour to clean air (fig. 2.2a). In subsequent experiments, the responding females had the choice between the odours of two host patches, one with females, one without. Females avoided the already exploited host patches, irrespective of whether conspecific (fig. 2.2b) or heterospecific (fig. 2.2c) females were present and preferred the host patch without wasps. Next, we replaced the living females on the host patch with an extract of *L. heterotoma* females. The host patch with the female extract was avoided by the females (fig. 2.2d), but the extract was not avoided if presented without a host patch (fig. 2.2e). The compound(s) responsible for this effect were present in the iridoid fraction of the female extract, whereas the CHC fraction was behaviourally inactive (figs. 2.2f–2.2g). Finally, we added synthetic (−)-iridomyrmecin and (+)-isoiridomyrmecin, respectively, to the host patch. In both cases, the responding females avoided the modified host patches and preferred the unmodified ones (figs. 2.2h–2.2i).

Hence, mated *L. heterotoma* females avoid the odour of exploited host patches. The effect is not species-specific and is mediated by (−)-iridomyrmecin and its naturally occurring epimer (+)-isoiridomyrmecin.

Female sex pheromone. Having demonstrated that pure (−)-iridomyrmecin mediates competition avoidance in *L. heterotoma* females, we asked the question whether (−)-iridomyrmecin also has a role in the attraction of males to virgin females. This hypothesis is based on the fact that (−)-iridomyrmecin is only produced by females of *L. heterotoma* but not by males (figs. 2.1b–2.1c). Again, we used a y-tube olfactometer to test this hypothesis and in a first experiment, we demonstrated that naive *L. heterotoma*

males were attracted by the odour of living virgin *L. heterotoma* females (fig. 2.3a). Next, we replaced the living females by a solvent extract of those, which was also highly attractive to the males (fig. 2.3b). CHCs from the female extract were not attractive to the males (fig. 2.3c), but the iridoid fraction strongly attracted the males (fig. 2.3d). However, pure synthetic (−)-iridomyrmecin (p5) containing its epimer (+)-isoiridomyrmecin (p6) as minor component was not attractive (fig. 2.3e). Likewise, a blend of the minor compounds p1, p2, p3, p6 and p7 was not attractive in the bioassay (fig. 2.3f), but adding synthetic (−)-iridomyrmecin to this mixture fully restored its attractiveness to males (fig. 2.3g). Using (+)-iridomyrmecin instead of (−)-iridomyrmecin , however, did not restore the attractiveness (fig. 2.3h). Males were still attracted by an iridoid fraction from which the compounds p3 and p4 had been removed by preparative GC (fig. 2.4a). In contrast, removing either (+)-isoiridomyrmecin (p6) or the minor iridoids p1, p2 and p7, respectively, from the iridoid fraction resulted in the loss of the attractiveness (figs. 2.4b–2.4d). Doses down to 1/40 of a *L. heterotoma* female equivalent were attractive for *L. heterotoma* males in the y-tube bioassay (fig. 2.5).

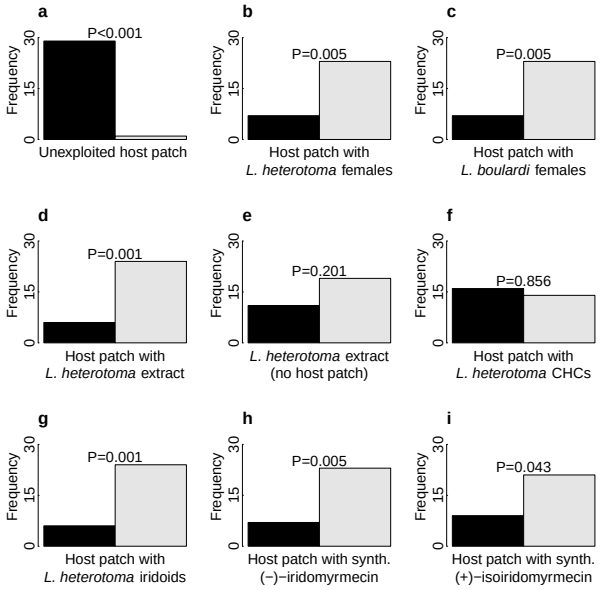

Figure 2.2.: Experiments to identify the competition avoidance cue. Frequency of decision for sample or control of mated *L. heterotoma* females in a y-tube experiment when choosing between the odour of (**a**) a host patch and clean air, (**b**) an unexploited host patch and the odour of a host patch with 10 living *L. heterotoma* females, (**c**) an unexploited host patch and the odour of a host patch with 10 living *L. boulardi* females, (**d**) an unexploited host patch and a host patch with extract of *L. heterotoma* females, (**e**) extract of *L. heterotoma* females and clean air, (**f**) an unexploited host patch and a host patch with the CHCs of *L. heterotoma* females, (**g**) an unexploited host patch and a host patch with the iridoids of *L. heterotoma* females, (**h**) an unexploited host patch and a host patch with synthetic (−)-iridomyrmecin, (**i**) an unexploited host patch and a host patch with synthetic (+)-isoiridomyrmecin. Bar colours indicate sample (dark) and control (clean air or unexploited host patch, light). *P*-values (rounded to the third decimal) are given for the two-sided binomial test. For each experiment $n = 30$.

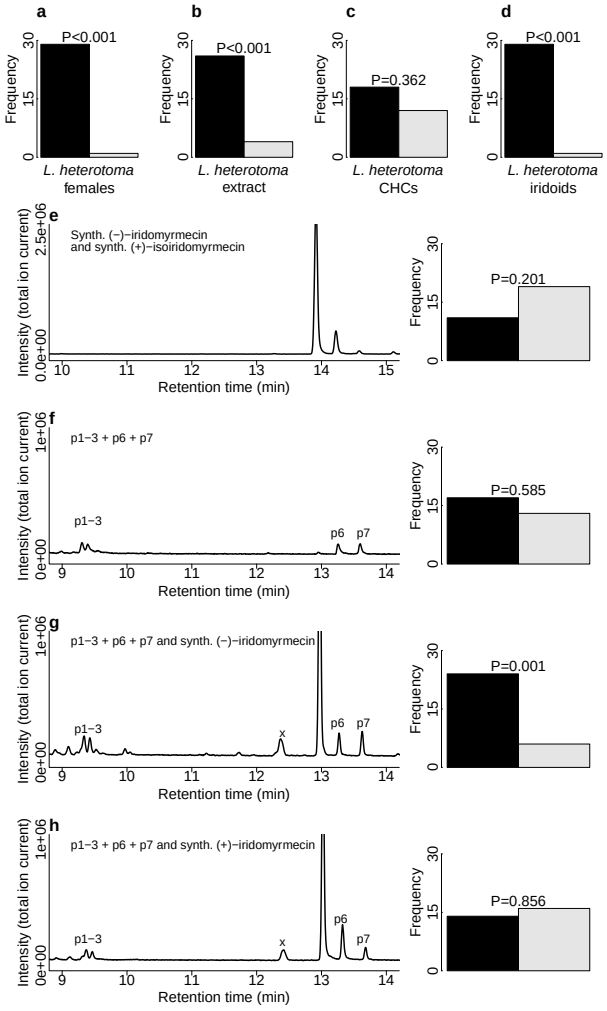

Figure 2.3.: Experiments to identify the female sex pheromone. Frequency of decision for sample or control of naïve *L. heterotoma* males in a y-tube experiment when choosing between the control and (**a**) the odour of virgin *L. heterotoma* females, (**b**) an extract of *L. heterotoma* females, (**c**) the CHCs or (**d**) the iridoid compounds from the female extract. (**e**)–(**h**) Total ion current chromatograms illustrating the manipulation of the iridoid fraction of the female extract and the frequency of decision in the corresponding y-tube experiment when testing the manipulated iridoid fraction against the control. Compounds p1 and p2 were tentatively identified as iridodials; p3 was behaviourally inactive and thus not identified; p6 is (+)-isoiridomyrmecin; p7 was tentatively identified as a third iridomyrmecin; x denotes contaminations. *P*-values (rounded to the third decimal) are given for the two-sided binomial test. Bar colours indicate sample (dark) and control (light). For each experiment $n = 30$.

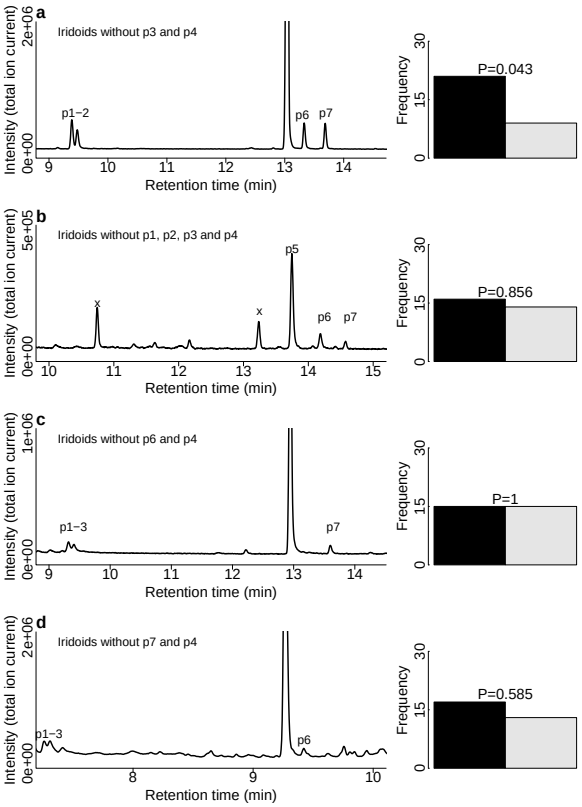

Figure 2.4.: Importance of minor components in the sex pheromone. (**a**–**d**) Total ion current chromatograms illustrating the manipulation of the iridoid fraction of the *L. heterotoma* female extract and the frequency of decision for sample or control in the corresponding y-tube experiment when testing the manipulated iridoid fraction against the control. Compounds p1 and p2 were tentatively identified as iridodials; p3 and p4 were behaviourally inactive and thus not identified; p5 is (−)-iridomyrmecin; p6 is (+)-isoiridomyrmecin; p7 was tentatively identified as a third iridomyrmecin; x denotes contaminations. *P*-values (rounded to third decimal) are given for the two-sided binomial test. Bar colours indicate sample (dark) and control (light). For each experiment $n = 30$.

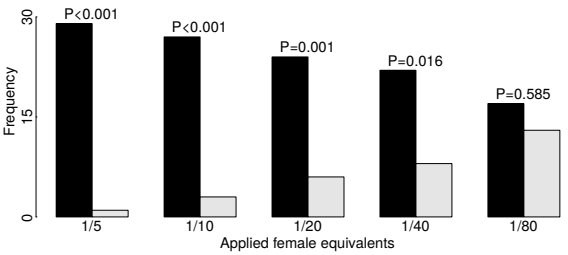

Figure 2.5.: Dose-response experiments for the sex pheromone. Frequency of decision for sample or control of naïve *L. heterotoma* males in a y-tube experiment when choosing between the odour of 1/5 to 1/80 female equivalent of virgin *L. heterotoma* female extract and the control. Bar colours indicate female extract (dark) and control (light). *P*-values (rounded to third decimal) are given for the two-sided binomial test. For each experiment $n = 30$.

We therefore conclude, that (−)-iridomyrmecin is the major component of the female sex pheromone in *L. heterotoma* and is perceived enantiospecifically. However, (+)-isoiridomyrmecin (p6), the two iridodials (p1, p2), as well as the third iridomyrmecin (p7) are also essential for bioactivity.

Nonetheless, mate attraction in *L. heterotoma* is not species-specific, as males were also attracted by an extract of *L. boulardi* females (fig. 2.6a). We therefore did an additional experiment to test whether the males discriminate between species at close range and observed the courtship behaviour (wing fanning) of *L. heterotoma* males towards filter paper discs impregnated with the test compounds. The duration of male wing fanning differed significantly between the test groups (Kruskal-Wallis test, $H = 30.4$, DF = 3, $P < 0.001$). *Post-hoc* tests indicated that males of *L. heterotoma* engaged in wing fanning behaviour significantly longer when exposed to paper discs treated with the iridoid fraction from conspecific females than to those treated with the iridoids from *L. boulardi* females, synthetic (−)-iridomyrmecin or the pure solvent (fig. 2.6c, see table A.1 for *P*-values).

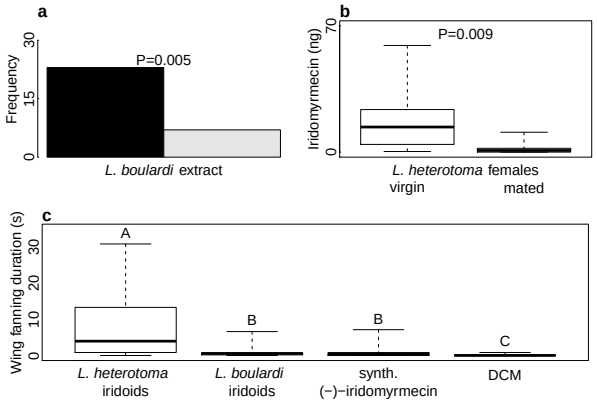

Figure 2.6.: Species specificity and released amounts of the sex-pheromone. (**a**) Frequency of decision for sample or control of naïve *L. heterotoma* males in a y-tube experiment when choosing between clean air (dark bar) and the extract of virgin L. boulardi females (light bar). The *P*-value (rounded to third decimal) is given for the two-sided binomial test; $n = 30$. (**b**) Box-and-whisker plots showing median (horizontal line), interquartile range (box) and maximum/minimum range (whiskers) of the duration (s) of the courtship behaviour (wing fanning) that naïve *L. heterotoma* males displayed towards the iridoid fraction of an extract of females of *L. heterotoma* and *L. boulardi*, respectively, synthetic (−)-iridomyrmecin and DCM. Different capital letters above box-and-whisker-plots indicate a significant difference between these plots (Kruskal-Wallis test followed by pairwise Mann-Whitney *U*-tests with Bonferroni-Holm correction, $P < 0.05$). For each experiment $n = 12$. (**c**) Box-and-whisker plots showing median (horizontal line), interquartile range (box) and maximum/minimum range (whiskers) of the amount (ng) of iridomyrmecins released by virgin and mated females of *L. heterotoma*. The *P*-value (rounded to third decimal) is given for the Mann-Whitney *U*-test; $n = 13$.

This shows that in contrast to mate attraction, mate recognition in *L. heterotoma* is species-specific. This specificity is attributed to the particular blend of iridoid compounds and not to differences in the composition of the CHCs.

Headspace analysis. In the last experiment, we used headspace collections and GC-MS analyses to measure the amount of (−)-iridomyrmecin released by females of *L. heterotoma* in a non-defensive context. Virgin females released a significantly higher amount of (−)-iridomyrmecin than mated females (Mann-Whitney U-test, $W = 136$, $P = 0.009$)(fig. 2.6b). Although the amount released by virgin females was much higher than in mated females, mated females still released (−)-iridomyrmecin.

Discussion

Our data clearly demonstrate that (−)-iridomyrmecin is not only a defensive agent in *L. heterotoma* but also serves as competition avoidance cue for host-searching females and as the major component of the female sex pheromone. (−)-iridomyrmecin is being used at three different levels of information transfer. As a defensive agent, it is not to be considered communicative. In competition avoidance, it serves as either cue or signal and as major component of the sex pheromone, (−)-iridomyrmecin is part of a true signal. This is exactly what current models on pheromone evolution predict: a primarily non-communicative compound may be utilized as a cue by eavesdroppers and, at the next stage, chemical ritualization may then transform the cue into a signal (Steiger et al., 2011; Bradbury and Vehrencamp, 2011; Wyatt, 2010). The hypothetical stages of this evolutionary scenario are illustrated in fig. 2.7 and discussed in detail below. Stage one of the hypothetical scenario is the use of a certain compound for a non-communicative purpose, in the case of *L. heterotoma* for defence (fig. 2.7a). *Leptopilina heterotoma* uses (−)-iridomyrmecin and (+)-isoiridomyrmecin as major component of their defensive secretions (Stökl et al., 2012). Actually, all stereoisomers of iridomyrmecin found in nature are effective repellents against ants and spiders (Stökl et al., 2012; Hübner and Dettner, 2000; Hübner et al., 2002) and thus probably against a broad range of insect predators. This suggests a conserved perception mechanism for this class of chemicals in insects, which might have been a preadaptation for the evolution of a communicative function. Apart from this, the volatility of (−)-iridomyrmecin and the fact that it is constantly available for perception from *Leptopilina* females (fig. 2.6b) might have favoured this compound as a good candidate for cue or pheromone evolution.

At stage two, the compound becomes a semiochemical cue. In our experiments, females foraging for hosts preferred non-exploited host patches over host patches already being exploited by conspecific or heterospecific females (fig. 2.7b). This competition avoidance can be elicited by treating a non-exploited host patch with (−)-iridomyrmecin alone (fig. 2.2h).

(−)-iridomyrmecin can be perceived by several insects (for example, ants) (Stökl et al., 2012) that have probably not been under selection to detect it. Likewise, the first eavesdropping females might have perceived (−)-iridomyrmecin without prior selection and thus could have used it as a cue. Our experiments suggest, however, that females additionally evolved a context-specific behaviour: (−)-iridomyrmecin triggers competition avoidance only when females perceive the cue with a background of host patch odour (figs. 2.2d–2.2e). This corroborates recent studies that have highlighted the importance of background odours for the chemical orientation of many insects in complex environments (as reviewed by Schröder and Hilker (2008)) and shows furthermore, that the avoidance behaviour of *L. heterotoma* females is not simply due to the repellent property of (−)-iridomyrmecin, but is indeed context-specific. We suggest that *L. heterotoma* females have also evolved a highly sensitive receptor, allowing them to reliably perceive even small amounts of iridomyrmecin and thus spot competitors.

At the stage of competition avoidance, (−)-iridomyrmecin is at least a semiochemical cue for the females, but it could also already be a signal. A cue has not evolved to transfer information and does not essentially benefit the sender. For something to be a signal, both the emission and the response need to have evolved (Wyatt, 2011). In our scenario, the receiver definitely benefits when favouring the unoccupied host patch over the occupied one: the sending females might already have started exploiting the resource and in *L. heterotoma*, earlier laid eggs have greater chances of success in cases of superparasitism (Bakker et al., 1985). On the other hand, females in an only partially exploited host patch would benefit if other host-searching females were to avoid this patch. They could then claim the remaining resources without competition. We do not know whether the females on the host patch release iridomyrmecin actively or inactively and the responding females might also behave differently if there is no unexploited host patch to choose alternatively. We can therefore not finally conclude whether the avoidance behaviour is mediated by a cue or a signal, most likely it is an intermediate evolutionary stage.

In any case, in its role as competition avoidance semiochemical, (−)-iridomyrmecin gained increased specificity (compared with its use as defensive agent)

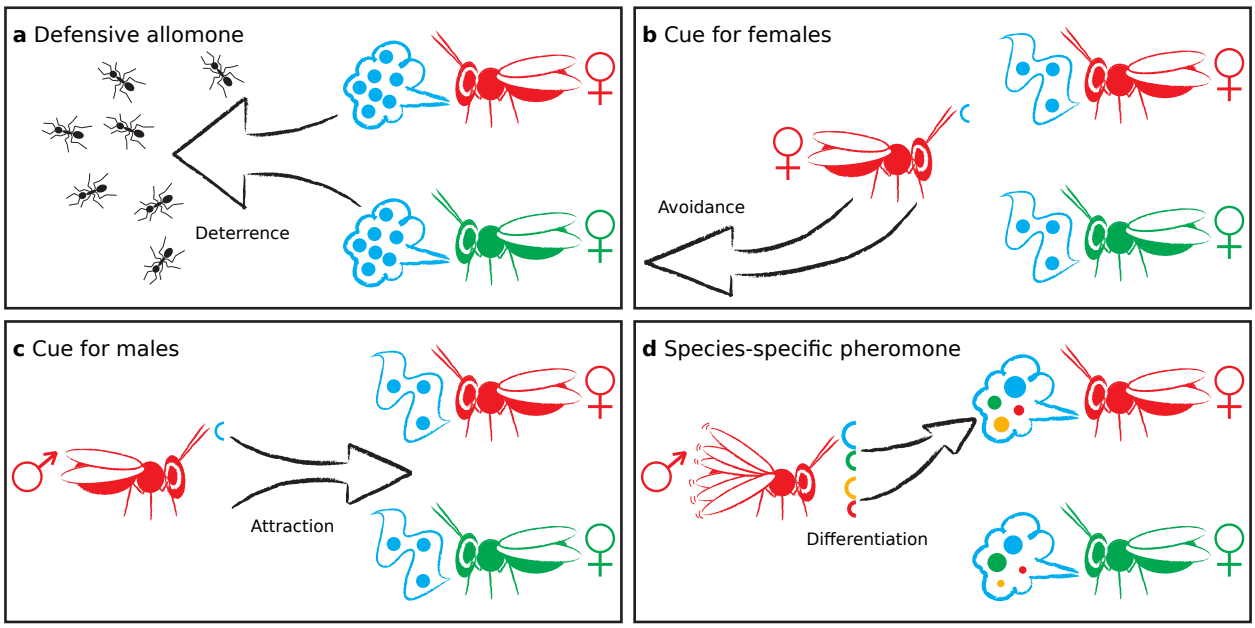

Figure 2.7.: Pheromone evolution in *Leptopilina*. Hypothetical stages for the gradual evolution of (−)-iridomyrmecin from a defensive compound via a semiochemical cue to a female sex pheromone in *L. heterotoma* as suggested by our experimental data. (**a**) (−)-iridomyrmecin is used as a defensive compound by females of *L. heterotoma* and *L. boulardi* (**b**) (−)-iridomyrmecin is leaking from the reservoir in small amounts in non-defensive contexts. Females of *L. heterotoma* use (−)-iridomyrmecin as a semiochemical cue to avoid competition with con- and heterospecific females. (**c**) Males of *L. heterotoma* use (−)-iridomyrmecin as a semiochemical cue to locate females. At this ancestral stage, males are not yet able to discriminate between the species based on the chemical cue. (**d**) Chemical ritualization turned the cue into a signal. (−)-iridomyrmecin and minor components are actively released by virgin females. The relative composition of (−)-iridomyrmecin and the minor components increases the reliability of the signal and creates a species-specific signal. Males of *L. heterotoma* discriminate between species and show courtship behaviour only towards conspecific females.

through behavioural adaptations on the receiver's side, making (−)-iridomyrmecin at least a cue.

Furthermore, the competition avoidance is mediated across species' borders. *Leptopilina heterotoma* females not only avoid conspecific competitors, but also heterospecific ones (*L. boulardi* in this study, *L. clavipes* in Janssen et al. (1995a)). These results are consistent with the fact that all three species occur sympatrically in Western Europe and share the same hosts (Hedlund et al., 1996; Pannebakker et al., 2008). Although there has been selection increasing the specificity of the cue/signal, there has been no selection for species-specificity in the response to the avoidance cue as responding females benefit by avoiding patches already exploited by females of either species.

The same mechanism that allows females to avoid other females would allow males to find them (fig. 2.7c). However, at this hypothetical intermediate stage, (−)-iridomyrmecin as sole cue is a rather unreliable means of finding a mate. Males need to distinguish between species and sexes to find a suitable mate. Using only (−)-iridomyrmecin as a cue might lead males to approach and court a different species that also produces (−)-iridomyrmecin (for example, *L. boulardi*). However, in this situation the females benefit from a reliable mate-finding mechanism as well. Selection is thus predicted to increase signal quality and modify the signal through chemical ritualization. In addition, during the cue stage, males that possess and leak (+)-isoiridomyrmecin instead of (−)-iridomyrmecin would benefit from not being courted by other males, whereas males that possess and leak (−)-iridomyrmecin would presumably be hampered in their own courtship by other males mistaking them for females. This could very well explain the sex-specific use of (−)-iridomyrmecin and (+)-isoiridomyrmecin in *L. heterotoma*.

The third and last stage in the hypothetical evolutionary scenario outlined here is (−)-iridomyrmecin becoming a true pheromone (fig. 2.7d). Our results show that (−)-iridomyrmecin is the major component of the female sex pheromone in *L. heterotoma*. However, contrary to its function in defence and competition avoidance, (−)-iridomyrmecin needs to be accompanied by several minor components to be effective as a sex pheromone (fig. 2.3g), (−)-iridomyrmecin alone neither attracted males (fig. 2.3e) nor elicited courtship (fig. 2.6c). The minor components are (+)-isoiridomyrmecin (p6), the two iridodials (p1, p2) and the third iridomyrmecin (p7). Hence, all minor pheromone compounds are struc-

turally closely related to (−)-iridomyrmecin and most probably originate from the same biosynthetic pathway. Subtle modifications of existing biosynthetic pathways may change the quantitative composition of established blends or give rise to novel pheromone components. This might lead to messenger diversification, increased signal specificity and even speciation (for example, (Roelofs and Rooney, 2003; Niehuis et al., 2013)). In the case of *Leptopilina*, the evolution of novel iridoid compounds allowed a diversification of the signal without hampering the original defensive function of (−)-iridomyrmecin.

A second adaptation on the sender's side is that virgin females release their sex pheromone actively (fig. 2.6b). We interpret the additional use of several minor components and the active release as measures of signal amplification (Steiger et al., 2011) to transform the former cue into a true and reliable signal. As the males are attracted only towards the complete pheromone blend, they have clearly adapted as well. Moreover, not only do they react to the minor components, but they also discriminate between enantiomers of iridomyrmecin. (figs. 2.3g–2.3h). The fact that 1/40 of a female equivalent (containing ~8 ng of (−)-iridomyrmecin) was still attractive in the y-tube assays demonstrates the high sensitivity of the olfactory receptors and the high degree of adaptation by males to the signal.

The increased complexity and specificity of the sex pheromone compared to the hypothetically preceding much simpler cue illustrates the chemical ritualization necessary for the evolution of highly specific signals (Steiger et al., 2011). At the level of mate attraction, males are attracted by *L. heterotoma* as well as *L. boulardi* females (fig. 2.3a, fig. 2.6a), only at close range do they discriminate conspecific and heterospecific females (fig. 2.6c). *Leptopilina heterotoma* males may be attracted towards *L. boulardi* females, but they will discriminate con- and heterospecific females during courtship. Our results show that this species-specificity is mediated by the iridoid compounds and unlike in other parasitic wasps (Ruther, 2013), CHCs are not required for species recognition. As both species produce the same iridoid compounds but in different ratios, we think that these quantitative differences are responsible for species recognition and can only be reliably detected at close range. Future selection processes might favour the evolution of a long range discrimination mechanism for the males.

The present study sheds new light on the chemical communication of insects by demonstrating how communicative semiochemicals evolve. Our data show the pre-adaptations that make defence chemicals likely candidates to become informative: owing to their primary function, they are available and perceivable. However, physiological adaptations are necessary on both the sender's and the receiver's side to fulfil the increasing demands with respect to specificity and reliability when defence chemicals evolve into cues and signals. The *Leptopilina* wasps studied here demonstrate that diversification of the chemical messengers and chemosensory adaptation to the modified information are a possible way for reliable and specific information transfer. Understanding the genetic and biochemical mechanisms underlying these processes will be a challenging task for future studies.

Methods

Insects. We reared *L. heterotoma* and *L. boulardi* using *D. melanogaster* as host species. *Drosophila melanogaster* was reared on a corn-based diet (504 ml water, 66 g sugar, 6 g baker's yeast, 2.3 g agar, 52 g cornmeal, 1.3 ml propanoic acid, 0.8 g nipagin) and kept at 25 °C, ~75 % humidity, and a 168 h L:D cycle. For each rearing, about 30 flies (mixed sexes) were placed into a jar containing fresh fly food. After 48 h, the flies were removed and ~10 mated *L. heterotoma* or *L. boulardi* females were put into the jar. Parasitized pupae were removed from the jars a few days before emergence and put singly into 1.5 ml microcentrifuge tubes to obtain unmated an naive wasps of known age.

Extraction and fractionation of compounds. We extracted wasps for 10 min in 5 µl dichloromethane (DCM) per wasp. To separate iridoid compounds from CHCs, we fractionated the extract either by solid-phase extraction as described or using SEC (Kühbandner et al., 2012), which proved to be the more convenient method.

For fractionation by solid-phase extraction, the DCM of the sample was evaporated completely under a gentle stream of nitrogen, and the sample was redissolved in hexane. Cyanopropyl-bonded silica gel columns (50 mg, DSC-CN, SigmaAldrich, Taufkirchen, Germany) were pre-conditioned by rinsing them with 2 ml each of DCM and hexane; subsequently, the sample was applied to the column and eluted successively with 0.3 ml of hexane and 0.3 ml of DCM. The concentration of the fractions was determined by GC-MS and re-adjusted to the concentration of the original extract.

For fractionation using SEC, we evaporated the solvent from the extract of 60 wasps, using a stream of nitrogen, reducing the sample volume to 25 µl. We then injected the sample onto a PLgel SEC column (300 mm x 7.5 mm, particle size 5 µm, pore size 100 Å, Agilent Technologies, Waldbronn, Germany) using a Rheodyne model 7125 HPLC injector equipped with a 25 µl sample loop (Rheodyne, Cotati, CA, USA). The column was connected to an LC-20AD HPLC pump (Shimadzu Europe, Duisburg, Germany) with

DCM as mobile phase at a flow rate of 1.00 ml s^{-1}. We collected two fractions: a fraction eluting between 6.55–7.30 min (SEC 1) and a fraction eluting between 7.30–8.40 min (SEC 2).

The SEC 1 fraction contained the same compounds as the hexane fraction obtained by solid-phase extraction, the SEC 2 fraction contained the same compounds as the DCM fraction (checked by GC-MS).

fig. 2.1a shows a total ion current chromatogram of *L. heterotoma* female extract and indicates how this extract was fractionated by the fractionation protocols described above. The female extract and the iridoid (DCM and SEC 2) fraction thereof contain (−)-iridomyrmecin (labelled "p5" in fig. 2.1b), (+)-isoiridomyrmecin ("p6" in fig. 2.1b) and the third stereoisomer of iridomyrmecin ("p7"), both iridodials ("p1" and "p2") and two unidentified compounds ("p3" and "p4"), whereas males possess only (+)-isoiridomyrmecin and traces of p1, p2, p3 and p4 (fig. 2.1c), but no (−)-iridomyrmecin (Stökl et al., 2012).

To investigate the role of compounds p1, p2, p3, p4 and p7 in the putative sex pheromone, we used preparative gas chromatography to isolate these compounds from the iridoid fraction. A Shimadzu GC-2010 gas chromatograph (Shimadzu Europe, Duisburg, Germany) equipped with a non-polar capillary column (RH5-ms, 30 m length, 0.32 mm inner diameter (i.d.), 0.25 µm film thickness, CZT, Kriftel, Germany) connected to an automated Prep 9000 fraction collection system (Brechbühler, Schlieren, Switzerland) and a Fisons GC-8000 series A equipped with a chiral cyclodextrin GC-column (BetaDEX 225, 30 m length, 0.25 mm i.d., 0.25 µm film thickness, Sigma-Aldrich, Taufkirchen, Germany) connected to a lab-made exhaustion apparatus were used for preparative gas chromatography. Carrier gas for both gas chromatographs was helium, and was employed either at a constant velocity of 50 cm s^{-1} (Shimadzu) or at a constant pressure of 1.2 bar (Fisons). The oven program for the Shimadzu GC-2010 started at 80 °C and the temperature was raised by 10 °C min^{-1} to 280 °C and held for 13 min. For the Fisons GC 8000, the oven program started at 100 °C and the temperature was raised by 10 °C min^{-1} to 200 °C and kept there for 5 min. On both systems we trapped the compounds of interest on 10 mg Carbotrap filters (Brechbühler, Schlieren, Switzerland) and eluted them with 80 µl DCM.

To obtain a natural sample of chrysomelidial as precursor for the production of iridodials, we extracted thirty larvae (L3) of *P. cochleariae* (Coleoptera: Chrysomelidae) with 500 µl methanol for 1 h. The larval defensive secretion of *P. cochleariae* contains only (3S,8S)-chrysomelidial ("chrysomelidial") as major component and minor amounts of (3S,8R)-chrysomelidial ('epi-chrysomelidial') (Pasteels et al., 1982).

Synthetic compounds. Synthetic reference samples of (+)- and (−)-iridomyrmecin and (+)-isoiridomyrmecin were synthesized in a diasteroselective manner from optically pure citronellal (Stökl et al., 2012). Briefly, citronellal was first converted to nepetalactol. Conversion of nepetalactol into iridomyrmecin and isoiridomyrmecin was accomplished by ionic hydrogenation, hydroxymercuration/demercuration, and silver(I) oxidation (Stökl et al., 2012). Final purification was accomplished by preparative GC on the BetaDEX 225 column as described above.

Hydrogenation of (epi-)/chrysomelidial to iridodial. To obtain iridodial stereoisomers as reference compounds, we subjected (epi-)/chrysomelidial to catalytic hydrogenation (fig. A.4). We added a spatula tip of a Pd/C catalyst (Sigma-Aldrich, Taufkirchen, Germany) to the methanol solution of (epi-)/chrysomelidial and exposed the mixture to a weak hydrogen flow for 2 min. To remove Pd/C from the solution, we centrifuged the sample for 2 min at 8000 rpm and then transferred the supernatant to a new vial.

Chemical analysis. (−)-iridomyrmecin and (+)-isoiridomyrmecin were identified in *L. heterotoma* in a previous study (Stökl et al., 2012). We used the same methods to identify the minor components p1, p2 and p7 as well as the iridodials obtained by hydrogenation of chrysomelidial, but instead of the BetaDEX 225 column we used a Gamma DEX 120 column (30 m length, 0.25 mm i.d., 0.25 µm film thickness, Sigma-Aldrich, Taufkirchen, Germany) for the identification of p7. Extracts of males and females of *L. boulardi* were analysed (including the identification of the absolute configuration of iridomyrmecin) by GC-MS. Compounds were identified by comparing the retention time (on polar, non-polar and cyclodextrin column) and mass spectra with those of reference compounds. The same methods were used to analyse the composition of the fractions obtained by solid-phase extraction or SEC.

Behavioural assays. We used a y-tube olfactometer to identify the competition avoidance agent and to investigate the composition of the female sex pheromone in *L. heterotoma*. To test the species-specificity of the chemical stimuli, we used the sympatric species *L. boulardi*. The y-tube was made from glass and rested at an angle of 30°, with the two arms pointing up the slope. The tube's inner diameter was 1.5 cm, the base and arms had a length of 6 cm and

9 cm, respectively. The two arms were oriented at an angle of 45°. An air pump (Fürguth, Tannheim, Germany) pumped humidified air at a combined rate of 150 ml min^{-1} into the tube's arms. The setup was illuminated from above by two neon tubes (8 W).

For all tests, we applied a sample to one arm and a control to the other arm. When testing extract/fractions/compounds, we always applied 1/10 female equivalent (which equals ~30 ng iridomyrmecin (Stökl et al., 2012)) or 30 ng of the synthetic compound in 2 µl solvent (test arm) or only 2 µl solvent (control arm) to small discs of filter paper. The paper discs were allowed to dry for 1 min and then placed in the upper end of each arm. To compensate for potential side preferences inherent to the experimental setup, we alternated sample and control arm regularly and turned the y-tube after every replicate. For each y-tube run, we carefully released a single individual into the y-tube's base. The test lasted for 5 min or until the individual passed a "decision line", which was marked in each arm 2 cm beyond the branching point. After every other test, the y-tube was rinsed with ethanol and water. We used each individual for only one test.

In the experiments conducted to identify the competition avoidance agent, we connected an Erlenmeyer flask to each of the y-tube's arms. Each flask contained an artificial host patch consisting of 5 g of *Drosophila* rearing substrate (incubated with dry yeast for 2 h at 35 °C) and 20 *Drosophila* larvae (L1 and L2). (Experiment 1.D is an exception to this, as we did not connect Erlenmeyer flasks to the setup, see table A.2.) Depending on the experiment, we put 5 mated females into the test arm's Erlenmeyer flask or discs of filter paper directly into the arms. In each experiment, we tested 7–10 d-old mated *L. heterotoma* females. To increase the number of responding females, these were allowed to lay eggs for 1 h directly before the tests. We conducted the tests 6 h after the start of the photophase, as females then show a peak in locomotor activity (Fleury et al., 1995). Each experiment was replicated 30 times. Detailed information for all conducted experiments is given in table A.2.

In the experiments conducted to identify the female sex pheromone, Erlenmeyer flasks were used only in experiment 2.A (table A.2). In all other experiments we placed the filter paper discs directly into the y-tube's arms. In each experiment, we tested 1–3 d-old naive *L. heterotoma* males. We conducted the tests 1–2 h after the start of the photophase. Each experiment was replicated 30 times. Detailed information for all conducted experiments is given in table A.2. We tested increasingly diluted samples of *L. heterotoma* female extract to determine the behavioural threshold for males. We used amounts equivalent to 1/5, 1/10, 1/20, 1/40, and 1/80 of a female equivalent (corresponding to ~60, 30, 15, 8 and 4 ng of iridomyrmecin).

To investigate the species-specificity of mate recognition in *L. heterotoma*, we observed the courtship behaviour of *L. heterotoma* males towards filter paper discs impregnated with the test compounds in an arena experiment. For each experiment a disc of filter paper (5 mm diameter) was placed on a glass plate and impregnated with the test compound (in the same concentrations as in the y-tube experiments). A naiven 1–4 d-old *L. heterotoma* male was placed next to the filter paper and both were covered with the lid of a glass Petri dish (6 cm diameter). The male was observed for 5 min and its location and courtship behaviour (wing fanning) recorded using the software "The observer XT 9". We tested the iridoid fractions of an extract of females of *L. heterotoma* and *L. boulardi*, respectively, synthetic (−)-iridomyrmecin and DCM. Amounts were equivalent to 1/10 of one female. Each experiment was replicated 12 times.

The sample sizes of the behavioural experiments were chosen to be sufficient for the corresponding statistical test.

Headspace analysis. To compare the amount of iridomyrmecins released by virgin and mated females, we put 10 mated 2 d-old females in a 100 ml Erlenmeyer flask equipped with a gas wash bottle insert (data for virgin females were taken from(Stökl et al., 2012)). Air was pumped through the flask at a a rate of 60 ml min^{-1}. Incoming air was cleaned by an activated charcoal filter; the effluent air stream passed a thermal desorption filter filled with a combined Tenax-TA/Carboxen adsorbent material (Sigma-Aldrich, Taufkirchen, Germany). For quantification, 5 ng of methyl decanoate (dissolved in methanol) were applied to the adsorbent, and the solvent was removed before volatile sampling by purging the filter for 5 min in a stream of nitrogen at a flow rate of 60 ml min^{-1}. Filters were thermally desorbed (8 min at 250 °C) using a Shimadzu TD20 automated thermal desorption system connected to the Shimadzu GC-MS system, described above, and analysed with the same GC and MS settings. A calibration curve was created by analysing known amounts (1–100 ng) of synthetic (−)-iridomyrmecin that were applied to the filter together with the internal standard. The experiment was replicated 13 times.

Statistical analysis. Behavioural data from the y-tube experiments were tested with the two-sided binomial test. Headspace data were analysed using the Mann-Whitney U-test. Data from the arena experiment were analysed with the Kruskal-Wallis

test followed by pairwise Mann-Whitney U-tests with Bonferroni-Holm correction. All statistical tests were performed using R version 2.2.1 (R Core Team, 2013).

Acknowledgements

The authors thank Thomas Hoffmeister, University of Bremen, and Roland Allemand, Université Claude Bernard Lyon 1, for sendung us a starter culture of *L. heterotoma* and *L. boulardi*, respectively. This study was funded by the German Research Councol (Deutsche Forschungsgemeinschaft, DFG; grant STO 966/1-1 to J.S.).

Additional information

Supplementary information. The supplementary information originally published at `http://www.nature.com/naturecommunications` is included in appendix A.

Competing financial interests. The authors declare no competing financial interests.

3. Mating frequency and post-mating attractiveness of *Leptopilina heterotoma* females

Solitary parasitic Hymenoptera are generally considered to be monandrous. Several authors reported that this is also the case in *Leptopilina heterotoma*, and that mated females are no longer attractive to males. Recent work on the defensive secretion and the female mate attraction pheromone of *L. heterotoma*, however, suggests that *L. heterotoma* mated females continue to release sex pheromones and thus attract males. Previous reports on post-mating attractiveness and mating frequency in *L. heterotoma* females also rather relied on incidental observations than on dedicated experiments.
To clarify mating frequency and post-mating attractiveness, an experimental setup is designed in which the response of naive males towards virgin and mated females is observed on several consecutive days.
Leptopilina heterotoma females are found to be indeed monandrous, as previously reported. Mated females, however, still elicit courtship in males and are thus attractive, contrary to what has been reported previously.

Introduction

Choosing a suitable mate is one of the most important tasks in the life of almost all animals, as the mate's quality directly and indirectly influences the fitness (Davies et al., 2012). The mate has to be fertile so offspring will be produced and the mate should possess traits that are advantageous for the offspring. In many insect species, one or both sexes will mate multiple times. Multiple matings can increase the female's fitness by up to 70 % (Arnqvist and Nilsson, 2000), and can thereby mitigate the negative effects of a badly chosen mate. If one sex, most commonly females, mates only once, however, mate choice is of utmost importance, as all the offspring will carry the mate's traits. Consequences are even worse if the chosen mate cannot reproduce. If e. g. the female sex mates only once, her fitness will be heavily reduced when the male that is accepted as mate cannot produce viable offspring. In the extreme, not a single offspring will be produced. This will happen when the mate is either sterile or a heterospecific that cannot interbreed. This fact is even exploited in pest control in the so-called 'sterile insect technique', in which large numbers of sterilized males are released into a population to mate with the females and to thereby reduce the population's offspring (Klassen, 2005).

The effects of a female choosing an infertile or incompatible mate can sometimes be mitigated. Most Hymenoptera e. g. reprocude by partial parthenogenesis, predominantly by arrhenotoky (unfertilized eggs will produce males) but sometime also by thelytoky (unfertilized eggs will produce females) (Heimpel and de Boer, 2008). In such a scenario, the female will still produce offspring, but only of one sex. The fitness might be reduced, but not entirely nullified. In an arrhenotokous species, however, a phenomenon called 'local mate competition' can further reduce the fitness resulting from a mating with an infertile or incompatible mate. Under local mate competition, females achieve the greatest fitness if they do not produce more males than necessary to ensure the fertilization of all daughters (Hamilton, 1976). Producing only sons will thus result in a fitness loss.

While mate choice is more important and has greater consequences in a species and sex that mates only once, monandry has its own advantages. Many species invest heavily into reproduction. These investments include the production of signals to attract the opposite sex, to be recognized by the mate, and to elicit courtship rituals. In parasitic Hymenoptera, sex pheromones are employed at all these three levels of sexual communication (Ruther, 2013). These pheromones are often costly to produce, and time spent searching or choosing a mate cannot be spent otherwise, e. g. foraging. If the advertising sex mates only once, it would thus be of advantage to stop signalling to the opposite sex after mating. A post-mating loss

of female attractiveness has been observed in many species and happens on an intermediate to short time scale after the mating. E. g. in the European web-spinning larch sawfly, *Cephalcia lariciphila*, females become unattractive to males within as few as 10 min after mating (Borden et al., 1978).

Parasitic wasps are—as are most Hymenoptera—mostly arrhenotokous (Cook, 1993) and females from most (solitary) species mate only once (Ridley, 1993). Additionally, local mate competition is a common phenomenon. This is also true for my study organism, *Leptopilina heterotoma* (Debout et al., 2002). *Leptopilina heterotoma* is a solitary larval parasitoid of *Drosophila* larvae, including *D. melanogaster* (Jenni, 1951; Hedlund et al., 1996). *Leptopilina heterotoma*—and other *Leptopilina* species—have been studied intensively (for a review see Fleury et al. 2009) over the past years. In a recent paper, we have shown that females utilize a sex pheromone to attract males and we have identified the five components of the pheromone (Weiss et al., 2013). These five components can also be found in the defensive secretion released by *L. heterotoma* females, and the major component, (−)-iridomyrmecin, has been demonstrated to be repellent towards ants (Stökl et al., 2012). Additionally, (−)-iridomyrmecin is used as a competition avoidance agent (Weiss et al., 2013) in host-searching *L. heterotoma* females. Releasing (−)-iridomyrmecin as a part of the sex pheromone to attract males thus results in less (−)-iridomyrmecin being available for competition avoidance and defence. Females should thus stop emitting their sex pheromone after mating to save resources and to avoid being molested by courting males. In fact, van den Assem (1968) reported that mated *L. heterotoma* females lose their attractiveness to males and Stökl et al. (2012) demonstrated that mated *L. heterotoma* females release less iridoids than virgin females. However, all sex pheromone components were found in the headspace of mated females, which are thus potentially attractive to males. I therefore investigate the post-mating attractiveness of mated *L. heterotoma* females. Because we assume that mated females would still be attractive, we also investigate whether females indeed mate only once. The goal of our work is to answer the following specific questions:

1. Are mated *L. heterotoma* females still attractive to conspecific males?
2. Do mated *L. heterotoma* females mate a second time?

Material & methods

Insects. We reared *L. heterotoma* using *D. melanogaster* as host species. *Drosophila melanogaster* was reared on a corn-based diet (504 ml water, 66 g sugar, 6 g baker's yeast, 2.3 g agar, 52 g cornmeal, 1.3 ml propanoic acid, 0.8 g nipagin) and kept at 25 °C, roughly 75 % humidity, and a 16:8 h L:D cycle. For each rearing, about 30 flies (mixed sexes) were placed into a jar containing fresh fly food. After 48 h, the flies were removed and about 10 *L. heterotoma* (both sexes) were put into the jar. Parasitized fly pupae were removed from the jars before emergence and put singly into 1.5 ml microcentrifuge tubes to obtain naive and virgin wasps of known age.

Mating trials with virgin females. Single 2-d-old female *L. heterotoma* were introduced into an arena ($\varnothing = 55$ mm; $h = 8.5$ mm) containing a single virgin, naive, 1-d-old *L. heterotoma* male. The behaviour of both male and female was observed and recorded with the software The Observer XT 9.0 (Noldus, Wageningen, The Netherlands). Specifically, we recorded whether the male courted the female and whether the male and the female mated. The observation continued for 5 min and ended early when mating occurred. Each male was used only once. Females were given multiple successive opportunities to mate. The interval between two mating opportunities was 24 h, and females were given at most four mating opportunities. Females that mated were transferred to the mating trials with mated females (see below). 30 replicates were conducted.

Mating trials with mated females. *Leptopilina heterotoma* females that mated in the mating trials with virgin females (see above) were transferred to this second series of mating trials. These mating trials started 24 h after the female had mated and followed the experimental protocol described above. 26 replicates with the 26 females that had mated in the mating trials with virgin females were conducted.

Statistical analysis. The count data for courtship in the mating trials with mated females were analysed with *Fisher's exact test*. Although the same 26 females were used in the successive mating trials, the data can be regarded as indepent because (1) all females should have the same predisposition to either lose or retain attractiveness and (2) attractiveness was measured with a different male for each trial, i. e. independently (Petermeier, personal communication). The statistical analysis was performed using R version 3.1.1 (R Core Team, 2014).

Results

Attractiveness of mated females. Males readily courted mated females. We repeated the mating trials every 24 h for a total of four mating opportunities for already mated females to see whether a putative

loss of attractiveness might be time-dependent. The statistical analysis showed no significant influence of the time that had passed since the mating (*Fisher's exact test*: $p = 0.1327$; fig. 3.1).

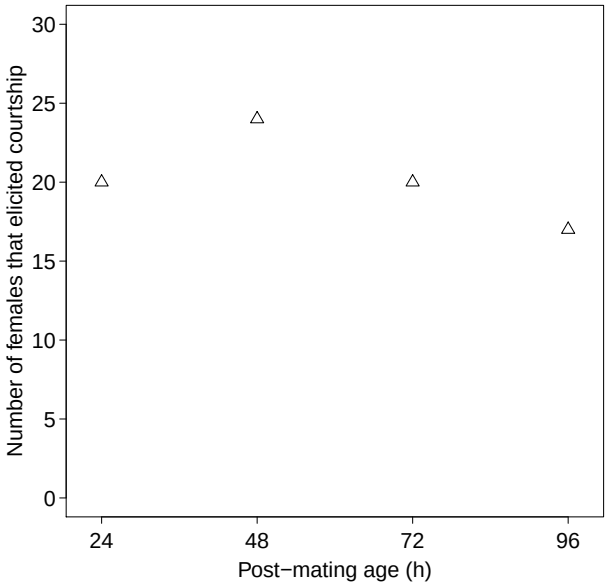

Figure 3.1.: Number of mated *L. heterotoma* females that elicited courtship in naive *L. heterotoma* males. Females were presented to new males every 24 h. No significant differences were found between females of different post-mating age (*Fisher's exact test*: $p = 0.1327$).

Female readiness to mate. Almost all (26/30) virgin females mated in our mating trials. Most of the females (22/26) had mated after the second mating opportunity. Opposed to that, not a single one of those 26 mated females mated for a second time in the four additional mating opportunities that each mated female was given (fig. 3.2).

Discussion

Our results clearly show, that *L. heterotoma* females mate only once. Mated females, however, are still attractive to males, which contradicts the findings of van den Assem (1968). In our experimental setup, the attractiveness of mated females was investigated for 4 consecutive days following the mating. In species in which the females' attractiveness ceases after mating, this loss happens on an intermediate (e. g. 24 h in the western corn rootworm beetle, *Diabrotica virgifera virgifera*; Hammack 1995) to short time scale (e. g. 10 min in the European web-spinning larch sawfly, *C. lariciphila*; Borden et al. 1978) after the mating. We thus assume that we would have detected a loss in attractiveness if it had existed.

Male courtship behaviour might not only cease because mated females lose their attractiveness to males. Mated females may also release antiaphrodisiac pheromones that inhibit male courtship behaviour. For Hymenoptera, Schiestl and Ayasse (2000) demonstrated this phenomenon in the solitary bee *Andrena nigroaenea* (Apoidea, Andrenidae), and Mowles et al. (2013) suggested that an antiaphrodisiac pheromone might exist in the parasitic wasp *Spalangia endius*. Schiestl and Ayasse found that only virgin females elicited courtship behaviour in males, but not mated females. They found that all-*trans*-farnesyl hexanoate and all-*trans*-farnesol were more abundant in cuticle extract of unattractive females and could demonstrate that synthetic farnesyl hexanoate and farnesol inhibit courtship behaviour in males. In *S. endius*, males prefer virgin over mated females (King et al., 2005; Mowles et al., 2013), and Mowles et al. suggested that female *S. endius* indicate their mating status by releasing methyl 6-methylsalicylate. Our data on *L. heterotoma*, however, does not suggest the existence of a female antiaphrodisiac pheromone in *L. heterotoma*.

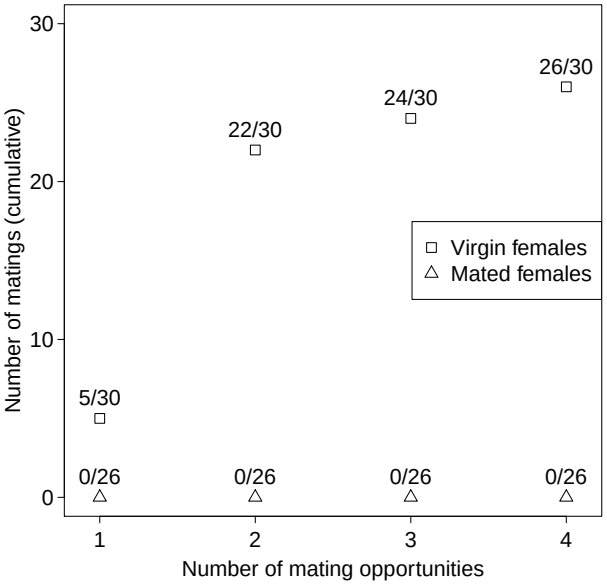

Figure 3.2.: Number of *L. heterotoma* females that mated after a given number of mating opportunities in mating trials with virgin (□) and mated (△) females. (Females in the mating trials with mated females had different numbers of mating opportunities in the mating trial with virgin females.) While most of the virgin females mated, none of the mated females mated for a second time.

While our experimental setup is suitable to show that mated females are still readily courted by males, it does not allow to infer the ecological implications of this courtship towards mated females. We did not investigate male behaviour in the presence of both mated and virgin females at the same time. In such a scenario, males might prefer the virgin female. Such preference of virgin females over mated females

has been described for several parasitic Hymenoptera, e. g. *S. endius* (King et al., 2005; Mowles et al., 2013). Further experiments are necessary to investigate whether virgin females are more attractive than mated females in *L. heterotoma*. Such experiments could possibly include the measurement of courtship intensity, e. g. male wing fanning duration, instead of a simple comparison of courtship frequencies.

In our experiments, males readily courted mated females, but never elicited receptiveness. Ruther et al. (2000) reported similar findings for the parasitic wasp *Lariophagus distinguendus*. In *L. distinguendus*, mated females elicited a strong wing fanning response even 5 d after the females had mated, but copulation never occurred. In fact, courtship elements other than wing fanning (i. e. mounting and antennal stroking) towards mated females were observed significantly less often than in courtship towards virgin females.

Courtship in *L. heterotoma* consists of a combination of three distinctinve behaviours (van den Assem, 1968): wing fanning (the male fans its wings at a high frequency), mounting (the male climbs on top of the female), and antennal stroking (the male rhythmically moves its antennae and touches the female antennae while on top of the female). When the male mounts the female, the female usually cooperates by remaining still and folding its antennae backwards, towards the male's head, regardless of the female's mating status (an observation already made by van den Assem). During courtship, the female is clearly hindered in its movements, and therefore the courted females can not search for hosts. Additionally, such sexual harassment by males may reduce the longevity and fitness of the harassed females. These costs of sexual harassment have been demonstrated in e. g. *Neacoryphus bicrucis* (Heteroptera: Lygaeidae) (McLain and Pratt, 1999), *Gryllus bimaculatus* (Orthoptera: Gryllidae) (Bateman et al., 2006), and *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) (Gay et al., 2009) . To lower the risk of sexual harassment, mated *L. heterotoma* females should thus clearly avoid attracting further males.

This raises the question why mated females are still attractive. Females will not mate a second time, so presumably they do not need to attract males. This attractiveness is possibly a negative side effect of the semiochemical parsimony found in *L. heterotoma*: females use the same compounds as sex pheromone, defensive secretion, and competition avoidance cue (Stökl et al., 2012; Weiss et al., 2013). Females that release iridoids to repel predators or reduce intraspecific competition for hosts will thus inevitably attract males. Semiochemical parsimony may thus not always be advantageous but may also imply costs.

In addition to the post-mating attractiveness, we found that females are monandrous. This was expected, as most solitary parasitic Hymenoptera are monandrous (Ridley, 1993). In monandrous species, mate choice is especially important for females. Accepting an inferior male as mate will reduce a female's fitness without the possibility to mitigate by mating with additional males. Females thus need to choose a mate as opposed to accepting any courting male. Our experimental setup, however, is not suited to determine whether female mate choice exists in *L. heterotoma*.

If females do indeed choose their mate, males need to communicate their quality to the females (Steiger and Stökl, 2014). Isidoro et al. (1999) demonstrated that antennal contact during courtship is necessary to elicit readiness to mate in *Leptopilina boulardi* females. They furthermore described male antennal glands in the 3rd and 4th antennomere, which are the antenommeres that touch the female antenna during antennal stroking. This suggests that males transfer a substance from the antennal glands onto the female antennae, which might enable females to assess the male quality as a mate. Our own findings (chapter 5) show that the transferred information is species specific, which corroborates the idea of a male antennal aphrodisiac pheromone in *L. heterotoma*. Antennal aphrodisiac pheromones have been proposed for a number of parasitic Hymenoptera (e. g. van den Assem et al. 1980; Isidoro and Bin 1995; Isidoro et al. 1999; Bin et al. 1999; Romani et al. 2005). However, to date, not a single male antennal aphrodisiac pheromone has been fully identified.

4. Species specificity and chemical diversity of mate recognition in *Leptopilina heterotoma*, *L. boulardi*, and *L. victoriae*

This chapter has been published as **Weiss, I., Hofferberth, J., Ruther, J., and Stökl, J. Varying importance of cuticular hydrocarbons and iridoids in the species-specific mate recognition pheromones of three closely related *Leptopilina* species. *Frontiers in Ecology and Evolution*, 3 (19), 2015. doi: 10.3389/fevo.2015.00019**.

Author contributions: I.W., J.R., and J.S. designed the study; I.W. performed the experiments; J.H. synthesized the compounds; I.W., J.H., J.R., and J.S. wrote the manuscript.

Finding a suitable mate for reproduction is one of the most important tasks for almost all animals. In insects this task is often facilitated by pheromone-mediated communication. While insect pheromones in general show enormous chemical diversity, closely related species often use structurally similar compounds in their pheromones. Despite this similarity, pheromones of congeneric species living in sympatry need to be species specific.

We investigated the pheromone-mediated mate recognition by males of three closely related species of *Leptopilina*, a genus of parasitoid wasps that utilize the larvae of *Drosophila* as hosts. The study species, *L. heterotoma*, *L. boulardi*, and *L. victoriae*, occur sympatrically and have a similar ecology and life history. We have found that mate recognition is species specific in all three species. This species specificity is achieved by a differing importance of cuticular hydrocarbons (CHCs) and iridoids in the female mate recognition pheromones. In *L. heterotoma* the iridoids are of major importance while CHCs play a negligible role. In *L. boulardi*, however, the CHCs are as important as the iridoids, while in *L. victoriae*, the CHCs alone elicit a full behavioral response of males.

Our results provide novel insights into pheromone evolution in insects by showing that selection on two completely different classes of chemical compounds may generate conditions where compounds from both classes contribute to a varying degree to the chemical communication of closely related species and that this variation also generates the species specificity of the signals.

Introduction

For successful reproduction sexual organisms need to find a suitable mate. This process can be divided into three distinct steps (Ruther, 2013): (1) mate attraction at long range, (2) mate recognition at short range, and (3) courtship and elicitation of receptiveness. Signals perceived by any sensory modality can be involved in all three steps, but in insects it is often sex pheromones that are crucial for these steps. Mate recognition requires high species specificity to ensure that individuals do not erroneously invest resources in courtship without potential for successful reproduction.

Species-specific sex pheromones enable insects to recognize conspecifics with a high reliability and to date, over 1500 chemical compounds used as sex pheromones are known (El-Sayed, 2014). This large number of compounds is accompanied by an impressive diversity in pheromone composition, which ranges from a single compound to a dozen or more pheromone components (Wyatt, 2014), and includes compounds from many different chemical classes. However, species from the same genus typically use structurally similar compounds in their pheromone communication (Wyatt, 2014), as has been found in numerous studies for instance in Lepidoptera, Cole-

optera, and Hymenoptera (Hardie and Minks, 1999; Ando et al., 2004).

Species-specific sex pheromones enable individuals to recognize conspecifics with high reliability, even if closely related heterospecifics occur within the same time and location, which can easily happen in sympatric species with similar ecology. In parasitoid wasps, species from the same genus may share the same host genus if not host species (e. g. *Leptopilina*; Nordlander 1980) and in microsympatry virgin males and females of different species might even emerge from the very same host (e. g. *Nasonia*; Grillenberger et al. 2009). In the jewel wasp genus *Nasonia*, the female cuticular hydrocarbon (CHC) profile is attractive to males (Steiner et al., 2006; Buellesbach et al., 2013). Buellesbach et al. showed by multivariate statistical methods that the female CHC profiles are chemically distinguishable in all four *Nasonia* species. Pre-zygotic reproductive isolation, however, is incomplete in *Nasonia* as interpecific matings can be regularly observed (Buellesbach et al., 2014). This is surprising, as pre-zygotic reproductive isolation would probably prevent fitness losses due to very effective post-zygotic reproductive isolation caused by *Wolbachia* (Saul, 1961; Breeuwer and Werren, 1990). Females that mate with a heterospecific male can only produce sons, but no daughters, because of the haplodiploid sex determination in Hymenoptera (Cook, 1993; Heimpel and de Boer, 2008). *Wolbachia* infection frequently occurs in insects (Hilgenboecker et al., 2008) and has been described in several parasitic hymenopterans (*Trichogramma*: Pintureau et al. 1999, 2002; *Nasonia*: Breeuwer and Werren 1990; Bordenstein and Werren 2007; *Leptopilina*: Fleury et al. 2000; Gueguen et al. 2012). The resulting post-zygotic reproductive isolation combined with the fact that most (solitary) parasitoid hymenopterans are monandrous (Ridley, 1993), should drive the evolution of a strong pre-zygotic reproductive isolation. Thus, hymenopteran parasitoids are excellent model organisms to study how pre-zygotic reproductive isolation through highly specific sex pheromones evolves and is maintained in sympatric species with similar ecologies.

In a recent study (Weiss et al., 2013), we have identified the female sex pheromone responsible for mate attraction in *Leptopilina heterotoma*, a larval parasitoid of *Drosophila*. The pheromone consists of five iridoid compounds: (−)-iridomyrmecin, (+)-iridomyrmecin, a third stereoisomer of iridomyrmecin, and two iridodials. (−)-iridomyrmecin, the major component of the pheromone, is also used for chemical defense in *L. heterotoma* (Stökl et al., 2012), and seems likely to have been the starting point for the evolution of the sex pheromone (Weiss et al., 2013). Apart from iridoids, we also considered CHCs as candidate pheromone components in *L. heterotoma*. CHCs did not attract males of *L. heterotoma* in y-tube experiments (Weiss et al., 2013), but a possible role in courtship is yet to be investigated.

The genus *Leptopilina* comprises 30 described species and has a worldwide distribution (Nordlander, 1980; Quinlan, 1988; Nordlander and Grijpma, 1991; Allemand et al., 2002; Novkovic et al., 2011; Forshage et al., 2013). All *Leptopilina* species investigated so far parasitize larvae of *Drosophila* and have a similar ecology and life history (Nordlander, 1980; Allemand et al., 2002; Novkovic et al., 2011).

In our previous study (Weiss et al., 2013) we have shown that males of *L. heterotoma* were also attracted by female-derived extracts of the sympatric species *L. boulardi*. At close range and upon contact, however, *L. heterotoma* males did not respond to iridoid extracts from *L. boulardi* females, while those from conspecific females elicited courtship behavior. The species specificity of the mate recognition pheromone in *L. boulardi* males, however, has not yet been investigated. *Leptopilina heterotoma*, *L. boulardi*, and a third closely related species, *L. victoriae*, have overlapping distribution (Allemand et al., 2002; Novkovic et al., 2011) and thus it is reasonable to expect species-specific mate recognition pheromones in these species. Male courtship in *Leptopilina* consists of several distinct behaviors that can be easily identified (van den Assem, 1968). Males typically start to show wing-fanning, a high-frequency vibration of the wings, as soon as they recognize an attractive female. Females are then followed and touched with the antennae, which is followed by mounting. After mounting the female, males start antennal stroking, moving their antennae in a circular pattern, thereby bringing their own proximal antennomeres into contact with the female's distal antennomeres in a rhythmical fashion. Wing fanning is usually maintained throughout courtship and stops only when copulation occurs or courtship is abandoned (van den Assem, 1968).

Due to their similar ecology, we expected all three species to produce iridoids and employ these in mate recognition. To ensure species specificity, however, it stands to reason that the composition of the iridoid profiles should differ significantly between the species or iridoid signals should be modulated by interaction with other pheromone chemicals such as CHCs.

In this study, we compare the sex pheromones responsible for mate recognition in *L. heterotoma*, *L. boulardi*, and *L. victoriae*. In an approach that combines chemical analysis and behavioral assays, we asked the following questions:

1. Is mate recognition species-specific in the three species?

2. Are iridoids produced by and used in mate re-

cognition in *L. boulardi* and *L. victoriae*?

3. Do CHCs play a role in mate recognition in the three species?

Material & methods

Insects. We reared all three wasp species on *Drosophila melanogaster* hosts. The flies were reared on a corn-based diet (504 ml water, 66 g sugar, 6 g baker's yeast, 2.3 g agar, 52 g cornmeal, 1.3 ml propanoic acid, 0.8 g nipagin). Both flies and wasps were kept at 25 °C, about 75 % humidity and a 16:8 h L:D cycle. For each rearing, about 30 flies of both sexes were put into a jar containing the freshly prepared diet. After 48 h, the flies were removed from the jar and about 10 mated females of either *L. heterotoma*, *L. boulardi* or *L. victoriae* were introduced to parasitize the fly larvae. Several days before emergence (about 3 weeks after oviposition), parasitized fly pupae were identified by their dark coloration and removed from the jars and put singly into 1.5 ml microcentrifuge tubes. The isolated pupae were screened daily for emerged wasps. In this way, unmated and naive wasps of known age were obtained. Emerged wasps were kept individually in the microcentrifuge tubes until they were used in an experiment. Each individual wasp was used for a single experiment only.

Species specificity of courtship. To determine the species specificity of the male courtship behavior, 1-d-old naive females of each species were presented to 1-d-old naive males of each species. For each trial, a female was carefully placed into a glass arena (15 mm diameter, 2 mm height). Shortly thereafter, a single male was introduced into the arena, which was then covered with a glass lid. Male behavior was recorded as digital video for 2 min and afterwards the total wing fanning duration of responding males was determined with the video module of the scientific observation software The Observer XT 11.0 (Noldus, Wageningen, The Netherlands). Wing fanning in *Leptopilina* consists of both continuous sequences as well as as intermittent bouts of wing vibration (personal observation). Thus, high frequency wing vibrations of any length were classified as wing fanning. The duration of the experiment was chosen according to our previous wing fanning experiments, which lasted 5 min and in which wing fanning was only rarely observed after 2 min. After each replicate the used arena was rinsed with ethanol and left to dry at room temperature. Each combination of species was tested 20 times.

Pheromone extraction and fractionation. To test whether the courtship behavior is elicited by pheromones and to disentangle the contribution of iridoids and CHCs to the pheromone function, we extracted female wasps of either species for 10 min in 5 µl dichloromethane (DCM) per wasp. To separate iridoid compounds from CHCs, we fractionated the extract either by solid-phase extraction (SPE; samples from *L. heterotoma* and *L. boulardi*) or size-exclusion chromatography (SEC; samples from *L. victoriae*), following the method of Kühbandner et al. (2012). Both SPE and SEC resulted in the same fractionation, i. e. an iridoid fraction and a CHC fraction. We switched from SPE to SEC to avoid the additional step of drying and redissolving the hexane fraction for bioassays (see below), which was required after the SPE fractionation.

Prior to SPE, the raw extracts were dried under a stream of nitrogen and the samples were then redissolved in 50 µl hexane. Cyanopropyl-bonded silica gel columns (50 mg, DSC-CN, Sigma-Aldrich, Taufkirchen, Germany) were pre-conditioned by rinsing them with 2 ml each of DCM and hexane. Then, the samples were applied to the column and eluted with 300 µl hexane followed by 300 µl DCM. Between elution with hexane and elution with DCM, the column was flushed with additional 300 µl hexane. The hexane fractions contained the CHCs and the DCM fraction contained the iridoids. For bioassays, hexane fractions were carefully dried under nitrogen and then redissolved in DCM.

Prior to SEC, raw extracts were reduced to about 25 µl under nitrogen. The samples were then injected onto a PLgel SEC column (300 mm x 7.5 mm, particle size 5 µm, pore size 100 Å, Agilent Technologies, Waldbronn, Germany) using a Rheodyne model 7125 HPLC injector equipped with a 25 µl sample loop (Rheodyne, Cotati, CA, USA). The column was connected to an LC-20 AD HPLC pump (Shimadzu Europe, Duisburg, Germany) with DCM as mobile phase at a flow rate of 1.00 ml min^{-1}. Two fractions were collected: fraction SEC 1, eluting between 6.75–7.17 min, and fraction SEC 2, eluting between 7.50–8.00 min. SEC 1 contained the CHCs and SEC 2 contained the iridoids.

The composition of all fractions was analyzed by GC-MS (see below). The concentration of fractions and extracts was adjusted to 1 female equivalent per 5 µl for chemical analysis and to 1 female equivalent per 20 µl for behavioral experiments.

Chemical analysis. Extracts and fractions were analyzed on a GC2010 gas chromatograph (GC) connected to a QP2010 plus mass spectrometer (MS; both Shimadzu, Duisburg, Germany). The GC was equipped with a non-polar capillary column (BPX-5, 30 m length, 0.25 mm inner diameter (i.d.), 0.25 µm film thickness; SGE Analytical Sciences, Milton

Keynes, UK). Helium was used as carrier gas with a constant linear velocity of 50 cm s^{-1}. The temperature of the GC oven started at 80 °C and was raised by 5 °C min^{-1} to 280 °C, where it was kept for 20 min. The MS was run in electron impact (EI) mode at 70 eV and set to a scan range from 35–600 mz^{-1}. Sample volumes of 1 µl were injected splitless at an injector temperature of 280 °C. For the enantioselective analysis of iridoids, the GC was equipped with a chiral β-cyclodextrin column (Beta DEX 225, 30 m length, 0.25 mm i.d., 0.25 µm film thickness; Sigma-Aldrich, Taufkirchen, Germany). For these analyses, the GC oven temperature started at 80 °C and was raised by 6 °C min^{-1} to 200 °C, where it was kept for 20 min. The MS settings were as described above. Sample volumes of 1 µl were injected splitless at an injector temperature of 200 °C.

Iridoids. The iridoids produced by female *L. heterotoma* have been identified in previous studies (Stökl et al., 2012; Weiss et al., 2013). These iridoids are (−)-iridomyrmecin, (+)-isoiridomyrmecin, a third iridomyrmecin of unknown absolute configuration, and two stereoisomers of iridodial, with (−)-iridomyrmecin making up about 80 % of the pheromone. *Leptopilina boulardi* females also possess these iridoids, albeit in different ratios (Weiss et al., 2013). Iridoids in *L. victoriae* were identified by comparing mass spectra and retention indices on both the non-polar and the cyclodextrin column to those of the *L. heterotoma* iridoids. Additionally, (+)-iridomyrmecin and (−)-iridomyrmecin as well as (+)-isoiridomyrmecin and (−)-isoiridomyrmecin were used as synthetic references (Fischman et al., 2013). Compounds that contributed less than 0.5 % to the total amount of iridoids were not considered.

Cuticular hydrocarbons. The *n*-alkanes in females of all three *Leptopilina* species were identified by comparing mass spectra and retention indices to those of synthetic reference compounds. Methyl-branched hydrocarbons were identified by interpretation of diagnostic ions resulting from the favored fragmentation at the branching points (Nelson, 1993) and comparison of linear retention indices with literature data (Carlson et al., 1998). Double bond positions of unsaturated compounds were identified by derivatization with dimethyl disulfide (DMDS; Carlson 1989). Derivatized samples were analyzed on the non-polar column as described above with a modified temperature program (final temperature 300 °C for 178 min) and scan range 35–800 m/z. Compounds that contributed less than 0.5 % to the total amount of CHCs were not considered.

Quantification. For quantification of both iridoids and CHCs, single females were extracted in 15 µl DCM, containing 5 ng $µl^{-1}$ methyl decanoate as an internal standard. GC-MS analyses were carried out with the non-polar column, as described above. A separate calibration curve (1–50 ng each) was established for iridoids and CHCs assuming that response factors would differ little within each structural class. For iridoids, we establised a calibration curve using (+)-iridomyrmecin as the standard. Hydrocarbons were quantified using *n*-tricosane as the standard. Quantification of the iridoids and CHCs was based on 10 individuals from each species.

Pheromone bioassays. Extracts from females and fractions thereof were tested for their ability to elicit wing fanning in conspecific males. For this purpose, 2 µl of extracts, fractions (equivalent to one 10th of a female) or the pure solvent control were applied to a small disc (5 mm diameter) of filter paper. The filter paper was then placed in an arena (dimensions as describe above for *L. boulardi* and *L. victoriae*; 55 mm diameter, 8.5 mm height for *L. heterotoma* (we used a bigger arena for *L. heterotoma* because only few *L. heterotoma* males had responded in preliminary pheromone bioassays in the smaller arena)) and the solvent was allowed to evaporate for 1 min. After solvent evaporation, a naive 1-d-old male was introduced into the arena which was then covered with a glass lid. The male's behavior was recorded as digital video for 2 min. After each replicate the used arena was rinsed with ethanol and left to dry at room temperature. Afterwards, the video files were analyzed with the video module of The Observer XT 11.0 to measure the wing fanning duration of responding males ($n = 20$ for *L. boulardi* and *L. victoriae*; $n = 25$ for *L. heterotoma*; sample size for *L. heterotoma* was increased because males had responded less frequently in the preliminary pheromone bioassays).

Statistical analysis. Wing fanning duration was analyzed using the *Kruskal-Wallis* test, followed by pairwise comparisons with the *Mann-Whitney* U-test with *Bonferroni-Holm* correction. Wing fanning duration was only compared within a species but not between species. All statistical analyses were performed using R 3.1.0 (R Core Team, 2014).

Results

Species specificity of courtship. In all three *Leptopilina* species male courtship behavior elicitation was shown to be species specific as demonstrated by significantly increased wing fanning durations towards conspecific females (fig. 4.1). Statistical details are given in table B.1. Although males of all three species showed short wing fanning bouts towards heterospe-

cific females, males very rarely tried to copulate and no interspecific matings were observed. In intraspecific trials, matings were regularly observed.

Fractionation. Fractions obtained from SPE and SEC, respectively, were analyzed with GC-MS to ensure that the fractions contained only the expected compounds. The analyses confirmed that the hexane and DCM fractions from the SPE fractionation contained the CHCs and iridoids, respectively. For SEC fractions, the analyses showed that the SEC1 fraction contained the CHCs and that the SEC2 fraction contained the iridoids. For simplification, the DCM fraction (SPE) and the SEC2 fraction will be referred to as 'iridoid fraction', and the hexane fraction (SPE) and the SEC1 fraction will be referred to as 'CHC fraction'.

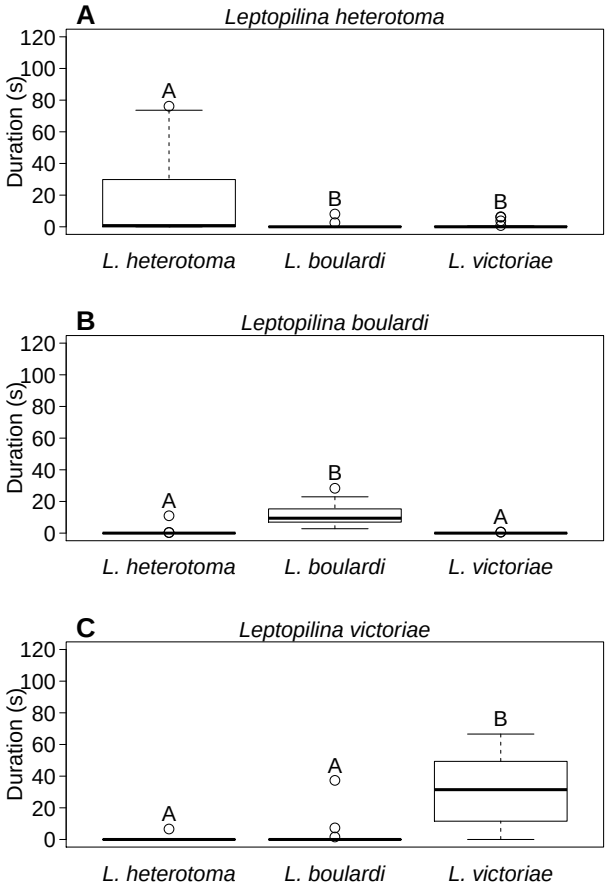

Figure 4.1.: (**A**) Total duration of wing fanning displayed by *L. heterotoma* males towards con- and heterospecific females ($n = 20$). (**B**) Total duration of wing fanning displayed by *L. boulardi* males towards con- and heterospecific females ($n = 20$). (**C**) Total duration of wing fanning displayed by *L. victoriae* males towards con- and heterospecific females ($n = 20$). Different letters indicate significant differences between median values at $p < 0.05$ (*Mann-Whitney* U-test with *Bonferroni-Holm* correction); comparisons were only made within but not between male species.

Chemical analysis

Iridoids. The major iridoid compound found in *L. heterotoma* and *L. boulardi* females is (−)-iridomyrmecin, whereas extracts from *L. victoriae* females contained (+)-iridomyrmecin as the major compound (table B.2). Extracts from all three species contained (+)-isoiridomyrmecin, two stereoisomers of iridodial, and a third iridomyrmecin stereoisomer of unknown absolute configuration (for more details on the structure of the latter three compounds see Weiss et al. (2013)). In addition to the mentioned iridoids, extracts from *L. boulardi* and *L. victoriae* females contained some additional putative iridoids. The total amount of iridoids is lower in *L. victoriae* than in *L. heterotoma* and *L. boulardi* (table B.2). Overall, the iridoid profiles differ both qualitatively and quantitatively between the three species.

Cuticular hydrocarbons. The CHCs found in all *Leptopilina* species were mainly methyl-branched and mono- or di-unsaturated alkenes. The *n*-alkanes were found only in low amounts. While all three species share a number of *n*-alkanes, 4-methyl alkanes, and mono-unsaturated *n*-alkenes, each species was characterized by a number of species-specific CHCs (table B.3).

Pheromone bioassays. Raw extracts from conspecific females elicited wing fanning behavior in males from all three *Leptopilina* species, indicating the presence of courtship eliciting mate recognition pheromones. The relative contribution of iridoids and CHCs to the behavioral activity of the extracts, however, differed significantly between the three species.

In *L. heterotoma* the iridoids elicited the same degree of wing fanning as the raw extract. In contrast, CHCs were only slightly attractive and elicited significantly less intense wing fanning than the raw extract and the iridoids, respectively (fig. 4.2A; statistical details in table B.4).

In *L. boulardi*, significant wing fanning responses were elicited in males by both iridoids and CHCs, which did not differ in their behavioral activity. Both fractions alone, however, were less active than the raw extract, while the recombined fractions were as active as the raw extract (fig. 4.2B; statistical details in table B.5).

Both fractions elicited wing fanning behavior also in males of *L. victoriae* but the response to CHCs was significantly stronger in this species. When compared to the raw extract, CHCs elicited the same degree of wing fanning in males while iridoids were significantly less active (fig. 4.2C; statistical details in table B.6).

Discussion

Species specificity of courtship. Our results show that male courtship behavior in *L. boulardi*, *L. heterotoma*, and *L. victoriae* is species specific and that this specificity is accompanied by a high chemical diversity of the female courtship-eliciting pheromone.

Females of most (solitary) parasitic wasps mate only once (Ridley, 1993) while males can mate multiple times. This is also true for *Leptopilina*, and although heterospecific matings probably do not occur, hybridization of species would still be prevented by *Wolbachia*-mediated cytoplasmatic incompatibility (Fleury et al., 2000; Gueguen et al., 2012). Females that mate with a heterospecific male are still able to produce male offspring, because of the haplodiploid sex determination in Hymenoptera (Cook, 1993; Heimpel and de Boer, 2008), but nevertheless experience a massive fitness loss. This fitness loss is even greater in species with (partial) local mate competition (which includes *Leptopilina*; Debout et al. 2002), which usually produce female-biased sex ratios (Hamilton, 1976). For males the sperm transferred to a heterospecific female is lost without any reward. It stands to reason that accurate conspecific mating represents a fitness advantage and our courtship elicitation experiments with both conspecific and heterospecific females indicate that species recognition is indeed highly accurate. Even though heterospecific females elicited short bouts of wing fanning in some males, conspecific females were courted significantly longer. Some compounds (iridoids and CHCs) were identified in the extracts of females from all three investigated species. This overlap in the chemical profiles may very well explain the observed heterospecific courtship elicitation. However, we cannot exclude that other than chemical cues or signals, like visual or tactile, elicited the short courtship of males towards heterospecific females.

All males that courted heterospecific females failed to elicit female receptiveness and no heterospecific matings were observed. Most likely, a species-specific male aphrodisiac pheromone ensures that females do not accept heterospecific males as a mate (Isidoro et al., 1999). *Leptopilina heterotoma*, *L. boulardi*, and *L. victoriae* occur sympatrically and even share a common host, *D. melanogaster*. Thus, the putative species-specific male aphrodisiac pheromone is of great importance as it provides a second species border beyond the highly, but not completely, species-specific female courtship pheromone.

Females of the jewel wasp *Nasonia*, a parasitoid of fly pupae, also show a high rejection rate of heterospecific mates, while males showed very little discrimination against heterospecific mates (Buellesbach et al., 2014). But in contrast to *Leptopilina*, heterospecific matings do occur in *Nasonia*, despite the presence of a male aphrodisiac pheromone which is necessary to elicit female receptiveness (van den Assem et al., 1980). This indicates that the male aphrodisiac pheromone of *Nasonia* might not be species-specific. On the contrary, preliminary results of our own group suggest that the male aphrodisiac pheromones in *L. boulardi* and other *Leptopilina* species are indeed species specific. The phenomenon of male aphrodisiac pheromones is widespread in hymenopteran parasitoids (e. g. van den Assem et al. 1980; Isidoro and Bin 1995; Isidoro et al. 1999; Bin et al. 1999; Romani et al. 2005), however, to date, no such putative pheromone has been fully identified and more work is needed to understand the evolutionary background of those male pheromones.

Overall, we showed that male courtship is highly species specific in the three investigated *Leptopilina* species. We assume that the chemical profiles of the females alone are sufficient for males to distinguish con- and heterospecific females but further studies are needed to identify the exact cues used for species recognition in *Leptopilina*.

Composition of courtship pheromone. The results of the pheromone bioassays indicate that the three investigated *Leptopilina* species possess different courtship eliciting female sex pheromones. While this result was indicated by the species specific courtship of the males, it is surprising, how much the female sex pheromones of the three species differ in their chemical composition. All three species have the same two classes of chemical compounds, iridoids and CHCs, available, but use them to very different extents in their mate recognition pheromone. In *L. heterotoma*, the iridoids elicited full wing fanning and thus are of major importance, while the CHCs elicited almost no wing fanning and contribute only marginally to the sexual signal in this species. This is in concordance with previous results from y-tube experiments, in which *L. heterotoma* males were attracted by the female-derived iridoids, but not by the female CHCs (Weiss et al., 2013). In *L. boulardi* the relative importance of iridoids and CHCs is different from *L. heterotoma*. Both iridoids and CHCs elicited significant wing fanning responses in males, but significantly less than the crude extract. Only the combination of iridoids and CHCs elicited the full behavioral response in *L. boulardi* males. This means, that in *L. boulardi*, iridoids and CHCs both convey important information in sexual communication. The picture is yet different in *L. victoriae*: CHCs alone elicited the full wing fanning response in males; hence, the importance of CHCs in sexual communication is even greater than in *L. boulardi*. In *L. victoriae*, the iridoids elicited a weak but significant wing fanning

response when presented alone, but unlike in *L. boulardi*, the iridoids are not required for the full courtship response.

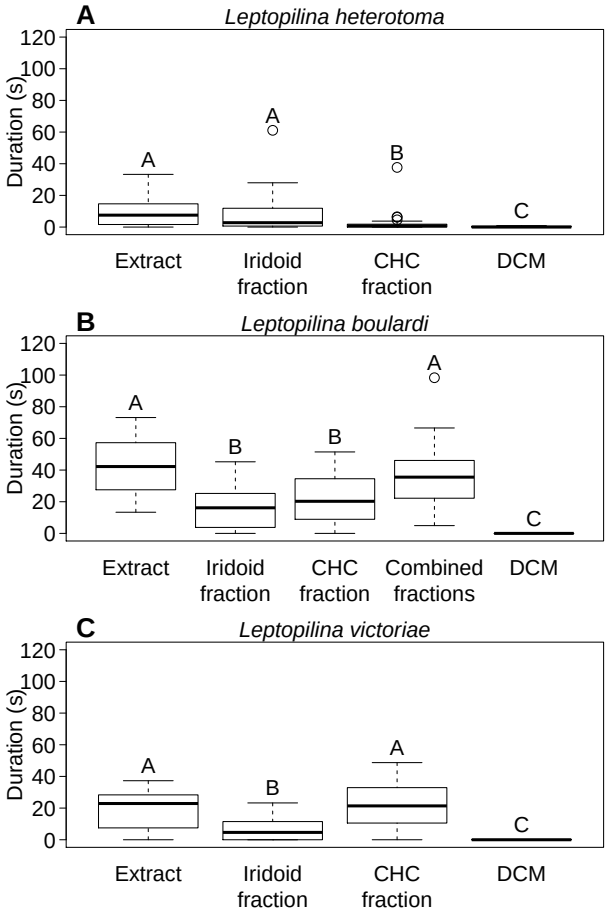

Figure 4.2.: (**A**) Total duration of wing fanning displayed by *L. heterotoma* males towards conspecific female extract, the DCM and hexane fractions thereof, and DCM only ($n = 25$). (**B**) Total duration of wing fanning displayed by *L. boulardi* males towards conspecific female extract, the DCM and hexane fractions thereof, the combined fractions, and DCM only ($n = 20$). (**A**) Total duration of wing fanning displayed by *L. victoriae* males towards conspecific female extract, the DCM and hexane fractions thereof, and DCM only ($n = 20$). The DCM fractions contain the iridoid compounds and the hexane fractions contain the CHCs. Different letters indicate significant differences between median values at $p < 0.05$ (*Mann-Whitney* U-test with *Bonferroni-Holm* correction).

To date, the female courtship eliciting pheromones of about a dozen parasitoid wasp species have been chemically identified (Ruther, 2013; Stökl et al., 2014). Those consists of both CHCs and non-CHC compounds, but a combination of both, like in *L. boulardi*, has only been found in *Lariophagus distinguendus* (CHCs and triacylglycerides, Kühbandner et al. 2012) and in *Asobara tabida* (Methyl 6-methyl salicylate, fatty alcohol acetates and CHCs, Stökl et al. (2014)). Although in *A. tabida* CHCs elicit courtship behavior, they are not necessary for a full response of the males.

CHCs are commonly used in the chemical communications of insects (Howard, 1993) and approximately half of the parasitoid wasps with identified female sex pheromones rely on CHCs for their sexual communication (Ruther, 2013). Iridoids are far less common than CHCs, but have been described in the defensive secretion of several ants, beetles and parasitoid wasps (e. g. Huth and Dettner 1990; Völkl et al. 1994; Do Nascimento et al. 1998; Stökl et al. 2012), and are also used as sex pheromone components by some species (e. g. aphids, Stewart-Jones et al. 2007). However, in parasitoid wasps iridoids have so far only been found in the genera *Alloxysta* and *Leptopilina*. Species from both genera use the iridoids for defense (Völkl et al., 1994; Stökl et al., 2012), but their use as sex pheromone has so far only been demonstrated for *L. heterotoma* (Weiss et al., 2013).

The reasons why one collection of available compounds is selected over another to compose a pheromone is one of the big questions in pheromone research. In a previous study we demonstrated, that in *L. heterotoma* (−)-iridomyrmecin most likely evolved primarily as a defensive compound against predators and later gained a second role in communication as sex pheromone (Weiss et al., 2013). At the moment we can only speculate about the reasons and evolutionary constraints, why *L. boulardi* and *L. victoriae* do not use iridoids for mate recognition to the same extent as *L. heterotoma*. It is interesting to note, that the observed low importance of iridoids in mate recognition in *L. victoriae* is correlated with a lower total amount of iridoids in *L. victoriae* compared to the other two species. Perhaps a lower investment into defense led to the selection of more abundant and therefore more reliable compounds, the CHCs, as the mate recognition pheromone in *L. victoriae*.

Molecular analyses of the genus *Leptopilina* have shown that *L. heterotoma* and *L. victoriae* are closely related, while *L. boulardi* is placed in a more distantly related species group (Allemand et al., 2002; Novkovic et al., 2011). This means that the two most closely related species use the most divergent sex pheromones while the distantly related *L. boulardi* uses a hybrid of the signals from the other two species. Therefore, it seems unlikely that there was a gradual evolution from only CHCs as sex pheromone to a pheromone consisting solely of iridoids, or vise versa. Future studies that elucidate the chemical composition of the pheromone of more species could be coupled with a more reliable phylogeny of the genus to test this hypothesis.

Vast differences in pheromones of sibling species can also be explained by saltational evolution (reviewed in Symonds and Elgar 2008). Pheromones that are under strong stabilizing selection, and thus

cannot evolve gradually, such as sex pheromones, might undergo quite drastic changes in a rather short time, leading to clearly different signals in sibling species. Examples for saltational evolution include aggregation pheromones in bark beetles (Symonds and Elgar, 2004) and sex pheromones in *Yponomeuta* moths (Löfstedt et al., 1991). Buellesbach et al. (2013) showed that male CHC profiles in *Nasonia* correlated with the *Nasonia* phylogeny. The female CHC profiles, however, are highly divergent and not correlated with the phylogeny. This is in strict accordance with the concept of gradual and saltational evolution: the male CHC profiles are not under strong stabilizing selection and can thus evolve gradually, leading to similar profiles in related species, while the female CHC profiles, with their role in sexual signaling, are under strong stabilizing selection and can thus only evolve through major shifts to establish reproductive isolation. Preliminary results show qualitative and quantitative differences in both the iridoid and the CHC profiles of males and females in most species of *Leptopilina*. Therefore, a comparative analysis might also be a useful approach to investigate the evolution of iridoids and CHCs in the chemical communication of *Leptopilina*. However, the currently available molecular phylogenies do not provide sufficient resolution and statistical support for such an analysis.

Our analysis of the iridoids found in the three species showed, to our surprise, that females of *L. victoriae* possess (+)-iridomyrmecin instead of (−)-iridomyrmecin. (−)-iridomyrmecin has been found in four species of *Leptopilina* (*L. heterotoma* and *L. boulardi*, Stökl et al. 2012; Weiss et al. 2013; *L. guineaensis* and *L. clavipes*, unpublished data), and therefore seems to represent the ancestral state. Weiss et al. (2013) demonstrated, that males of *L. heterotoma* are able to discriminate between (−)-iridomyrmecin and (+)-iridomyrmecin, and thus males of *L. victoriae* probably can do so as well. And although the biosynthetic pathway of iridomyrmecin has not been investigated in detail so far, it is plausible to assume that the modification of a single enzyme in the biosynthetic pathway can lead to the production of (+)-iridomyrmecin instead of (−)-iridomyrmecin. The shift from (−)-iridomyrmecin to (+)-iridomyrmecin in *L. victoriae* females could thus be an example of such a saltational evolution in sex pheromones. A detailed analysis of the biosynthetic pathways of the different iridoids is required to clarify how the shift from one enantiomer to the other may have happened.

It is furthermore noteworthy, that (−)-iridomyrmecin proved to be a more potent repellent than (+)-iridomyrmecin in bioassays with ants (Stökl et al., 2012). This and the finding that *L. victoriae* produces lower amounts of iridoids compared to *L. heterotoma* and *L. boulardi*, leads us to the conclusion, that chemical defense might be of less importance for *L. victoriae*. This is surprising, as all three species have a very similar ecology and detailed studies will be needed to better understand the differences in the chemical ecology of *Leptopilina* species.

Wing fanning is a courtship element commonly found in parasitic Hymenoptera (van den Assem, 1968, 1986). In several species, the male wing fanning performance has been found to be correlated with the outcome of the male courtship. For example males of *Lysiphlebus testaceipes* that produced high-frequency wing fanning had a higher mating success than males that fanned at a lower frequency (Benelli et al., 2015). Similarly, in males of *Lariophagus distinguendus* the frequency of wing fanning observed before successful courtship has been found to be significantly higher than the frequency before unsuccessful courtship (Benelli et al., 2013). Thus, male wing fanning may be an indicator of male fitness in parasitic Hymenoptera. The videos recorded in this study do not allow to determine wing fanning characteristics such as frequency or amplitude. It would, however, be interesting to compare these features between the studied species and correlate the features with the outcome of the courtship, especially since *L. boulardi* males seem to elicit female receptiveness more often than male from the other species (personal observation).

Drosophila species, the host species of *Leptopilina*, are usually no pests. As a consequence, predators and parasitoids of *Drosophila* had not been investigated regarding their potential application to control *Drosophila* populations. This situation has changed with the appearance of *Drosophila suzukii*, a pest species that originates from Asia (Cini et al., 2012) and only recently emerged in Europe and North America (Hauser, 2011; Calabria et al., 2012). Ovipositing *D. suzukii* females frequently damage fruit and thereby ruin crops (Cini et al., 2012); the appearance of *D. suzukii* has thus led to first initial research into the application of *Drosophila* predators (Cuthbertson et al., 2014) and parasitoids (Chabert et al., 2012) to control *D. suzukii* populations. Chabert et al. (2012) reported that *L. heterotoma* developed only very rarely in *D. suzukii* larvae. In a more recent article, Kasuya et al. (2013) reported that several larval parasitoids, including *Leptopilina japonica*, successfully parasitize *D. suzukii* in the field. The potential role of *Leptopilina* in controlling the emerging pest species *D. suzukii* stresses the importance of a profound understanding of the parasitoid's ecology, especially aspects regarding the efficient rearing and deployment of parasitoids.

Coming back to our introductory questions, we conclude that (1), mate recognition is species spe-

cific in *L. heterotoma*, *L. boulardi*, and *L. victoriae*; (2), iridoids, including iridomyrmecin, are produced by females of all three species, but contribute to a different extent to the sex pheromone; and (3) CHCs are used as sex pheromones by *L. boulardi* and *L. victoriae*. However, further comparative studies including more *Leptopilina* species are necessary to generalize our findings and to understand the selective forces acting on iridoids and CHCs which create the unexpectedly high pheromone diversity within the genus *Leptopilina*.

Acknowledgement

The authors thank Thomas Hoffmeister (University of Bremen), Roland Allemand (Université Claude Bernard Lyon 1), and Leo W. Beukeboom (University of Groningen) for sending us a starter culture of *L. heterotoma*, *L. boulardi*, and *L. victoriae*, respectively, and Michael Brummer for rearing the insects. This study was funded by the German Research Council (Deutsche Forschungsgemeinschaft, DFG; grant STO 966/1-1 to JS).

5. Species specificity of the putative male antennal aphrodisiac pheromone in *Leptopilina heterotoma*, *L. boulardi*, and *L. victoriae*

Male antennal aphrodisiac pheromones have been suggested to elicit female receptiveness in several parasitic Hymenoptera. None of the proposed pheromones, however, has been fully identified to date. A male aphrodisiac pheromone has also been proposed in *Leptopilina boulardi*. Due to the species specificity of mate recognition and courtship elicitatition in *Leptopilina*, however, the species specificity of the proposed male aphrodisiac pheromone could not be investigated.
In this study, an experimental design is presented that allows the investigation of the species specificity of the male aphrodisiac pheromone in several *Leptopilina* species. This is achieved by chemically manipulating the odour profile of heterospecific females, so that males perceive them as conspecifics. The putative male antennal aphrodisiac pheromones are found to be species specific. Additionally, it is proposed that the experimental setup can be employed to investigate the behavioural activity of candidate compounds for the aphrodisiac pheromone.

Introduction

Chemical senses are widespread in nature and chemical communication was very likely the first mechanism to transfer information between individuals (Wyatt, 2003). Chemical compounds that transfer information can be divided into allelochemicals and pheromones (Nordlund and Lewis, 1976). Wyatt (2010) defines 'pheromones' as

> 'molecules that are evolved signals which elicit a specific reaction, for example, a stereotyped behavior and/or a developmental process in a conspecific.'

Sex pheromones are thus signals that are involved in behaviours or processes that relate to mating.

In parasitic Hymenoptera, sex pheromones are important in three different levels of sexual communication (Ruther, 2013).

1. Mate attraction: one sex attracts the other over some distance with a volatile pheromone.

2. Mate recognition: less volatile pheromones facilitate reliable recognition of sex and species to identify a specimen as a suitable mate and elicit courtship.

3. Courtship: during courtship, males release aphrodisiac pheromones to elicite female receptiveness.

We recently investigated the role of pheromones in *Leptopilina* in both mate attraction (Weiss et al., 2013) and mate recognition (chapter 4). Aphrodisiac pheromones are often employed by males to elicit receptiveness in females and their involvement in courtship has been extensively investigated in parasitic Hymenoptera. In several species, including *Leptopilina*, antennal or oral male aphrodisiac pheromones have been proposed (van den Assem et al., 1980; Isidoro and Bin, 1995; Isidoro et al., 1999; Bin et al., 1999; Romani et al., 2005). Such male aphrodisiac pheromones can allow females to identify a courting male as conspecific, if the pheromone is species specific. In some species, e. g. in the genus *Nasonia*, the male aphrodisiac pheromone lacks species specificity. Thus, heterospecific courting males may be accepted as a mate by a female. *Nasonia* species also possess very similar sex pheromones, which leads to interspecific courtship (Buellesbach et al., 2014). In other genera, such as *Leptopilina*, mate recognition is highly species specific (chapter 4), which prevents interspecific courtship.

Isidoro et al. (1999) proposed that a male antennal aphrodisiac pheromone also exists in *L. boulardi*. In their work, antennal contact between males and females during courtship was demonstrated to be required to elicit receptiveness in females. Additionally, Isidoro et al. (1999), described glands and gland openings in the third and fourth male antennomeres. These antennomeres are brought into contact with

the distal part of the female antennae during courtship. Isidoro et al. (1999) thus assumed that a substance is transferred from the male antennae onto the female antennae to elicit female receptiveness. The species specificity of the proposed aphrodisiac pheromone, however, could not be investigated in *L. boulardi* and *L. heterotoma*, as interspecific courtship rarely occurs.

Preliminary experiments conducted in our group with *L. heterotoma* and *L. victoriae* suggest that antennal contact is an essential element of courtship in these species as well and it thus seems likely that a male antennal aphrodisiac pheromone exists. Our own recent work on mate recognition in *Leptopilina* (chapter 4) allowed us to develop an experimental protocol, in which males readily court heterospecific females. Using this experimental setup, we investigate the species specificity of the putative male aphrodisiac pheromones in *L. heterotoma*, *L. boulardi*, and *Leptopilina victoriae*.

Material & methods

Insects. We reared *L. boulardi*, *L. heterotoma*, and *L. victoriae* using *D. melanogaster* as the host species. *Drosophila melanogaster* was reared on a corn-based diet (504 ml water, 66 g sugar, 6 g baker's yeast, 2.3 g agar, 52 g cornmeal, 1.3 ml propanoic acid, 0.8 g nipagin) at 25 °C ambient temperature, with roughly 75 % humidity, and a 16:8 h L:D cycle. About 30 flies (mixed sexes) were placed into a jar for each rearing. The jar contained fresh fly food. The flies were removed from the jar after 48 h, and about 10 wasps (both sexes) were put into the jar. Parasitized fly pupae were removed from the jars before the adult wasps emerged and put singly into 1.5 ml microcentrifuge tubes to obtain naive and virgin wasps of known age.

Extraction. Virgin 1-d-old females were extracted in batches of 30–50 with 5 µl dichloromethane (DCM) per female for 10 min. Afterwards, the DCM was evaporated under a gentle stream of nitrogen. Then, the residue was redissolved in 1 µl acetone per 5 µl original volume. The final concentration of the extract thus equalled 1 female per 1 µl.

Mating trials. To investigate whether males from each species could elicit readiness to mate in con- and heterospecific females, mating trials were conducted. For this, naive, virgin males were allowed to court naive, virgin females and we recorded whether females showed readiness to mate. Trials were conducted in a small plexiglass arena (15 mm diameter, 2 mm height) covered with a glass lid and lasted 120 s. Trials were terminated early when the female showed readiness to mate. As males court only conspecific females, heterospecific female odour profiles had to be chemically manipulated, so the females were perceived as conspecifics by the males. The female odour profiles were manipulated by applying 0.1 µl (equalling 0.1 female equivalents) female extract redissolved in acetone from the male's species to the female. Previous studies (Abdel-latief et al., 2008; Blaul et al., 2014) have revealed that many parasitic wasps tolerate the application of acetone extracts without any visible intoxication. Conspecific females were also treated with extract. The extract was applied using a on-column GC syringe (Hamilton, Bonaduz, Switzerland). After applying the extract, females were allowed to recover for 120 s. Females that did not recover after the application were discarded from the experiment (only 2 of all treated females did not recover within 120 s). After recovering, females were carefully placed into the arena and a single male was added. We recorded whether the male courted the female and whether the female showed readiness to mate. For each possible combination of male and female species, we conducted experiments until male courtship including antennal stroking was observed in 10 replicates.

Statistical analysis. The number of mating trials conducted until courtship including antennal stroking was observed 10 times in each combination of male and female species was analysed with the chi-squared test. All statistical tests were performed using R version 3.1.1 (R Core Team, 2014).

Results

Males of all three species readily courted both conspecific females and heterospecific females treated with extract of conspecific females (fig. 5.1). The statistical analysis of the number of conducted replicates indicated no significant differences between all combinations of male and female species (chi-squared test: $\chi^2 = 0.2527$, $df = 4$, $p = 0.9927$). The manipulation of the females' odour profiles was an effective means to reliably elicit interspecific courtship. However, males elicited readiness to mate only in conspecific females but never in heterospecific females (fig. 5.2).

Discussion

We have found that the male courtship signal (putatively an antennal aphrodisiac pheromone) in *Leptopilina* is species specific. Males of the species *L. heterotoma*, *L. boulardi*, and *L. victoriae* can elicit readiness to mate only in conspecific, but not in heterospecific females.

Isidoro et al. (1999) demonstrated that antennal contact during courtship is essential in *L. boulardi*. They showed elegantly by amputation of antennae that males cannot elicit readiness to mate in females if

antennal contact is prevented. In their experiments, they used individuals that had one of their antennae amputated. When females and males had the antennae amputated on the same side, males were still able to elicit receptiveness in the females; when females and males had the antennae amputated on different sides, males failed to elicit receptiveness. However, Isidoro et al. (1999) could not investigate the species specificity of the assumed male antennal aphrodisiac pheromone, as *L. heterotoma* males did not court *L. boulardi* females—and vice versa—in the bioassays. The absence of cross-specific courtship is no surprise, as mate recognition is species specific in *Leptopilina* (chapter 4). We could overcome this problem by chemically manipulating the odour profile of heterospecific females, so males perceived them as conspecifics. Males of the three investigated species readily courted these manipulated females, which allowed us to investigate the species specificity of the male antennal pheromone.

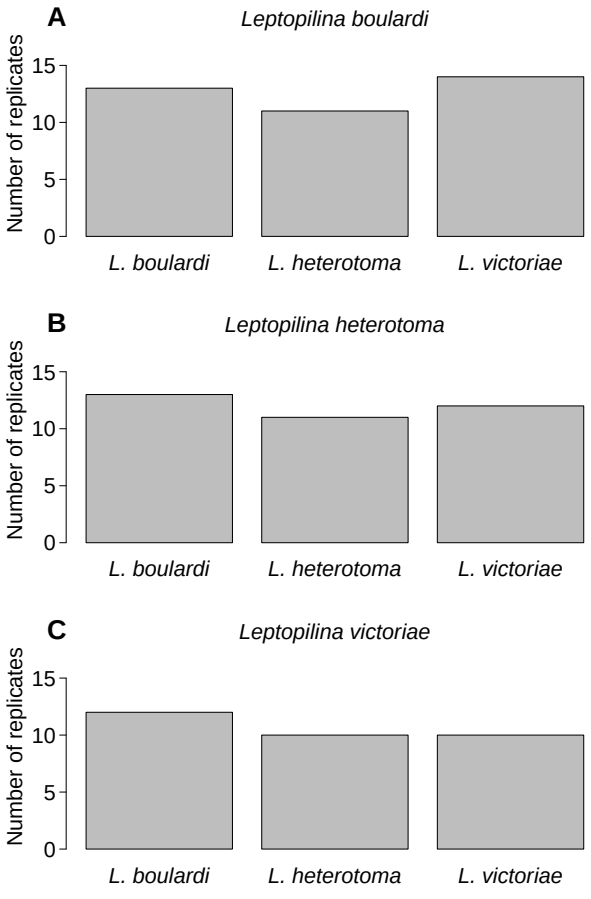

Figure 5.1.: Number of replicates conducted until courtship including antennal stroking was observed 10 times in intraspecific and interspecific mating trials. A statistical analysis of the number of conducted replicates showed no significant differences between the different species combinations (chi-squared test: $\chi^2 = 0.2527$, $df = 4$, $p = 0.9927$).

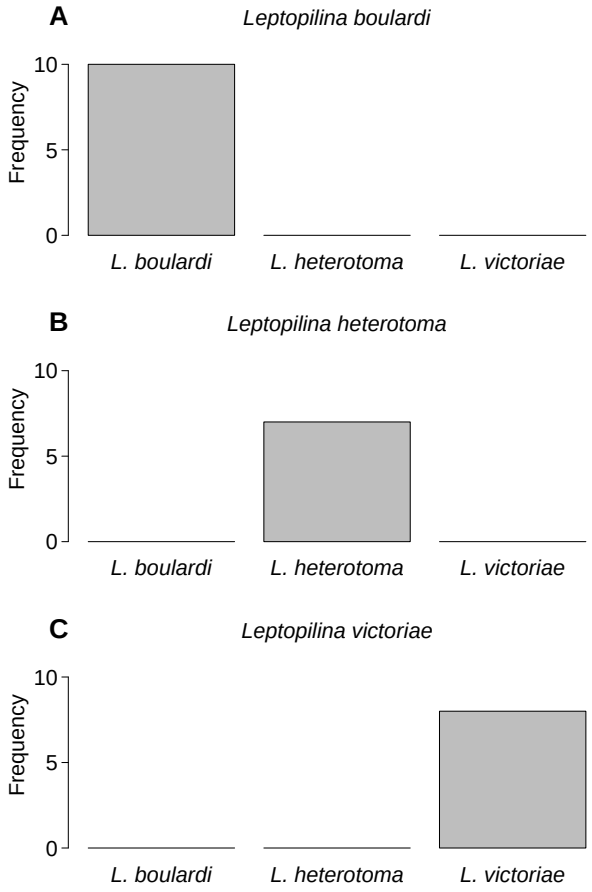

Figure 5.2.: Number of (**A**) *L. boulardi* males, (**B**) *L. heterotoma* males, and (**C**) *L. victoriae* males that elicited readiness to mate in mating trials with conspecific and heterospecific females. For each experiment $n = 10$.

Another possible explanation for the required antennal contact demonstrated by Isidoro et al. (1999) is, that the signal is not a chemical one, but a physical one. Males from different species could show different stroking patterns and e. g. the stroking speed could signal mate quality. Such signalling is known from e. g. cucumber beetles, in which the female decides to reject or accept the male's spermatophore based on antennal stroking speed (Tallamy et al., 2003). In the mating trials conducted in the present study, however, no obvious species-specific antennal stroking patterns could be observed. Antennal glands in males, on the other hand, are a common feature in Hymenoptera (Romani et al., 2008), and they are putatively involved in courtship in a number of parasitic Hymenoptera. Strong evidence for male antennal aphrodisiac pheromones has been found in e. g. *Amitus spiniferus* (Hymenoptera: Platygastridae) (Isidoro and Bin, 1995), *Pimpla turionellae* (Hymenoptera: Ichneumonidae) (Bin et al., 1999), *Trichopria drosophilae* (Hymenoptera: Diapriidae) (Romani et al., 2008), and also *L. boulardi* (Isidoro

et al., 1999). We thus assume, that the species-specific signal in *Leptopilina* is indeed a pheromone.

We found, that the putative male antennal aphrodisiac pheromone is indeed species specific. This is noteworthy, as a species-specific aphrodisiac pheromone establishes a barrier to heterospecific matings, even though mate recognition is already species specific (chapter 4) and should thus prevent heterospecific courtship and matings. The species specificity of the male pheromone might, however, reflect the great selective pressure against heterospecific matings. *Leptopilina* females are, like most (solitary) parasitic Hymenoptera, monandrous (Ridley 1993; chapter 3). In combination with the arrhenotoky found in *Leptopilina* and the *Wolbachia*-mediated cytoplasmatic incompatibility (Fleury et al., 2000; Gueguen et al., 2012), this imposes a great fitness loss upon females that accept a heterospecific mate. Only male offspring will be produced from heterospecific matings, which results in a reduced fitness as compared to producing female and male offspring. This is especially true for species that experience local mate competition, which is true for at least *L. heterotoma* (Debout et al., 2002).

During courtship, the female has to fold back its antennae to allow proper antennal stroking by the male. When the female folds back its antennae, the male's head comes to lie between the female antennae, and so the male's proximal antennomeres can be brought into contact with the female's distal antennomeres. Both conspecific and heterospecific females readily cooperated during courtship by folding back their antennae when mounted by a male. We assume that females would not cooperate if they were able to identify a male as heterospecific without antennal contact. Thus, the putative antennal pheromone is probably of low volatility and has to be transferred by physical contact of the antennae.

We found only cuticular hydrocarbons in dichloromethane extracts of male antennae in the species *L. heterotoma*, *L. boulardi*, and *L. victoriae* (unpublished data) and identified several diunsaturated alkenes as candidate compounds for the aphrodisiac pheromone. These alkenes are partially species specific, so they would be suitable to establish a species-specific courtship signal. Their chain length ranges from 31 to 37, they thus fit the assumption that the pheromone contains compounds of rather low volatility.

Despite the mentioned range of literature showing the great interest in such male aphrodisiac pheromones in parasitic Hymenoptera, however, no such putative pheromone has been identified to date. Our experimental setup allows us to have males court heterospecific females, in which they can not elicit readiness to mate. In subsequent experiments, the identified pheromone candidate compounds can now be applied to the male antennae to investigate their ability to elicit female receptiveness.

6. General discussion

In this doctoral thesis, new insights into the chemical communication of *Leptopilina* were gained.

I not only identified the female mate attraction pheromone in *L. heterotoma*, but also suggested an evolutionary route that led to the inclusion of the defensive allomone (−)-iridomyrmecin into the sex pheromone. A similar evolutionary route very likely led to the use of (−)-iridomyrmecin as the female competition avoidance agent in *L. heterotoma* (and probably other *Leptopilina* species). The use of (−)-iridomyrmecin as semiochemical for three different functions is a prime example for semiochemical parsimony. (Chapter 2.)

I demonstrated that *L. heterotoma* females are monandrous, thus reliable and specific species and mate recognition is pivotal for their fitness. Contrary to previous reports, mated females are still attractive to males—i. e. mated females elicit courtship in males. This is probably due to the parsimonous use of iridoids in the defensive secretion as well as the mate attraction pheromone. (Chapter 3.)

I showed that mate recognition in the sympatric species *L. heterotoma*, *L. boulardi*, and *L. victoriae* is species specific. The species specificity is achieved by a high chemical diversity of the female mate recognition pheromones. The use of different combinations of two strucural classes of chemicals (iridoids and cuticular hydrocarbons, CHCs) probably evolved through saltational evolution. (Chapter 4.)

I demonstrated that the elicitation of female receptiveness in *L. heterotoma*, *L. boulardi*, and *L. victoriae* is species specific. The experimental setup devised to investigate the species specificity can very likely also be employed to identify the putative male antennal aphrodisiac pheromones responsible for receptiveness elicitation. (Chapter 5.)

From zero to cue to signal

To date, thousands of chemical compounds have been identified as pheromones (El-Sayed, 2014). For only a few of them, an evolutionary route has been suggested, and for even fewer, experimental data supporting such a route is available. One of the main questions regarding pheromone evolution is, why a certain compound evolved into a pheromone, and not another, when probably hundreds of chemical compounds would be equally well suited.

One theory is, that pheromones evolve from compounds that fulfill other than communicative roles (Wyatt, 2010; Steiger et al., 2011; Bradbury and Vehrencamp, 2011), e. g. CHCs that serve as desiccation barrier. Such compounds are obviously already available, and given the right circumstances (compounds have to be detectable and released into the environment), individuals can eavesdrop on them. The least that can be inferred from the presence of a certain chemical compound is the presence of the compound's source. Depending on the function of the compound, even more information may be inferred. For example, Sorensen and Stacey (1999) showed, that a hormone that is excreted into the environment, does not only supply information about the organism's presence, but also about the organism's physiological state. As such, certain compounds might be more likely to evolve into pheromones than others. Key characteristica of candidate compounds include availability, volatility, and perceptibility. Additionally, the information that can be inferred from the compound must be beneficial to the receiving individual. If this is the case, a specific response to the compound may evolve in the receiver, the compound can now be considered a cue, but not yet a signal. Only when the receiver's response is beneficial to the sender, the cue will evolve into a signal. Selection will lead to the inclusion or exclusion of compounds as well as to changes in the ratio of compounds. Compounds are now selected for a reliable and specific information transfer, as a sender that provides more reliable and specific information will gain greater benefits than other senders. This process of increasing the signal's quality by modification is called chemical ritualization (Tinbergen, 1952; Steiger et al., 2011). On the receiver's side, evolution will lead to the selection of more efficient perception mechanisms. Through the selection for information transfer, the cue has now become a signal. Signal, perception mechanisms, and response to the signal have been formed through evolution, as defined by Smith and Harper (2003). Experimental data supporting such evolutionary routes, however, is scarce.

In chapter 2, the theoretical evolutionary route is supported by experimental data for *L. heterotoma*. A single compound, (−)-iridomyrmecin, that has been shown to be the defensive secretion of *L. heterotoma* (Stökl et al., 2012), is also the main component of

the female mate attraction pheromone (Weiss et al., 2013). By definition, a compound can not be a cue and a signal for the same function at the same time, so a cue stage for (−)-iridomyrmecin as mate attraction pheromone can not be demonstrated for *L. heterotoma*. However, in its role as competition avoidance agent in *L. heterotoma*, (−)-iridomyrmecin can be regarded as a cue. Although this is a different function than as sex pheromone, the use of (−)-iridomyrmecin as a cue in one scenario renders a previous cue stage during the evolution of the female mate attraction pheromone more likely.

We also showed, that *L. heterotoma* males are not only attracted towards extracts of conspecific females, but also towards extracts of *L. boulardi* females (Weiss et al., 2013). This implies, that the evolution of the mate attraction pheromone has not yet lead to a reliable species-specific signal. However, species-specific sex pheromones are required to prevent heterospecific matings.

The need for species-specific pheromones

Hymenoptera are haplodiploid organisms (Cook, 1993; Heimpel and de Boer, 2008) and generally arrhenotokous, thus fertilised eggs develop into females and unfertilised eggs develop into males. Monandrous Hymenoptera females are thus under high evolutionary pressure to choose a suitable mate, that will allow the fertilisation of their eggs. Most solitary parasitic Hymenoptera are monandrous (Ridley, 1993), and monandry has also been demonstrated for *L. heterotoma* (chapter 3).

Choosing an incompatible mate will result in reduced fitness, because only male offspring will be produced. Females will gain the greatest fitness if they produce the best ratio of female to male offspring. In species that experience local mate competition, females are expected to procude just enough sons to fertilize their daughters (Hamilton, 1976). This means, the greatest fitness gain is achieved by females that produce a female-biased sex ratio; producing only sons will thus inevitably lead to a massive fitness loss. Local mate competition has been found in *L. heterotoma* (Debout et al., 2002), although the local mate competition is only partial. About 80 % of the males and about 75 % of the females mate on patch, while the remaining males and females may mate off patch (Debout et al. 2002, inferred from data given by Fauvergue et al. 1999). Nevertheless, the larger part of *L. heterotoma* offspring will experience local mate competition, thus females can be expected to produce a female-biased sex ratio to ensure a high fitness.

Many insect species are infected with *Wolbachia* (Hilgenboecker et al., 2008). *Wolbachia* are parasitic or mutualistic bacteria that manipulate their host's reproduction. Several *Wolbachia*-induced changes in insects have been described, including cytoplasmatic incompatibility (Werren et al., 2008). Cytoplasmatic incompatibility occurs between individuals that are infected with different *Wolbachia* strains (bidirectional cytoplasmatic incompatibility) and when an infected male mates with an uninfected female (unidirectional cytoplasmatic incompatibility). When cytoplasmatic incompatibility occurs, sperm and egg are unable to develop into viable offspring, thus, in the case of haplodiploid species, only unfertilized eggs will develop. In *Leptopilina*, *Wolbachia*-induced cytoplasmatic incompatibility has been found in *L. heterotoma* (Fleury et al., 2000) and *L. victoriae* (Gueguen et al., 2012), whereas *L. boulardi* has been found to be free of *Wolbachia* and is assumed to be resistant to infection (Fleury et al., 2000).

In *Leptopilina*, all the factors mentioned above come together and females are thus under strong selective pressure to mate with a conspecific male. One way through which strong prezygotic isolation can occur are species-specific sex pheromones. In contrast to the female mate attraction pheromone in *L. heterotoma*, which is not species-specific (Weiss et al. 2013; chapter 2), the mate recognition pheromones (chapter 4) and the putative male antennal aphrodisiac pheromones (chapter 5) have been demonstrated to be species specific.

The evolution of species-specific pheromones

In chapter 2, an evolutionary route for the selection of certain compounds as pheromone components has been outlined. The mate attraction pheromone that evolved from the defensive secretion, however, lacks species specificity, and thus potentially seriously impedes reproduction. This lack of species specificity is mitigated by two other sex pheromones involved in reproduction, the female mate recognition pheromone (chapter 4) and the putative male antennal aphrodisiac pheromone (chapter 5). Their demonstrated species specificiy prevents heterospecific matings and they thus impose a strong prezygotic barrier. How does such species specificity evolve?

For a long time, it has been assumed, that pheromones evolve through small, gradual changes in the structures and proportions of the chemical compounds constituting the pheromone (Roelofs and Brown, 1982). This is, however, contradicted by the assumption, that a strong selection against signal modification should be imposed by their high specificity (Symonds and Elgar, 2008). It has thus been suggested that pheromone evolution does not occur through gradual changes but rather major shifts in the pheromone composition (Löfstedt, 1993). These major shifts have later been described as 'saltational shifts' by Baker (2002). In 2002, Roelofs et al. pub-

lished the first evidence for such saltational shifts. They showed that a saltational shift in the sex pheromones of two *Ostrinia* species required only the activation of a single gene transcript. Later, Symonds and Elgar (2004) investigated the correlation between the composition of the aggregation pheromones of 34 bark beetle species and their phylogeny. They found that the pheromone composition in sibling species is substantially different, which suggests that their aggregation pheromones indeed evolved through saltational shifts rather than gradual changes. A more recent review by Symonds and Elgar (2008) suggests, that both modes of evolution, gradual changes and saltational shifts, exist in pheromone evolution.

Our own data on the mate recognition pheromones in *Leptopilina* (chapter 4) are in concordance with the theory of saltational shifts. If their mate recognition pheromones had evolved through gradual changes, the pheromones should show at least some if not considerable similarity, as the species are regarded as closely related. The differences we found between the female mate recognition pheromones of *L. heterotoma*, *L. boulardi*, and *L. victoriae*, however, can hardly be explained by evolution through gradual changes. The three species have two classes of chemical compounds at their disposal: iridoids and CHCs. In *L. heterotoma*, mate recognition is mainly mediated by iridoids, and CHCs only play a negligibile role. *Leptopilina victoriae*, however, relies heavily on CHCs for mate recognition and iridoids are only of minor importance. In the third species, *L. boulardi*, mate recognition is mediated by both iridoids and CHCs. The different degree alone, to which the available chemical classes of compounds are incorporated into the mate recognition pheromone, contradicts evolution through gradual changes. A saltational shift in the response behaviour of the males, however, can explain how the two different classes of chemical compounds are incorporated to different degrees into the female mate recognition pheromones. Additionally, the species' iridoid and CHC profiles are species specific. The sister species *L. heterotoma* and *L. victoriae* even differ in the major component of their iridoid profiles. In *L. heterotoma* females, the major component is (−)-iridomyrmecin, whereas *L. victoriae* females possess the enantiomer, (+)-iridomyrmecin, as the major component. The respective enantiomer is not present in either species. Although the biosynthetic pathways of (−)-iridomyrmecin and (+)-iridomyrmecin are unknown, it is plausible to assume that a rather minor change in single enzyme could lead to the production of one enantiomer instead of the other. Such a change would fit the idea of saltational evolution: a small change in the biosynthetic pathway leads to a saltational shift in the resulting iridoid profile.

The morphological and molecular phylogenies that have been described for *Leptopilina*, offer only low resolution and insufficient statistical support. Should this situation be rectified, our work on the mate recognition pheromones in the genus *Leptopilina* would provide an excellent starting point to further support the theory of pheromone evolution through saltational shifts.

Competition avoidance—cue or signal?

In several *Leptopilina* species, host-searching females avoid host patches that are already exploited by heterospecific or conspecific females (chapter 2; Janssen et al. 1995a,b). This competition avoidance is odour-mediated; in *L. heterotoma*, the semiochemical used for competition avoidance is (−)-iridomyrmecin. Whether the substance mediating competition avoidance is a cue or a signal, however, is unclear. The main difference between a cue and a signal is that selection must operate on both the sender and the receiver for a signal to evolve, while for a cue to evolve selection of the receiver is sufficient (Scott-Phillips, 2008).

It is obvious, that the receiver benefits from competition avoidance. Females that oviposit at an already exploited host patch will find fewer hosts for their own eggs. *Leptopilina heterotoma* is a solitary parasitoid, so females generally need unexploited hosts to oviposit. Superparasitism, however, may occur if hosts are rare. In cases of superparasitism, most of the eggs that have been laid second, will be outcompeted by the eggs that have been laid first (Bakker et al., 1985). Thus, choosing an unexploited host patch over an already exploited host patch will avoid a decrease in fitness. This is not necessarily true for multiparasitism, e. g. *L. boulardi* may even benefit from additional parasitism by *L. heterotoma* (Carton et al., 1991). The data given by Carton et al., however, does not allow to conclude whether *L. boulardi* benefits only if the host has been parasitized first by *L. boulardi*. It would thus be highly interesting to investigate whether *L. boulardi* females avoid host patches that are already being exploited by *L. heterotoma* females.

Competition avoidance, however, also benefits the first female at a host patch. If a second egg is laid into an already parasitized host, the first egg is likely to outcompete the second egg (Bakker et al., 1985). The resources available for development, however, are limited, and the resources consumed by the partial development of the second egg impedes the development of the first egg, e. g. the emerging adult will likely be smaller than compared to development without competition. If the host patch is exploited by only one single female, all males are brothers. The exploitation of a host patch by more than one female can lead

to a reduced fitness, because additional, non-brother males will compete for the females.

Whether the competition avoidance benefits the first female enough to drive the evolution from cue to signal, is unclear. To clarify this, further competition avoidance experiments need to be conducted. These could e. g. investigate whether ovipositing females actively release (−)-iridomyrmecin. If this is the case, (−)-iridomyrmecin could be considered a true signal.

Male courtship pheromones

In a number of parasitic Hymenoptera, male courtship pheromones have been proposed, e. g. *Nasonia vitripennis* (van den Assem et al., 1980; Ruther et al., 2010), *Amitus spiniferus* (Isidoro and Bin, 1995), and *Pimpla turionellae* (Bin et al., 1999). The assumption of the existence of these pheromones is mostly based on the observation that stereotyped behaviour including the mouthparts ('head nodding' accompanied by chewing behaviour) or the antennae ('antennal stroking') can be observed during courtship and that oral or antennal glands have been described in many species showing such behaviour. In some cases the secretion from these glands has long been suspected to elicit receptiveness in females (e. g. *N. vitripennis*, van den Assem et al., 1980). These glands are believed to produce secretions that are released and sometimes transferred onto the female antennae during courtship and elicit female receptiveness. These courtship pheromones can thus be regarded primarily as male aphrodisiac pheromones.

Only recently, an additional function has been demonstrated for such a courtship pheromone. Ruther and Hammerl (2014) showed that in *N. vitripennis*, the male courtship pheromone terminates the female response to the male-produced sex attractant. This is the first pheromone-mediated behavioural switch that is not caused by the transfer of bioactive molecules with the male ejaculate, but by the secretion of the male cephalic glands. The male courtship pheromone of *N. vitripennis* is also the first cephalic courtship pheromone in parasitic Hymenoptera that has been chemically identified. Ruther and Hammerl (2014) identified the bioactive compounds as a blend of ethyl oleate, ethyl linoleate, and ethyl α-linolenate. These compounds do, however, not elicit receptiveness in females, thus to date, no male aphrodisiac pheromone in parasitic Hymenoptera has been fully identified.

In the genus *Leptopilina*, a male antennal aphrodisiac pheromone had so far only been proposed for *L. boulardi*. Isidoro et al. (1999) demonstrated by amputation of one antenna of the male and one antenna of the female that antennal contact during courtship is required to elicit receptiveness in females. In their experiments, males that had their antenna amputated on the same side as the females still elicited receptiveness in the females; when females and males had the antennae amputated on different sides, however, males failed to elicit receptiveness. They also described a gland and gland openings in the third and fourth male antennomeres. Their assumption was thus, that these glands produce a secretion that is transferred onto the female antennae during courtship and elicits female receptiveness. Isidoro et al. (1999) tried to investigate the species specificity of the proposed pheromone in cross-specific courtship experiments with *L. boulardi* and *L. heterotoma*. Mate recognition and courtship elicitation, however, is species specific in *Leptopilina* (chapter 4), thus males will not court heterospecific females. However, interspecific courtship is required to investigate the species specificity of the male antennal pheromone.

Preliminary investigations in our own group suggested the existence of a male courtship signal—putatively a pheromone—not only in *L. boulardi*, but also in *L. heterotoma* and *L. victoriae*. By chemically manipulating the odour profiles of heterospecific females so that they were perceived as conspecifics, cross-specific courtship could be observed, and thus the species specificity of the male courtship signal could be investigated (chapter 5). The demonstrated species specificity of the male courtship signal further corroborates the assumption that the male courtship signal is indeed a pheromone. The identification of the putative pheromone has not yet been undertaken, but candidate compounds have been identified as species-specific diunsaturated alkenes (Stökl, unpublished data), and the experimental setup described in chapter 5 seems well-suited for the investigation of their bioactivity.

Semiochemical parsimony

Semiochemical parsimony describes the idea that organisms use one or more chemical compounds for more than just one semiochemical function (Blum, 1996). This is believed to be advantageous, as e. g. a reduced number of biosynthetic pathways is required if the same semiochemicals are produced for several functions instead of a number of different semiochemicals. Blum described a great number of examples he assumed to represent semiochemical parsimony and which thus lead to the impression that semiochemical parsiomy is a phenomenon well-supported by conclusive data. That is, however, not the case. Many of the numerous examples Blum compiled from the literature, are indeed inconclusive, mostly because semiochemical functions had been ascribed to blends of several compounds without investigating the role of the individual compounds. In other cases, only one function had been demonstrated for certain com-

pounds in one species and a second function was simply assumed because it had been found in other species. This is not to say that the examples given by Blum are not indeed examples for semiochemical parsimony, many of the examples have just not been unambiguously demonstrated.

One example given by Blum is the sex pheromone of the mullein bug, *Campylomma verbasci*. The work cited by Blum is a prime example for the identification of a two-component sex pheromone (Smith et al., 1991). Smith et al. unambiguously demonstrated that the sex pheromone in *C. verbasci* consists of two components, butyl butyrate and (*E*)-crotyl butyrate. Blum then continues to name these compounds as 'typical defense constituents ... of heteropterans ...' and thus assumes semiochemical parsimony in *C. verbasci*. Neither does Blum give a source for this nor does the original publication (Smith et al., 1991) mention a defensive function. To date, no defensive function of the two compounds has been demonstrated in *C. verbasci*. In many cases cited by Blum, one or more putative functions of a semiochemical have not been clearly demonstrated, and thus, semiochemical parsimony is not supported by data to the extent implied by Blum.

Nevertheless, semiochemical parsimony is a very plausible phenomenon and several examples have been unambiguously demonstrated. Blum (1996) e. g. mentions undecane as both sex pheromone and alarm pheromone in *Formica lugubris*. Both functions have been unambiguously demonstrated by Walter et al. (1993).

A prime example for a three-fold semiochemical parsimony is demonstrated in *L. heterotoma* in this thesis. In *L. heterotoma*, (−)-iridomyrmecin has been shown to fulfill three different semiochemical roles. Firstly, Stökl et al. (2012) showed that (−)-iridomyrmecin is released, in combination with additional iridoids, when *L. heterotoma* females encouter potential predators. Additionally, Stökl et al. demonstrated, that (−)-iridomyrmecin alone is repellent to ants. Thus, (−)-iridomyrmecin definitely has defensive properties (which is not to say that the additional released iridoids do not also have defensive properties). Secondly, (−)-iridomyrmecin has been demonstrated to mediate competition avoidance in host-searching *L. heterotoma* females (Weiss et al. 2013, chapter 2). In several *Leptopilina* species (Janssen et al., 1995a,b; Weiss et al., 2013), host-searching females avoid host patches that are already being exploited by con- or heterospecific females. The same avoidance behaviour could be elicited by manipulating a host patch with synthetic (−)-iridomyrmecin in choice experiments with *L. heterotoma* (Weiss et al. 2013, chapter 2). (−)-iridomyrmecin thus mediates competition avoidance in addition to its defensive properties. Thirdly, (−)-iridomyrmecin has been incorporated into the female mate attraction pheromone in *L. heterotoma* (Weiss et al. 2013, chapter 2). The mate attraction pheromone is a blend of five iridoids, with (−)-iridomyrmecin constituting roughly 90 % of the amount of iridoids. The removal of (−)-iridomyrmecin from the iridoid blend rendered the remaining four iridoids unattractive to males. This unambiguously demonstrates the third semiochemical role of (−)-iridomyrmecin. The threefold use of (−)-iridomyrmecin as a semiochemical in *L. heterotoma* females (defensive secretion, competition avoidance agent, and mate attraction pheromone) is thus one of the few unambiguous examples for semiochemical parsimony.

Leptopilina—a potential biological control agent of *D. suzukii*?

Drosophila species, the host species of *Leptopilina*, are usually no pests. In almost all *Drosophila* species, the females do not possess a serrated ovipositor and can therefore not penetrate the surface of healthy fruit or mushrooms to lay their eggs. Instead, they depend on already damaged, rotten substrate for oviposition and can thus only exploit fruit that are already lost as crop. As a consequence, predators and parasitoids of *Drosophila* had not been investigated regarding their potential application to control *Drosophila* populations.

This situation has changed with the appearance of *Drosophila suzukii*, a pest species that originates from Asia (Cini et al., 2012) and only recently emerged in Europe and North America (Hauser, 2011; Calabria et al., 2012). In contrast to almost all other *Drosophila* species, *D. suzukii* females possess a serrated ovipositor that enables them to penetrate the surface of healthy fruit (Cini et al., 2012). Ovipositing *D. suzukii* females thus frequently damage fruit and thereby ruin crops (Cini et al., 2012). The appearance of *D. suzukii* and the economic damage caused by it has led to first initial research into the application of *Drosophila* predators (Cuthbertson et al., 2014) and parasitoids (Chabert et al., 2012) to control *D. suzukii* populations. Chabert et al. (2012) reported, that only pupal parasitoids were able to successfully parasitize *D. suzukii*, where as larval parasitoids, including *L. heterotoma* and *L. boulardi* did not develop in *D. suzukii* larvae. They also reported that in a second experiment, however, 3 out of 180 *L. heterotoma* eggs had successfully developed in *D. suzukii* larvae. In a more recent article, however, Kasuya et al. (2013) reported, that several larval parasitoids, including *Leptopilina japonica*, successfully parasitize *D. suzukii* in the field. Owing to the sympatric distribution with *D. suzukii*, *L. japonica* probably evolved to overcome the strong immune

response that prevents the development of other larval parasitoids in *D. suzukii*. As *Leptopilina* species such as *L. heterotoma* and *L. boulardi* already readily accept *D. suzukii* larvae as host, and are, albeit only very rarely, in principle able to overcome the immune response (Chabert et al., 2012), these species will probably evolve to successfully develop in *D. suzukii*.

Employing parasitoids as biological control agents of pest species is an established practice (van Lenteren, 2000). It thus stands to reason, that *Leptopilina* species could potentially be established as antagonists of *D. suzukii* populations. For that matter, in-depth knowledge of the chemical ecology—including the sexual communication—of *Leptopilina* is essential to efficiently rear and deploy *Leptopilina* populations to control *D. suzukii* in the field. While the presented insights into the sexual communication of *L. heterotoma*, *L. boulardi*, and *L. victoriae* presented in this thesis are a first step, further research into the sexual communication of *Leptopilina*, including species that already successfully parasitize *D. suzukii*, is definitely required.

Another promising approach is the exploitation of an avoidance behaviour found in *Drosophila* females. Ovipositing females of several *Drosophila* species—including *D. suzukii*—avoid food and oviposition sources that had been enriched with the odour of *L. boulardi* (Knaden et al., unpublished). This avoidance behaviour is elicited by (−)-iridomyrmecin (Knaden et al., unpublished)—which mediates competition avoidance in host-searching *L. heterotoma* females (Weiss et al., 2013). It is highly likely that *Drosophila* females eavesdrop on the parasitoid's chemical communication to avoid parasitisation. Knowledge on the chemical communication of *Leptopilina* can thus help identifying candidate compounds to control the emerging pest species *D. suzukii*. In this very example, (−)-iridomyrmecin could potentially be employed to repel *D. suzukii* females from crops and thus prevent crop damage.

Conclusions

Two classes of chemical compounds drive the chemical communication in *Leptopilina*: iridoids and CHCs. The extent to which these chemicals convey information differs between the investigated species, *L. heterotoma*, *L. boulardi*, and *L. victoriae*.

For mate attraction and mate recognition, *L. heterotoma* seem to rely solely on iridoids. The sister species, *L. victoriae*, relies on CHCs for mate recognition, and *L. boulardi* employs a combination of both iridoids and CHCs. These differences point to a saltational evolution of the mate recognition pheromones in *Leptopilina*.

The composition of the putative male aphrodisiac pheromones of the three species has not been investigated, but preliminary results suggest that CHCs may play an important role. The elicitation of female receptiveness, however, is species specific, even though the aphrodisiac pheromones may be composed of structurally related chemicals.

In *L. heterotoma*, iridoids seem to predominate the chemical communication. Not only are mate attraction and mate recognition mediated by iridoids, but also competition avoidance. Additionally, the defensive secretion of *L. heterotoma* females is composed of iridoids. Besides illustrating the great semiochemical parsimony in *L. heterotoma*, this also gives strong support to the theory, that communicative functions evolve for compounds that are already used in non-communicative contexts.

The results obtained in this thesis provide new insight into chemical communication and pheromone evolution in *Leptopilina*. However, the genus is comprised of more than 30 species, and so far, the pheromones of only very few species have been investigated. The genus *Leptopilina* with its numerous members provides ample opportunity to further investigate pheromone evolution, especially the evolution of species specificity. Additionally, the emergence of the pest *D. suzukii* raises more interest than the so far mainly academic interest in the (chemical) ecology of the genus *Leptopilina*.

References

Abdel-latief, M., Garbe, L. A., Koch, M., and Ruther, J. An epoxide hydrolase involved in the biosynthesis of an insect sex attractant and its use to localize the production site. *Proceedings of the National Academy of Sciences of the United States of America*, 105(26):8914–8919, 2008. doi: 10.1073/pnas.0801559105.

Allemand, R., Lemaître, C., Frey, F., Boulétreau, M., Vavre, F., Nordlander, G., van Alphen, J. J. M., and Carton, Y. Phylogeny of six African *Leptopilina* species (Hymenoptera: Cynipoidea, Figitidae), parasitoids of *Drosophila*, with description of three new species. *Annales de la Société entomologique de France (N.S.)*, 38(4):319–332, 2002. doi: 10.1080/00379271.2002.10697346.

Andersson, M. *Sexual Selection*. Princeton University Press, Princeton, NJ, 1994.

Ando, T., Inomata, S.-i., and Yamamoto, M. Lepidopteran sex pheromones. In Schulz, S., editor, *The Chemistry of Pheromones and Other Semiochemicals I*, volume 239 of *Topics in Current Chemistry*, pages 51–96. Springer Berlin Heidelberg, 2004. doi: 10.1007/b95449.

Arnqvist, G. and Nilsson, T. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, 60(2):145–164, 2000. doi: 10.1006/anbe.2000.1446.

Baker, T. C. Mechanism for saltational shifts in pheromone communication systems. *Proceedings of the National Academy of Sciences of the United States of America*, 99(21):13368–13370, 2002. doi: doi:10.1073/pnas.222539799.

Bakker, K., van Alphen, J. J. M., van Batenburg, F. H. D., van der Hoeven, N., Nell, H. W., van Strien-van Liempt, W. T. F. H., and Turlings, T. C. J. The function of host discrimination and superparasitization in parasitoids. *Oecologica*, 67(4): 572–576, 1985. doi: 10.1007/BF00790029.

Bateman, P. W., Ferguson, J. W. H., and Yetman, C. A. Courtship and copulation, but not ejaculates, reduce the longevity of female field crickets (*Gryllus bimaculatus*). *Journal of Zoology*, 268(4):341–346, 2006. doi: 10.1111/j.1469-7998.2006.00054.x.

Benelli, G., Bonsignori, G., Stefanini, C., Dario, P., and Canale, A. Male wing fanning performance during successful and unsuccessful mating in the parasitic wasp *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae). *Journal of Insect Behavior*, 26(2):228–237, 2013. doi: 10.1007/s10905-012-9356-2.

Benelli, G., Kavallieratos, N. G., Donati, E., Giunti, G., Stefanini, C., and Canale, A. Singing on the wings! male wing fanning performance affect female willingness to copulate in the aphid parasitoid *Lysipheblus testaceipes* (Hymenoptera: Braconidae: Aphidiinae. *Insect science*, 2015. doi: 10.1111/1744-7917.12201.

Bin, F., Wäckers, F., Romani, R., and Isidoro, N. Tyloids in *Pimpla turionellae* (L.) are release structures of male antennal glands involved in courtship behaviour (Hymenoptera: Ichneumonidae). *International Journal of Insect Morphology and Embryology*, 28(1–2):61–68, 1999. doi: 10.1016/S0020-7322(99)00015-X.

Blaul, B., Steinbauer, R., Merkl, P., Merkl, R., Tschochner, H., and Ruther, J. Oleic acid is a precursors of linoleic acid and the male sex pheromone in *Nasonia vitripennis*. *Insect Biochemistry and Molecular Biology*, 51:33–40, 2014. doi: 10.1016/j.ibmb.2014.05.007.

Blum, M. S. Semiochemical parsimony in the arthropoda. *Annual Review of Entomology*, 41:353–374, 1996. doi: 10.1146/annurev.en.41.010196.002033.

Boppré, M. Insects pharmacophagously utilizing plant chemicals (pyrrolozidine alkaloids). *Naturwissenschaften*, 73(1):17–26, 1986. doi: 10.1007/BF01168801.

Borden, J. H., Billany, D. J., Bradshaw, J. W. S., Edwards, M., Baker, R., and Evan, D. A. Pheromone response and sexual behaviour of *Cephalica lariciphila* Wachtl (Hymenoptera: Pamphilidae). *Ecological Entomology*, 3(1):13–23, 1978. doi: 10.1111/j.1365-2311.1978.tb00899.x.

Bordenstein, S. R. and Werren, J. H. Bidirectional incompatibility among divergent *Wolbachia* and incompatibility level differences among closely related *Wolbachia* in *Nasonia*. *Heredity*, 99:278–287, 2007. doi: 10.1038/sj.hdy.6800994.

Bradbury, J. W. and Vehrencamp, S. L. *Principles of Animal Communication*. Sinauer Associates, 2nd edition, 2011.

Breeuwer, J. A. J. and Werren, J. H. Microorganisms associated with chromosome destruction and reproductive isolation between two insec species. *Nature*, 346:558–560, 1990. doi: 10.1038/346558a0.

Buellesbach, J., Gadau, J., Beukeboom, L. W., Echinger, F., Raychoudhury, R., Werren, J. H., and Schmitt, T. Cuticular hydrocarbon divergence in the jewel wasp *Nasonia*: evolutionary shifts in chemical communication channels? *Journal of Evolutionary Biology*, 26(11):2467–2478, 2013. doi: 10.1111/jeb.12242.

Buellesbach, J., Greim, C., Raychoudhury, R., and Schmitt, T. Asymmetric assortative mating behaviour reflects incomplete pre-zygotic isolation in the *Nasonia* species complex. *Ethology*, 2014. doi: 10.1111/eth.12250.

Butenandt, A., Beckmann, R., Stamm, D., and Hecker, E. T. Über den Sexuallockstoff des Seidenspinners *Bombyx mori* – Reindarstellung und Konstitution. *Zeitschrift für Naturforschung Part B – Chemie Biochemie Biophysik Biologie Und Verwandten Gebiete*, 14(4):283–284, 1959.

Calabria, G., Máca, J., Bächli, G., Serra, L., and Pascual, M. First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *Journal of Applied Entomology*, 136(1–2):139–147, 2012. doi: 10.1111/j.1439-0418.2010.01583.x.

Cardé, R. T., Doane, C. C., and Roelofs, W. L. Diel periodicity of male sex pheromone response and female attractiveness in the gypsy moth (Lepidoptera: Lymnatriidae). *The Canadian Entomologist*, 106(5):479–484, 1974. doi: 10.4039/Ent106479-5.

Cardé, R. T., Comeau, A., Baker, T. C., and Roelofs, W. L. Moth mating periodicity: Temperature regulates the circadian gate. *Experientia*, 31(1):46–48, 1975. doi: 10.1007/BF01924672.

Carlson, D. A. Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. *Analytical Chemistry*, 61:1564–1571, 1989. doi: 10.1021/ac00189a019.

Carlson, D. A., Bernier, U. R., and Sutton, B. D. Elution patterns from capillary GC for methyl-branched-alkanes. *Journal of Chemical Ecology*, 24(11):1845–1865, 1998. doi: 10.1023/A:1022311701355.

Carton, Y., Haouas, S., Marrakchi, M., and Hochberg, M. Two competing parasitoid species coexist in sympatry. *Oikos*, 60:220–230, 1991.

Castrovillo, P. J. and Cardé, R. T. Environmental regulation of female calling and male pheromone response periodicities in the codling moth (*Laspeyresui pomonella*). *Journal of Insect Physiology*, 25(8):659–667, 1979. doi: 10.1016/0022-1910(79)90116-1.

Chabert, S., Allemand, R., Poyet, M., Eslin, P., and Gilbert, P. Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, *Drosophila suzukii*. *Biological Control*, 31(1):40–47, 2012. doi: 10.1016/j.biocontrol.2012.05.005.

Cini, A., Ioratti, C., and Anfora, G. A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. *Bulletin of Insectology*, 65(1):149–160, 2012. URL `http://hdl.handle.net/10449/21029`.

Cook, J. M. Sex determination in the Hymenoptera: a review of models and evidence. *Heredity*, 71:421–435, 1993. doi: 10.1038/hdy.1993.157.

Cuthbertson, A. G. S., Blackburn, L. F., and Audsley, N. Efficacy of commercially available invertebrate predators against *Drosophila suzukii*. *Insects*, 5:952–960, 2014. doi: 10.3390/insects5040952.

Davies, N. B., Krebs, J. R., and West, S. A. *An Introduction to Behavioural Ecology*, chapter Sexual Selection, Sperm Competition and Sexual Conflict, pages 179–222. Wiley-Blackwell, 4th edition, 2012.

Debout, G., Fauvergue, X., and Fleury, F. The effect of foundress number on sex ratio under partial local mate competition. *Ecological Entomology*, 27:242–246, 2002. doi: 10.1046/j.1365-2311.2002.00402.x.

Do Nascimento, R. R., Billen, J., Sant'ana, A. E. G., Morgan, E. D., and Harada, A. Y. Pygidial gland of *Azteca* nr. *bicolor* and *Azteca chartifex*: Morphology and chemical identification of volatile components. *Journal of Chemical Ecology*, 24(10):1629–1637, 1998. doi: 10.1023/A:1020864427854.

Eggleton, P. and Belshaw, R. Insect parasitoids: An evolutionary overview. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 337(1279):1–20, 1992. doi: 10.1098/rstb.1992.0079.

Eggleton, P. and Belshaw, R. Comparisons of dipteran, hymenopteran and coleopteran parasitoids: provisional phylogenetic explanations. *Biological Journal of the Linnean Society*, 48(3):213–226, 1993. doi: 10.1111/j.1095-8312.1993.tb00888.x.

Eggleton, P. and Gaston, K. J. "parasitoid" species and assemblages: convenient definitions or misleading compromises. *Oikos*, 59(3):417–421, 1990.

El-Sayed, A. M. The Pherobase: Database of pheromones and semiochemicals, 2009–2013. URL `http://www.pherobase.com`.

El-Sayed, A. M. The Pherobase: Database of pheromones and semiochemicals, 2014. URL `http://www.pherobase.com`.

Endler, J. A. Some general comments on the evolution and design of animal communication systems. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 340(1292):215–225, 1993. doi: 10.1098/rstb.1993.0060.

Fauvergue, X., Fleury, F., Lemaître, C., and Allemand, R. Parasitoid mating structures when hosts are patchily distributed: field and laboratory experiments with *Leptopilina boulardi* and *Leptopilina heterotoma*. *Oikos*, (86):344–356, 1999.

Fischman, C. J., Adler, S., and Hofferberth, J. E. Divergent diastereoselective synthesis of iridomyrmecin, isoiridomyrmecin, teucrimulactone, and dolicholactone from citronellol. *Journal of Organic Chemistry*, 78(14):7318–7323, 2013. doi: 10.1021/jo400884g.

Fleury, F., Allemand, R., Fouillet, P., and Boulétreau, M. Genetic variation in locomotor activity rhythm among populations of *Leptopilina heterotoma* (Hymenoptera: Eucoilidae), a larval parasitoid of *Drosophila* species. *Behavior Genetics*, (25):81–89, 1995. doi: 10.1007/BF02197245.

Fleury, F., Vavre, F., Ris, N., Fouillet, P., and Boulétrau, M. Physiological cost induced by the maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid *Leptopilina heterotoma*. *Parasitology*, 121(5):493–500, 2000. doi: 10.1017/S0031182099006599.

Fleury, F., Gibert, P., Ris, N., and Allemand, R. *Advances in Parasitology*, volume 70, chapter Ecology and Life History Evolution of Frugivorous *Drosophila* Parasitoids, pages 3–44. Academic Press, 2009. doi: 10.1016/S0065-308X(09)70001-6.

Forshage, M., Nordlander, G., and Buffington, M. L. Eucoilinae of North America: A revised catalog of genera and described species. *Proceedings of the Entomological Society of Washington*, 115(3):225–255, 2013. doi: 10.4289/0013-8797.115.3.225.

Gay, L., Eady, P. E., Vasudev, R., Hosken, D. J., and Tregenze, T. Costly sexual harassment in a beetle. *Physiological Entomology*, 34(1):86–92, 2009. doi: 10.1111/j.1365-3032.2008.00656.x.

Geiselhardt, S., Jakobschy, D., Ockenfels, P., and Peschke, K. A sex pheromone in the desert tenebrionid beetle *Parastizopus armaticeps*. *Journal of Chemical Ecology*, (34):1065–1071, 2008.

Gibbs, A. G. Water-proofing properties of cuticular lipids. *American Zoologist*, (38):471–482, 1998.

Godfray, H. C. J. *Parasitoids: Behavioral and Evolutionary Ecology*. Princeton University Press, Princeton, 1994.

Grillenberger, B. K., van de Zande, L., Bijlsma, R., Gadau, J., and Beukeboom, L. W. Reproductive strategies under multiparasitism in natural populations of the parasitoid wasp *Nasonia* (Hymenoptera). *Journal of Evolutionary Biology*, 22(3):460–470, 2009. doi: 10.1111/j.1420-9101.2008.01677.x.

Gueguen, G., Onemola, B., and Govind, S. Association of a new *Wolbachia* strain with, and its effects on, *Leptopilina victoriae*, a virulent waps parasitic to *Drosophila* spp. *Applied and Environmental Microbiology*, 78(16):5962–5966, 2012. doi: 10.1128/AEM.01058-12.

Hamilton, W. D. Extraordinary sex ratios. *Science*, 156(3477):477–488, 1976. doi: 10.1126/science.156.3774.477.

Hammack, L. Calling behavior in female western corn rootworm beetles (Coleoptera: Chrysomelidae). *Annals of the Entomological Society of America*, 88(4):562–569, 1995.

Hardie, J. and Minks, A. K., editors. *Pheromones of non-lepidopteran insects associated with agricultural plants*. CABI Publishing, Wallingford, UK, 1999.

Hare, D. J. *Methods in Chemical Ecology Volume 2*, chapter Bioassay Methods with Terrestrial Invertebrates, pages 212–270. Springer US, 1998. doi: 10.1007/978-1-4615-5411-0_5.

Hauser, M. A historic account of the invasion of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the continental United States, with remarks on their identification. *Pest Management Science*, 67(11):1352–1357, 2011. doi: 10.1002/ps.2265.

Haynes, K. F. and Birch, M. C. Temporal reproductive isolation between two species of plume moths (Lepidoptera: Prerophoriae). *Annals of the Entomological Society of America*, 79(1):210–251, 1986.

Hedlund, K., Vet, L. E. M., and Dicke, M. Generalist and specialist parasitoid strategies of using odours of adult drosophilid flies when searching for larval hosts. *Oikos*, (77):390–398, 1996.

Heimpel, G. E. and de Boer, J. G. Sex determination in the Hymenoptera. *Annual Review of Entomology*, 53:209–230, 2008. doi: 10.1146/annurev.ento.53.103106.093441.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., and Werren, J. H. How many species are infected with *Wolbachia*? – a statistical analysis of current data. *FEMS Microbiology Letters*, 281(2):215–220, 2008. doi: 10.1111/j.1574-6968.2008.01110.x.

Hilgraf, R., Zimmermann, N., Lehmann, L., Tröger, A., and Francke, W. Stereoselective synthesis of trans-fused iridoid lactones and their identification in the parasitoid wasp *Alloxysta victrix*, part ii; iridomyrmecins. *Beilstein Journal of Organic Chemistry*, (8):1256–1264, 2012.

Howard, R. W. *Insect Lipids: Chemistry, Biochemistry, and Biology*, chapter Cuticular Hydocarbons and Chemical Communication, pages 179–226. University of Nebraska Press, Lincoln, NE, 1993.

Howard, R. W. and Blomquist, G. J. Ecologival, behavioural and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology*, (50):371–393, 2005.

Huth, A. and Dettner, K. Defense cchemical from abdominal glands of 13 rover beetle species of subtribe staphylinina (Coleoptera: Staphylinidae, Staphylininae). *Journal of Chemical Ecology*, 16(9):2691–2711, 1990. doi: 10.1007/BF00988079.

Hübner, G. and Dettner, K. Hyperparasitoid defense strategies against spiders: the role of chemical and morphological protection. *Entomologia Experimentalis et Applicata*, (97):64–74, 2000.

Hübner, G., Völkl, W., Francke, W., and Dettner, K. Mandibular gland secretions in alloxystine wasps (Hymenoptera, Cynipoidea, Charipidae): do ecological or phylogenetical constraints influens occurrence or composition. *Biochemical Systematics and Ecology*, (30):505–523, 2002.

Isidoro, N. and Bin, F. Male antennal gland of *Amitus spiniferus* (Brethes) (Hymenoptera: Platygastridae), likely involved in courtship behavior. *International Journal of Insect Morphology and Embryology*, 24(4):365–373, 1995. doi: 10.1016/0020-7322(95)00014-U.

Isidoro, N., Bin, F., Romani, R., Pujade-Villar, J., and Palmira, R.-F. Diversity and function of male antennal glands in Cynipoidea (Hymenoptera). *Zoologica Scripta*, 28(1–2):165–174, 1999. doi: 10.1046/j.1463-6409.1999.00013.x.

Janssen, A., van Alphen, J. J. M., Sabelis, M. W., and Bakker, K. Specificity of odour-mediated avoidance of competition in *Drosophila* parasitoids. *Behavioral Ecology and Sociobiology*, (36):229–235, 1995a. doi: 10.1007/BF00165831.

Janssen, A., van Alphen, J. J. M., Sabelis, M. W., and Bakker, K. Odour-mediated avoidance of competition in *Drosophila* parasitoids: The ghost of competition. *Oikos*, 73:356–366, 1995b.

Jenni, W. Beitrag zur Morphologie und Biologie der Cynipide *Pseudeucoila bochei* Weld, eines Larvenparasiten von *Drosophila melanogaster* Meig. *Acta Zoologica*, (32):177–254, 1951.

Kaiser, L., Couty, A., and Perze-Maluf, R. *Advances in Parasitology*, chapter Dynamic Use of Fruit Odours to Locate Host Larvae: Individual Learning, Physiological State and Genetic Variability as Adaptive Mechanisms, pages 67–95. Academic Press, 2009. doi: 10.1016/S0065-308X(09)70003-X.

Kasuya, N., Mitsui, H., Shinsuke, I., Watada, M., and Kimura, M. T. Ecological, morphological and molecular studies on *Ganaspis* individuals (Hymenoptera: Figitidae) attacking *Drosophila suzukii* (Diptera: Drosophilidae). *Applied Entomology and Zoology*, 48(1):87–92, 2013. doi: 10.1007/s13355-012-0156-0.

King, B. H., Saporito, K. B., Ellison, J. H., and Bratzke, R. M. Unattractiveness of mated females to males in the parasitoid wasp *Spalangia endius*. *Behavioral Ecology and Sociobiology*, 57:350–356, 2005. doi: 10.1007/s00265-004-0863-9.

Klassen, W. *Sterile Insect Technique*, chapter Area-Wide Integrated Pest Management and the Sterile Insect Technique, pages 39–68. Springer Netherlands, 2005. doi: 10.1007/1-4020-4051-2_2.

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

Laurent, P., Braekman, J.-C., and Daloze, D. *The Chemistry of Pheromones and Other Semiochemicals II*, chapter Insect Chemical Defense, pages 167–229. Springer, 2005.

Löfqvist, J. Formic acid and saturated hydrocarbons as alarm pheromones for the ant *Formica rubra*. *Journal of Insect Physiology*, (22):1331–1346, 1976.

Löfstedt, C. Moth pheromone genetics and evolution. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 340(1292):167–177, 1993. doi: 10.1098/rstb.1993.0055.

Löfstedt, C., Herrebout, W. M., and Menken, S. B. J. Sex pheromones and their potential role in the evolution of reproductive isolation in smale ermine moths (Yponomeutidae). *Chemoecology*, 2(1): 20–28, 1991. doi: 10.1007/BF01240662.

Ma, M. and Burkholder, W. E. Sex pheromone releasing behavior of *Anthrenus flavipes* (furniture carpet beetles) females (Coleoptera: Dermestidae). *Annals of the Entomological Society of America*, 71 (1):129–133, 1978.

McLain, D. K. and Pratt, A. E. The cost of sexual coercion and heterospecific sexual harassment on the fecundity of a host-specific, seed-eating insect (*Neacoryphus bicruis*). *Behavioral Ecology and Sociobiology*, 46(3):164–170, 1999. doi: 10.1007/s002650050606.

McNeil, J. M. and Brodeur, J. Pheromone-mediated mating in the aphid parasitoid, *Aphidius nigripes* (Hymenoptera: Aphididae). *Journal of Chemical Ecology*, 21(7):959–972, 1995. doi: 10.1007/BF02033801.

Mowles, S. L., King, B. H., Linforth, R. S. T., and Hardy, I. C. W. A female-emitted pheromone component is associated with reduced male courtship in the parasitoid wasp *Spalangia endius*. *PLOS ONE*, 8(11):e82010, 2013. doi: 10.1371/journal.pone.0082010.

Nelson, D. R. *Insect Lipids – Chemistry, Biochemistry and Biology*, chapter Methyl-branched lipids in insects, pages 271–315. University of Nebraska Press, Lincoln, NE, 1993.

Niehuis, O., Buellesbach, J., Gibson, J. D., Pothmann, D., Hanner, C., Mutti, N. S., Judson, A. K., Gadau, J., Ruther, J., and Schmitt, T. Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature*, (494):345–348, 2013.

Nordlander, G. Revision of the genus *Leptopilina* Förster, 1869, with notes on the status of some other genera (Hymenoptera, Cynipoidea: Eucoilidae). *Entomologica Scandinavia*, 11(4):428–453, 1980. doi: 10.1163/187631280794710024.

Nordlander, G. and Grijpma, P. Systematics and biology of *Rhoptromeris strobigena* sp. n., a parasitoid of chloropids inhabiting conifer cones (Hymenoptera: Cynipoidea: Eucoilidae). *Entomologica Scandinavia*, 22(2):209–218, 1991. doi: 10.1163/187631291X00084.

Nordlund, D. A. and Lewis, W. J. Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *Journal of Chemical Ecology*, 2(2):211–220, 1976. doi: 10.1007/BF00987744.

Novkovic, B., Mitsui, H., Suwito, A., and Kimura, M. T. Taxonomy and phylogeny of *Leptopilina* species (Hymenoptera: Cynipoidea: Figitidae) attacking frugivorous drosophilid flies in Japan, with description of three new species. *Entomological Science*, 14(3):333–346, 2011. doi: 10.1111/j.1479-8298.2011.00459.x.

Ohmura, W., Hishiyama, S., Nakashima, T., Kato, A., Makihara, H., Ohira, T., and Irei, H. Chemical composition of the defensive secretion of the longhorned beetle, *Chloridolum loochooanum*. *Journal of Chemical Ecology*, (35):250–255, 2009.

Pannebakker, B. A., Garrido, N. R. T., Zwaan, B. J., and van Alphen, J. J. M. Geographic variation in host-selection behaviour in the *Drosophila* parasitoid *Leptopilina clavipes*. *Entomologia Experimentalis et Applicata*, (127):48–54, 2008.

Pasteels, J. M., Braekman, J. C., Daloze, D., and Ottinger, R. Chemical defence in chrysomelid larvae and adults. *Tetrahedron*, (38):1891–1897, 1982.

Pintureau, B., Chapelle, L., and Delobel, B. Effects of repeated thermic and antibiotic treatments on a *Trichogramma* (Hym., Trichogrammatidae) symbiont. *Journal of Applied Entomology*, 123(8):473–483, 1999. doi: 10.1046/j.1439-0418.1999.00412.x.

Pintureau, B., Lassablière, F., Daumal, J., and Grenier, S. Does a cyclic natural thermal cure occur in *Wolbachia*-infected *Trichogramma* species? *Ecological Entomology*, 27(3):466–372, 2002. doi: 10.1046/j.1365-2311.2002.00416.x.

Quicke, D. L. J. *Parasitic wasps*. Chapman & Hall, London, 1997.

Quinlan, J. A revision of some aftrotropical genera of Eucoilidae (Hymenoptera). *Bulletin of the British Museum (Natural History) Entomology*, 56: 171–229, 1988.

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

R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2014. URL `http://www.R-project.org/`.

Ridley, M. Clutch size and mating frequency in parasitic Hymenoptera. *The American Naturalist*, 142 (5):893–910, 1993.

Roelofs, W. L. and Brown, R. L. Pheromones and evolutionary relationships of Tortricidae. *Annual Review of Ecology and Systematics*, 13:395–422, 1982. doi: 10.1146/annurev.es.13.110182.002143.

Roelofs, W. L. and Rooney, A. P. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proceedings of the National Academy of Sciences of the United States of America*, (100): 9179–9184, 2003.

Roelofs, W. L., Liu, W., Hao, G., Jiao, H., Rooney, A. P., and Linn, C. E. Jr. Evolution of moth sex pheromones via ancestral genes. *Proceedings of the National Academy of Sciences of the United States of America*, 99(21), 2002. doi: 10.1073/pnas.152445399.

Romani, R., Isidoro, N., Riolo, P., Bin, F., Fortunato, A., Turillazzi, S., and Beani, L. A new role for antennation in paper wasps (Hymenoptera, Vespidae): antennal courtship and sex dimorphic glands in antennomeres. *Insectes Sociaux*, 52(1): 92–102, 2005. doi: 10.1007/s00040-004-0780-y.

Romani, R., Rosi, M. C., Isidoro, N., and Bin, F. The role of the antennae during courtship behaviour in the parasitic wasp *Trichopria drosophilae*. *The Journal of Experimental Biology*, 211:2486–2491, 2008. doi: 10.1242/âĂŃjeb.013177.

Ruther, J. *Chemical Ecology of Insect Parasitoids*, chapter Novel insights into pheromone-mediated communication in parasitic hymenopterans, pages 112–144. Wiley-Blackwell, Hoboken, NJ, 2013.

Ruther, J. and Hammerl, T. An oral male courtship pheromone terminates the response of *Nasonia vitripennis* females to the male-produced sex attractant. *Journal of Chemical Ecology*, 40(1):52–62, 2014. doi: 10.1007/s10886-013-0372-2.

Ruther, J. and Steiner, S. Costs of female odour in males of the parasitic wasp *Lariophagus distinguendus* (Hymenoptera: Pteromalidae). *Naturwissenschaften*, (95):547–552, 2008. doi: 10.1007/s00114-008-0357-0.

Ruther, J., Homann, M., and Steidle, J. L. M. Female-derived sex pheromone mediates courtship behaviour in the parasitoid *Lariophagus distinguendus*. *Entomologia Experimentalis et Applicata*, 96(3):265–274, 2000. doi: 10.1046/j.1570-7458.2000.00705.x.

Ruther, J., Reinecke, A., Tolasch, T., and Hilker, M. Make love not war: a common arthropod defence compound as sex pheromone in the forest cockchafer *Melolontha hippocastani*. *Oecologica*, (128):44–47, 2001.

Ruther, J., Thal, K., Blaul, B., and Steiner, S. Behavioural switch in the sex pheromone response of *Nasonia vitripennis* females is linked to receptivity signalling. *Animal Behaviour*, 80(6):1035–1040, 2010. doi: 10.1016/j.anbehav.2010.09.008.

Saul, G. B. 2nd. An analysis of non-reciprocal cross incompatibility in *Mormoniella vitripennis* (Walker). *Zeitschrift für Vererbungslehre*, 92(1): 28–33, 1961. doi: 10.1007/BF01854097.

Schiestl, F. P. and Ayasse, M. Post-mating odor in females of the solitary bee, *Andrena nigroaenea* (Apoidea, Andrenidae), inhibits male mating behavior. *Behavioral Ecology and Sociobiology*, 48 (4):303–307, 2000. doi: 10.1007/s002650000241.

Schilthuizen, M., Nordlander, G., Stouthamer, R., and van Alphen, J. J. M. Morphological and molecular phylogenetics in the genus *Leptopilina* (Hymenoptera: Cynipoidea: Eucoilidae). *Systematic Entomology*, 23(3):253–264, 1998. doi: 10.1046/j.1365-3113.1998.00049.x.

Schreiber, S. L., Meyers, H. V., and Wiberg, K. B. Stereochemistry of the intramolecular enamine/enal (enone) cycloaddition reaction and subsequent transformations. *Journal of the American Chemical Society*, (108):8274–8277, 1986. doi: 10.1021/ja00286a034.

Schröder, R. and Hilker, M. The relevance of background odor in resource location by insects: A behavioral approach. *BioScience*, (58):308–316, 2008. doi: 10.1641/B580406.

Scott-Phillips, T. C. Defining biological communication. *Journal of Evolutionary Biology*, 21(2):387–395, 2008. doi: 10.1111/j.1420-9101.2007.01497.x.

Smith, J. M. and Harper, D. *Animal Signals*. Oxford University Press, 2003.

Smith, R. F., Pierce, H. D. Jr., and Borden, J. H. Sex pheromone of the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *Journal*

of Chemical Ecology, 17(7):1437–1447, 1991. doi: 10.1007/BF00983775.

Sorensen, P. W. and Stacey, N. E. *Advances in Chemical Signals in Vertebrates*, pages 15–47. Kluwer Academic Publishers, 1999.

Steiger, S. and Stökl, J. The role of sexual selection in the evolution of chemical signals in insects. *Insects*, 5(2):423–438, 2014. doi: 10.3390/insects5020423.

Steiger, S., Schmitt, T., and Schaefer, H. M. The origin and dynamic evolution of chemical information transfer. *Proceedings of the Royal Society of London B: Biological Sciences*, (278):970–979, 2011. doi: 10.1098/rspb.2010.2285.

Steiner, S., Steidle, J. L. M., and Ruther, J. Female sex pheromone in immature insect males – a case of pre-emergence chemical mimicry? *Behavioral Ecology and Sociobiology*, (58):111–120, 2005.

Steiner, S., Hermann, N., and Ruther, J. Characterization of a female-produced courtship pheromone in the parasitoid *Nasonia vitripennis*. *Journal of Chemical Ecology*, 32(8):1687–1702, 2006. doi: 10.1007/s10886-006-9102-3.

Stewart-Jones, A., Dewhirst, S. Y., Durrant, L., Fitzgerald, J. D., Hardie, J., Hooper, A. M., Pickett, J. A., and Poppy, G. M. Structure, rratio and patterns of release in the sex pheromone of an aphid, *Dysaphis plantaginea*. *Journal of Experimental Biology*, 210:4335–4344, 2007. doi: 10.1242/jeb.009944.

Stökl, J., Hofferberth, J., Pritschet, M., Brummer, M., and Ruther, J. Stereoselective chemical defense in the *Drosophila* parasitoid *Leptopilina heterotoma* is mediated by (−)-iridomymrecin and (+)-isoiridomyrmecin. *Journal of Chemical Ecology*, 38(4):331–339, 2012. doi: 10.1007/s10886-012-0103-0.

Stökl, J., Dandekar, A.-T., and Ruther, J. High chemical diversity in a wasp pheromone: a blend of methyl 6-methylsalicylate, fatty alcohol acetates and cuticular hydrocarbons releases courtship behavior in the *Drosophila* parasitoid *Asobara tabida*. *Journal of Chemical Ecology*, 40(2):159–168, 2014. doi: 10.1007/s10886-014-0378-4.

Symonds, M. R. E. and Elgar, M. A. The mode of pheromone evolution: evidence from bark beetles. *Proceedings of the Royal Society of London B: Biological Sciences*, 271(1514):839–846, 2004. doi: 10.1098/rspb.2003.2647.

Symonds, M. R. E. and Elgar, M. A. The evolution of pheromone diversity. *Trends in Ecology and Evolution*, 23(4):220–228, 2008. doi: 10.1016/j.tree.2007.11.009.

Tallamy, D. W., Darlington, M. B., Pesek, J. D., and Powell, B. E. Copulatory courtship signals male genetic quality in cucumber beetles. *Proceedings of the Royal Society of London B: Biological Sciences*, 270(1510):77–82, 2003. doi: 10.1098/rspb.2002.2198.

Tillman, J. A., Seybold, S. J., Jurenka, R. A., and Blomquist, G. J. Insect pheromones—an overview of biosynthesis and endocrine regulation. *Insect Biochemistry and Molecular Biology*, 29(6):481–514, 1999. doi: 10.1016/S0965-1748(99)00016-8.

Tinbergen, N. 'derived' activities; their causation, biological significance, origin and emancipation during evolution. *Quarterly Review of Biology*, 27 (1):1–32, 1952.

van Alphen, J. J. M., Nordlander, G., and Eijs, I. Host habitat finding and host selection of the *Drosophila* parasitoid *Leptopilina australis* (Hymenoptera, Eucoilidae), with a comparison of the niches of European *Leptopilina* species. *Oecologica*, 87(3): 324–329, 1991. doi: 10.1007/BF00634586.

van den Assem, J. Reproductive behaviour of *Pseudeucoila bochei* (Hymenoptera: Cynipidae). *Netherlands Journal of Zoology*, 19(4):641–649, 1968. doi: 10.1163/002829669X00080.

van den Assem, J. *Insect Parasitoids*, chapter Mating Behaviour in Parasitic Wasps, pages 137–167. Academic Press, London, 1986.

van den Assem, J., Jachmann, F., and Simbolotti, P. Courtship behaviour of *Nasonia vitripennis* (Hym., Pteromalidae): Some qualitative, experimental evidence for the role of pheromones. *Behaviour*, 75 (3):301–307, 1980. doi: 10.1163/156853980X00456.

van Lenteren, J. C. *Biological Control: Measures of Success*, chapter Success in Biological Control of Arthropods by Augmentation of Natural Enemies, pages 77–103. Springer Netherlands, 2000. doi: 10.1007/978-94-011-4014-0_3.

Völkl, W., Hübner, G., and Dettner, K. Interactions between *Alloxysta brevis* (Hymenoptera, Cynipoidea, Alloxystidae) and honeydew-collecting ants: How an aphid hyperparasitoid overcomes ant aggression by chemical defense. *Journal of Chemical Ecology*, 20(11):2901–2915, 1994. doi: 10.1007/BF02098397.

Wachi, N., Nomano, F. Y., Mitsui, H., Kasuya, N., and Kimura, M. T. Taxonomy and evolution of putative thelytokous species of *Leptopilina* (Hymenoptera: Figitidae) from Japan, with description of two new species. *Entomological Science*, 18(1):41–54, 2015. doi: 10.1111/ens.12089.

Walter, F., Fletcher, J. C., Chautems, D., Cheric, D., Keller, L., Francke, W., Fortelius, W., Rosengren, R., and Vargo, E. L. Identification of the sex pheromone of an ant, *Formica lugubris* (Hymenoptera, Formicidae). *Naturwissenschaften*, 80(1): 30–34, 1993. doi: 10.1007/BF01139755.

Weiss, I., Rössler, T., Hofferberth, J., Brummer, M., Ruther, J., and Stökl, J. A nonspecific defensive compound evolves into a competition avoidance cue and a female sex pheromone. *Nature Communications*, 4, 2013. doi: 10.1038/ncomms3767.

Weiss, I., Hofferberth, J., Ruther, J., and Stökl, J. Varying importance of cuticular hydrocarbons and iridoids in the species-specific mate recognition pheromones of three closely related *Leptopilina* species. *Frontiers in Ecology and Evolution*, 3(19), 2015. doi: 10.3389/fevo.2015.00019.

Werren, J. H., Baldo, L., and Clark, M. E. *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6:741–751, 2008. doi: 10.1038/nrmicro1969.

Wiskerke, J. S., Dicke, M., and Vet, L. E. M. Larval parasitoid uses aggregation pheromone of adult hosts in foraging behaviour: a solution to the reliability-detectability problem. *Oecologica*, 93(1): 145–148, 1993. doi: 10.1007/BF00321204.

Wyatt, T. D. *Pheromones and Animal Behaviour: Communication by Smell and Taste*. Cambridge University Press, 2003.

Wyatt, T. D. Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative Physiology A*, (196):685–700, 2010. doi: 10.1007/s00359-010-0564-y.

Wyatt, T. D. *Chemical Communication in Crustaceans*, chapter Pheromones and Behavior, pages 23–38. Springer, 2011. doi: 10.1007/978-0-387-77101-4_2.

Wyatt, T. D. *Pheromones and Animal Behaviour: Chemical Signals and Signatures*. Cambridge University Press, Cambridge, UK, 2nd edition, 2014.

Acknowledgements

I thank all those who contributed—directly or indirectly—to my dissertation.

First and foremost, I thank Johannes Stökl, my supervisor. I thank Joachim Ruther, leader of the Chemical Ecology group and first mentor of the dissertation. Johannes and Joachim, thank you for giving me the opportunity for this dissertation, for guiding me through it, and for helping me to overcome any obstacle that surfaced. You gave me the opportunity to find my own way and encouraged me to pursue my own ideas. I always felt that I could rely on your help when I was stuck, though.

I thank Michael Gebhardt, second mentor of the dissertation. Michael, thank you for providing valuable input and discussion.

I thank all those who, through the course of their own work in the Chemical Ecology group, contributed to my dissertation, especially Thomas Rössler and Michael Brummer.

I thank all members of the Chemical Ecology group. Working with you was a very pleasant experience. You provided not only discussion and ideas, but also the laughter and the warmth that made me feel at home in the group.

I thank John Hofferberth for providing synthetic samples of several iridomyrmecins, as well as for his contributions to chapter 2 and chapter 4.

I also thank Thomas Hoffmeister, Roland Allemand, and Leo W. Beukeboom for providing starter cultures for *L. heterotoma*, *L. boulardi*, and *L. victoriae*, respectively.

I thank Johannes Petermeier for his advice on the statistical analysis in chapter 3.

I thank Sarah Weiß for proofreading the unpublished and unsubmitted parts of this dissertation.

Last but not least, I thank the Deutsche Forschungsgemeinschaft for the funding (grant STO 966/1-1 to Johannes Stökl).

A. Supplementary information for chapter 2

Table A.1.: Species specificity of mate recognition. Test statistics for pairwise Mann-Whitney U-tests of wing fanning duration from the mate recognition experiment (fig. 2.6). P-values (rounded to third decimal) were corrected using the Bonferroni-Holm method. Uncorrected P-values (rounded to third decimal) are gigen in parantheses. For each experiment $n = 12$.

	L. heterotoma iridoids (DCM) fraction	*L. boulardi* iridoids (DCM) fraction	synth. (−)-iridomyrmecin	DCM
L. heterotoma iridoids (DCM fraction)	-	$U = 80$	$U = 63.5$	$U = 28.5$
L. boulardi iridoids (DCM fraction)	$P = 0.005$ ($P = 0.001$)	-	$U = 176.5$	$U = 103$
synth.(−)-iridomyrmecin	$P = 0.001$ ($P < 0.001$)	$P = 0.529$ ($P = 0.529$)	-	$U = 112$
DCM	$P < 0.001$ ($P < 0.001$)	$P = 0.016$ ($P = 0.005$)	$P = 0.021$ ($P = 0.011$)	-

Table A.2.: Behavioural assays. Y-tube experiments conducted to identify the competition avoidance agent (1.A–1.I) and the female sex pheromone (2.A–2.I and 2S.A–2S.E) in *L. heterotoma*.

Experiment	Test arm	Control arm	Responding individual
Identification of the competition avoidance agent.			
1.A	Host patch	Empty Erlenmeyer flask	*L. heterotoma* mated female
1.B	Host patch with 5 mated *L. heterotoma* females	Host patch	*L. heterotoma* mated female
1.C	Host patch with 5 mated *L. boulardi* females	Host Patch	*L. heterotoma* mated female
1.D	*L. heterotoma* female extract	Solvent	*L. heterotoma* mated female
1.E	Host patch plus extract of virgin *L. heterotoma* females	Host patch plus solvent	*L. heterotoma* mated female
1.F	Host patch plus CHC fraction of female extract	Host patch plus solvent	*L. heterotoma* mated female
1.G	Host patch plus iridoid fraction of female extract	Host patch plus solvent	*L. heterotoma* mated female
1.H	Host patch plus synth. (−)-iridomyrmecin	Host patch plus solvent	*L. heterotoma* mated female
1.I	Host patch plus synth. (+)-isoiridomyrmecin	Host patch plus solvent	*L. heterotoma* mated female
Identification of the female sex pheromone.			
2.A	10 virgin *L. heterotoma* females in Erlenmeyer flask	Empty Erlenmeyer flask	*L. heterotoma* virgin male
2.B	Extract of virgin *L. heterotoma* females	Solvent	*L. heterotoma* virgin male
2.C	CHC fraction of female extract	Solvent	*L. heterotoma* virgin male
2.D	Iridoid fraction of female extract	Solvent	*L. heterotoma* virgin male
2.E	synth. (−)-iridomyrmecin and (+)-isoiridomyrmecin	Solvent	*L. heterotoma* virgin male
2.F	Iridoid fraction of female extract, p5 (=(−)-iridomyrmecin) removed	Solvent	*L. heterotoma* virgin male
2.G	Iridoid fraction of female extract, p5 replaced with synth. (−)-iridomyrmecin	Solvent	*L. heterotoma* virgin male
2.H	Iridoid fraction of female extract, p5 replaced with synth. (+)-iridomyrmecin	Solvent	*L. heterotoma* virgin male
2.I	Extract of virgin *L. boulardi* females	Solvent	*L. heterotoma* virgin male
2S.A	Iridoid fraction of female extract, p4 removed	Solvent	*L. heterotoma* virgin male
2S.B	Iridoid fraction of female extract, p1, p2, p3, and p4 removed	Solvent	*L. heterotoma* virgin male
2S.C	Iridoid fraction of female extract, p4 and p6 (=(+)-isoiridomyrmecin) removed	Solvent	*L. heterotoma* virgin male
2S.D	Iridoid fraction of female extract, p6 and p7 removed	Solvent	*L. heterotoma* virgin male
2S.E	Iridoid fraction of female extract, p3 and p4 removed	Solvent	*L. heterotoma* virgin male

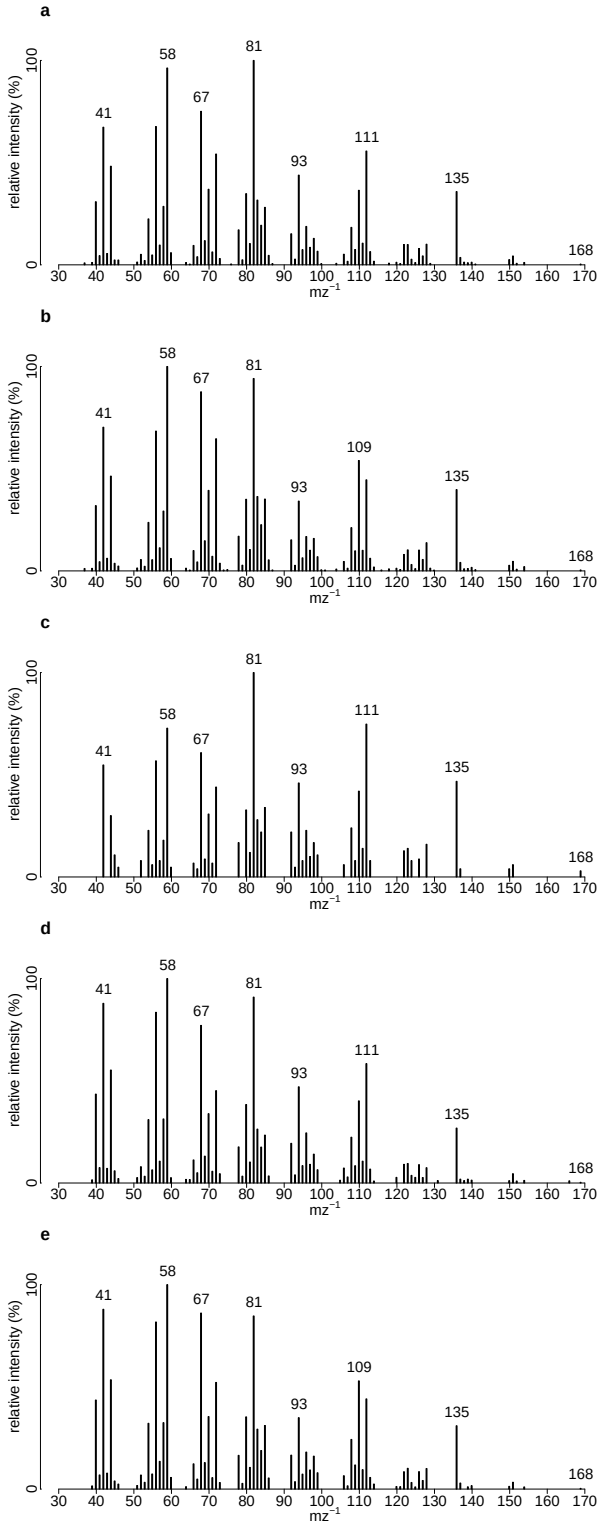

Figure A.1.: Tentative identification of p1 and p2. Mass spectra (EI) of (**a**) compound p1 and (**b**) compound p2 from *L. heterotoma* females, (**c**) iridodial (Ohmura et al., 2009), and (**d**), and (**e**) iridodials derived from (epi-)/chrysomelidial.

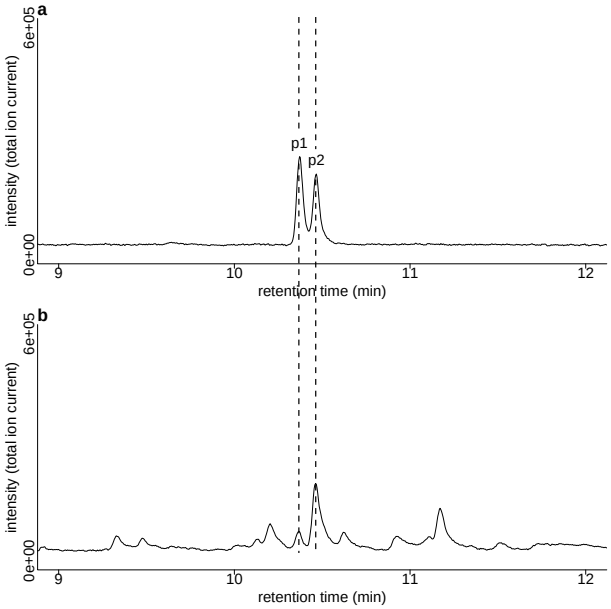

Figure A.2.: Tentative identification of p1 and p2. Total ion current chromatograms on a non-polar column of (**a**) the female sex pheromone of *L. heterotoma* and (**b**) the iridodials derived from (epi-)/chrysomelidial.

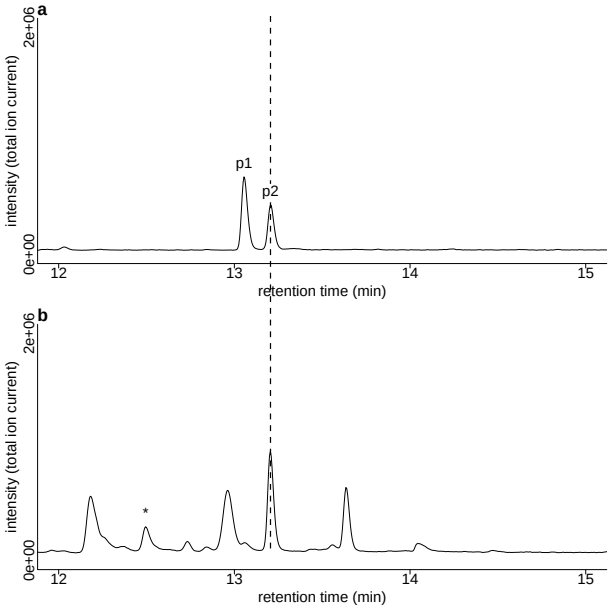

Figure A.3.: Tentative identification of p1 and p2. Total ion current chromatograms on a cyclodextrin (Beta DEX 225) column of (**a**) the female sex pheromone of *L. heterotoma* and (**b**) the iridodials derived from (epi-)/chrysomelidial. The asterisk denotes the compound that coeluted with p1 on the non-polar column.

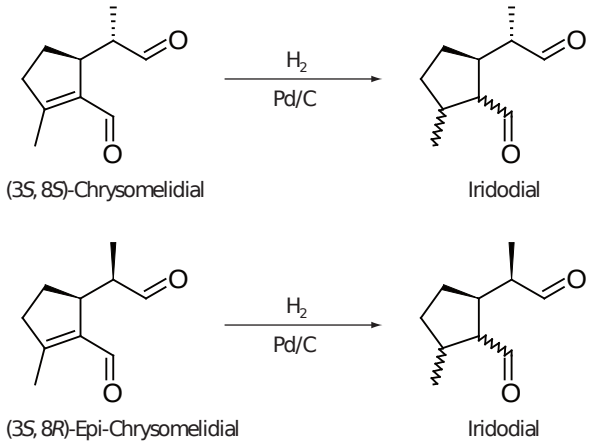

Figure A.4.: Hydrogenation of (epi-)/chrysomelidial. Catalytic hydrogenation of (epi-)/chrysomelidial yields multiple stereoisomers of iridodial through the addition of hydrogen at the carbon-carbon double bond.

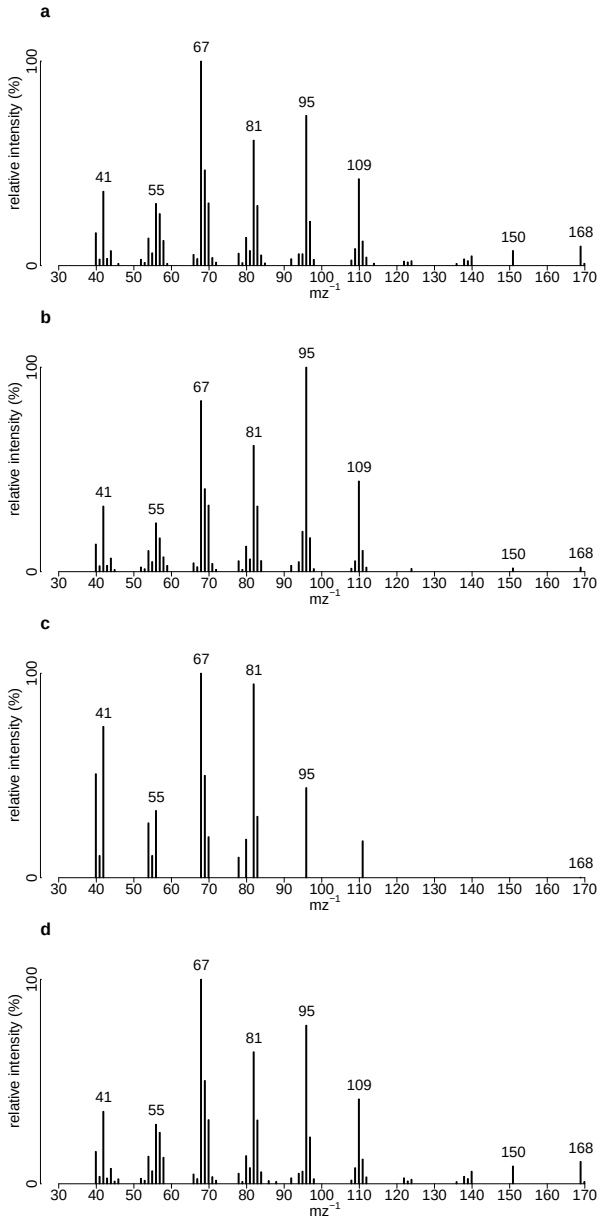

Figure A.5.: Tentative identification of p7. Mass spectra (EI) of (**a**) compound p7 from *L. heterotoma* females, (**b**) synth. (−)-iridomyrmecin(), (**c**) a trans-fused iridomyrmecin (Hilgraf et al., 2012), and (**d**) the minor product in the synthesis of (−)-iridomyrmecin.

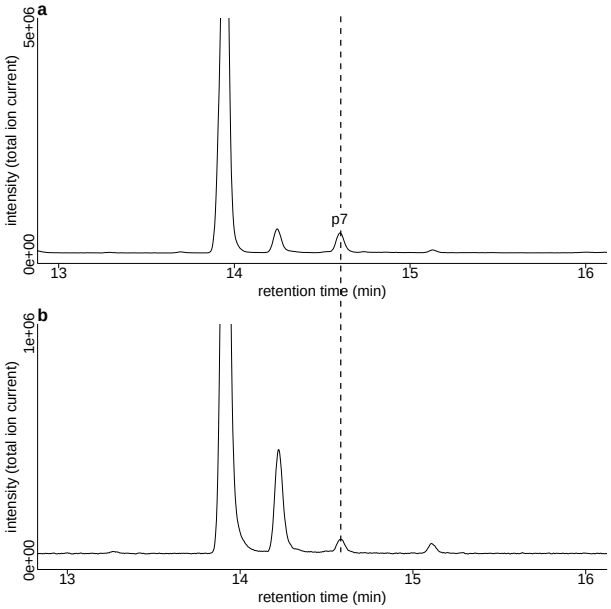

Figure A.6.: Tentative identification of p7. Total ion current chromatograms on a non-polar column of (**a**) an extract of virgin *L. heterotoma* females and (**b**) the synthetic sample of (−)-iridomyrmecin with minor compounds.

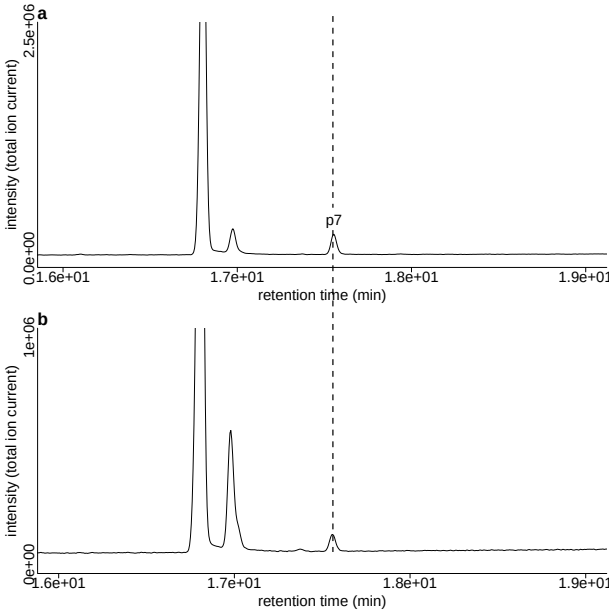

Figure A.7.: Tentative identification of p7. Total ion current chromatograms on a cyclodextrin (Gamma DEX 120) column of (**a**) an extract of virgin *L. heterotoma* females and (**b**) the synthetic sample of (−)-iridomyrmecin with minor compounds.

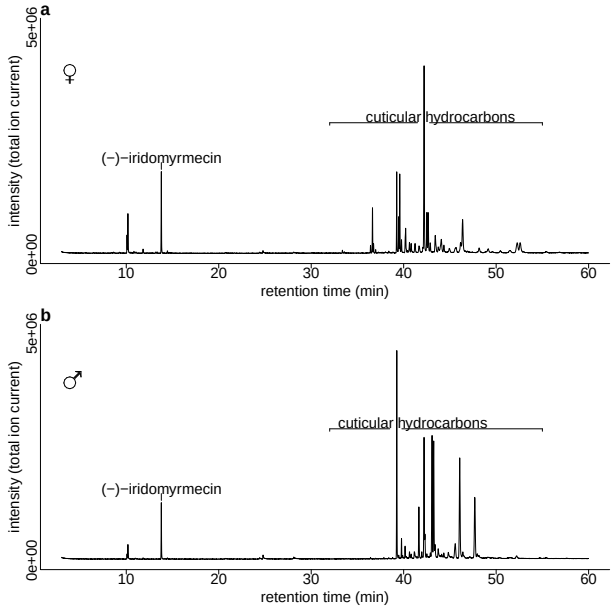

Figure A.8.: Chemical compounds produced by *L. boulardi*. Total ion current chromatograms of an extract of (**a**) virgin *L. boulardi* females and (**b**) *L. boulardi* males.

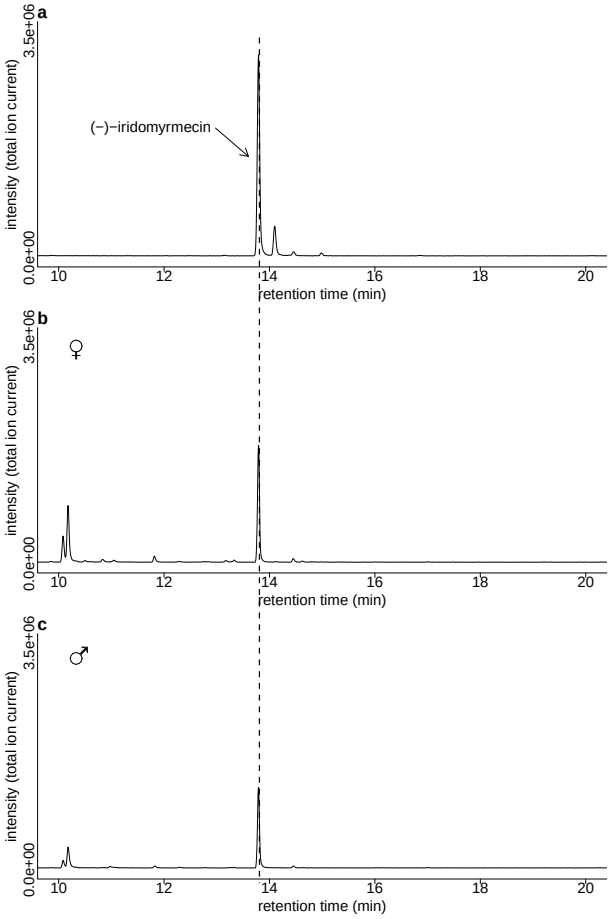

Figure A.9.: Identification of (−)-iridomyrmecin in *L. boulardi*. Total ion current chromatogram on a non-polar column of (**a**) synthetic (−)-iridomyrmecin, (**b**) the iridoid fraction of *L. boulardi* female extract, and (**c**) the iridoid fraction of *L. boulardi* male extract.

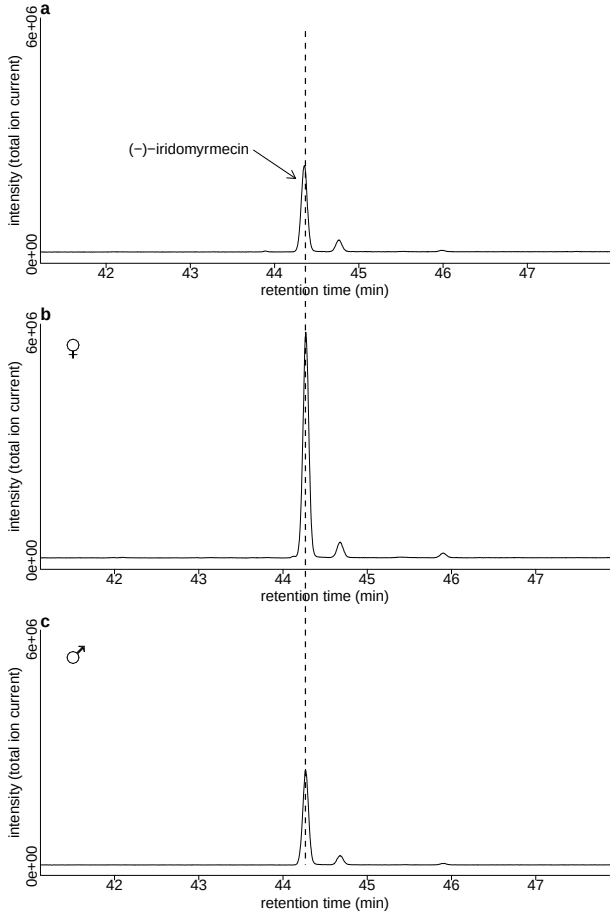

Figure A.10.: Identification of (−)-iridomyrmecin in *L. boulardi*. Total ion current chromatogram on a polar (RH-WAX) column of (**a**) synthetic (−)-iridomyrmecin, (**b**) the iridoid fraction of *L. boulardi* female extract, and (**c**) the iridoid fraction of *L. boulardi* male extract, both (**a**) and (**b**) coinjected with synthetic (−)-iridomyrmecin.

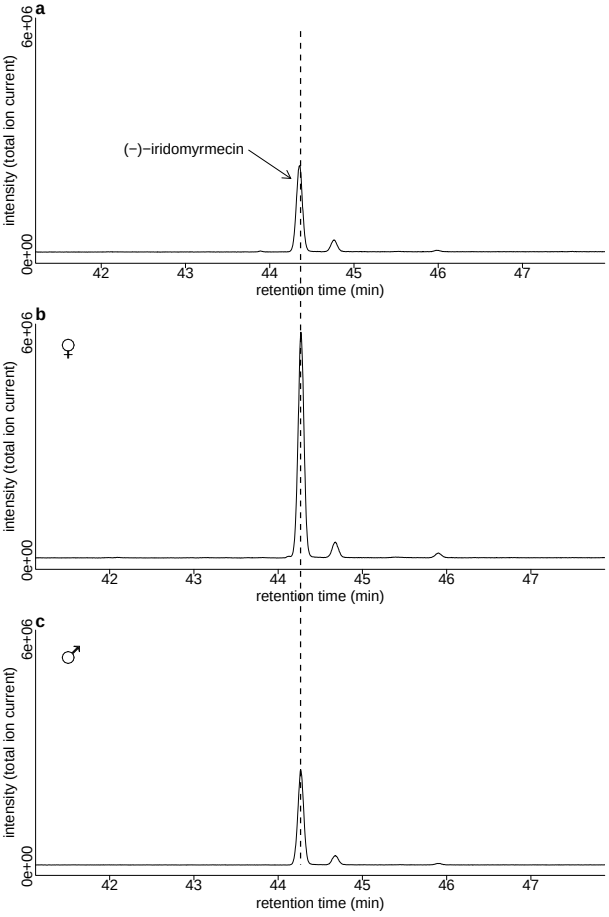

Figure A.11.: Identification of (−)-iridomyrmecin in *L. boulardi*. Total ion current chromatogram on a cyclodextrin (Beta DEX 225) column of (**a**) synthetic (−)-iridomyrmecin, (**b**) the iridoid fraction of *L. boulardi* female extract, and (**c**) the iridoid fraction of *L. boulardi* male extract, both (**a**) and (**b**) coinjected with synthetic (−)-iridomyrmecin.

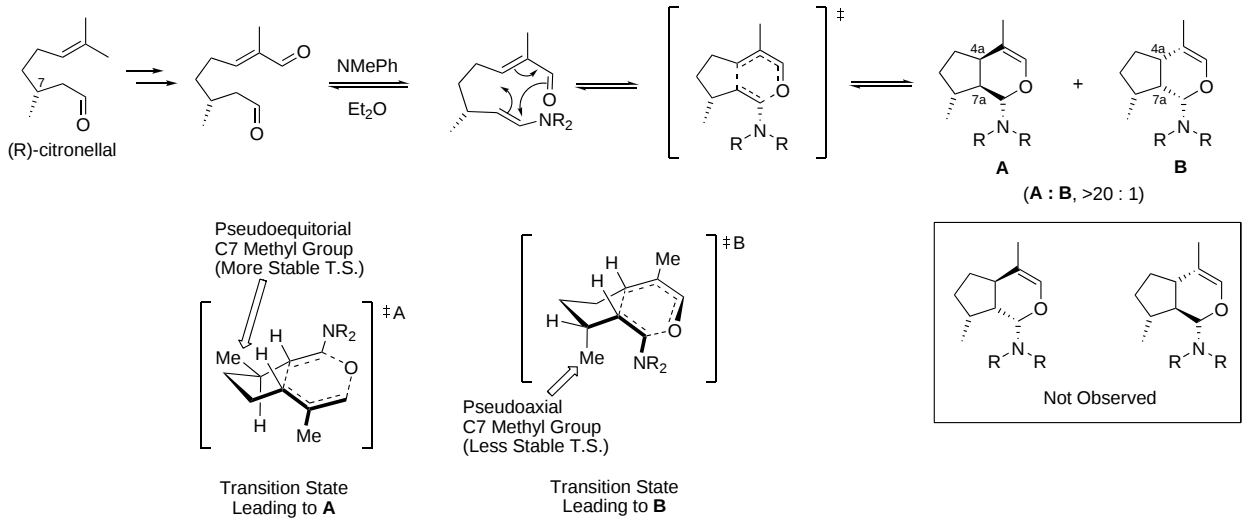

Figure A.12.: Stereochemical analysis of the Diels-Alder reaction. The putative mechanism of the Diels-Alder cycloaddition employed in the synthesis of (−)-iridomyrmecin and (+)-isoiridomyrmecin suggests that the formation of two cycloadducts, **A** (major) and **B** (minor), is likely.

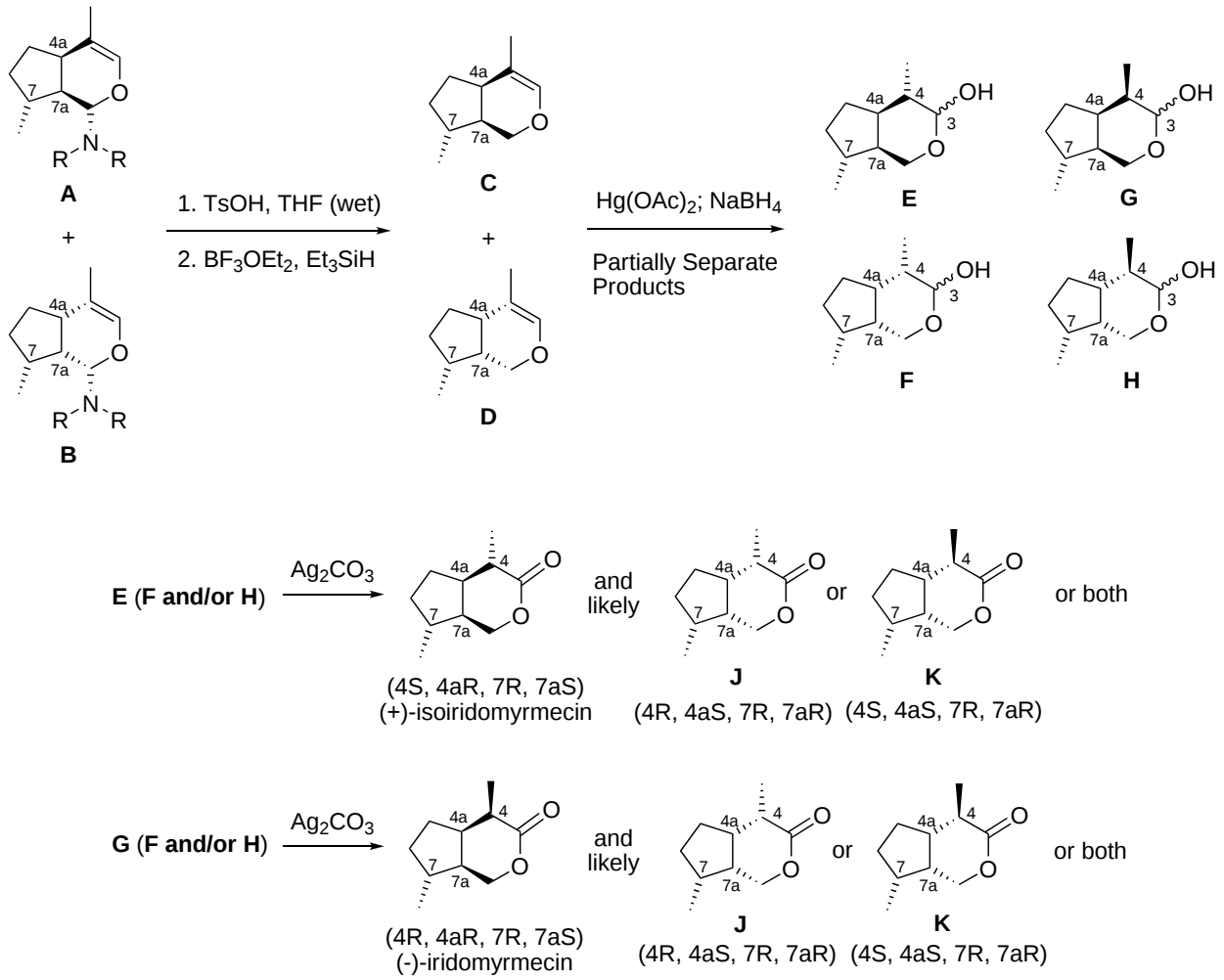

Figure A.13.: J and **K** are likely impurities in the authentic standard of (-)-iridomyermecin. The nonselective introduction of the C4 stereocenter late in the synthesis of the authentic standard would ultimately provide two possible side products, **J** and **K**, either or both of thes products could be a minor component of the authentic standard of (−)-iridomyrmecin.

B. Supplementary information for chapter 4

Table B.1.: Statistical details for the pairwise comparisons of courtship duration displayed by **(A)** *L. heterotoma*, **(B)** *L. boulardi*, and **(C)** *L. victoriae* males towards conspecific and heterospecific females. Data were compared using the *Mann-Whitney* U-test with *Bonferroni-Holm* correction. All p-values are rounded to the third digit, uncorrected p-values are given in parantheses. Comparisons were only made within male species, but not between.

(A) *L. heterotoma* males

	L. heterotoma	*L. boulardi*	*L. victoriae*
L. heterotoma	-	$U = 106$	$U = 124.5$
L. boulardi	$p = 0.007$ ($p = 0.002$)	-	$U = 172$
L. victoriae	$p = 0.044$ ($p = 0.022$)	$p = 0.261$ ($p = 0.261$)	-

(B) *L. boulardi* males

	L. heterotoma	*L. boulardi*	*L. victoriae*
L. heterotoma	-	$U = 11$	$U = 191$
L. boulardi	$p < 0.001$ ($p < 0.001$)	-	$U = 0$
L. victoriae	$p = 0.690$ ($p = 0.690$)	$p < 0.001$ ($p < 0.001$)	-

(C) *L. victoriae* males

	L. heterotoma	*L. boulardi*	*L. victoriae*
L. heterotoma	-	$U = 179.5$	$U = 15$
L. boulardi	$p = 0.300$ ($p = 0.300$)	-	$U = 54$
L. victoriae	$p < 0.001$ ($p < 0.001$)	$p < 0.001$ ($p < 0.001$)	-

Supplementary information for "Varying importance of cuticular hydrocarbons and iridoids in the species-specific mate recognition pheromones of three closely related *Leptopilina* species"

Table B.2.: Iridoid compounds and amounts thereof found in females of *L. heterotoma*, *L. boulardi*, and *L. victoriae*

Compound	RI BPX-5	RI Beta DEX 225	Diagnostic ions	Mean amount (ng ± SD) per female		
				L. heterotoma	*L. boulardi*	*L. victoriae*
Synthetic (-)-iridomyrmecin	1466	1639	M^+ 168, 95, 109	—	—	—
Synthetic (+)-iridomyrmecin	1464	1675	M^+ 168, 95, 109	—	—	—
Synthetic (-)-isoiridomyrmecin	1477	1697	M^+ 168, 95, 109	—	—	—
Synthetic (+)-isoiridomyrmecin	1477	1727	M^+ 168, 95, 109	—	—	—
Unidentified iridoid	1242			—	—	4.61±3.77
Unidentified iridoid	1251		M^+ 168, 109	—	1.37±0.61	—
Unidentified iridoid	1252			—	—	1.65±1.31
Unidentified iridoid	1259			—	—	4.92±3.98
Unidentified iridoid	1302			—	—	1.14±0.58
Iridodial 1	1312	1492	M^+ 168, 111, 135	10.85±2.80	26.71±10.44	1.01±1.06
Iridodial 2	1316	1499	M^+ 168, 109, 135	4.98±1.58	47.03±16.37	1.15±0.86
Unidentified iridoid	1334			—	—	1.14±0.21
Unidentified iridoid	1340			—	—	0.76±0.18
Unidentified iridoid	1343		M^+ 164, 109, 137	—	3.58±1.31	—
Unidentified iridoid	1352		M^+ 166, 137	—	3.20±1.06	—
Unidentified iridoid	1383	1516	M^+ 166, 123	—	5.46±1.46	5.27±3.35
Unidentified iridoid	1439			—	2.36±0.97	—
Unidentified iridoid	1446			—	3.55±1.10	—
(-)-iridomyrmecin	1466	1639	M^+ 168, 95, 109	110.12±16.55	91.46±23.38	—
(+)-iridomyrmecin	1464	1674	M^+ 168, 95, 109	—	—	11.86±9.42
Unidentified iridoid	1470	1640		—	—	2.03±1.72
(+)-isoiridomyrmecin	1477	1728	M^+ 168, 95, 109	5.78±2.98	0.83±0.25	2.58±1.97
Iridomyrmecin[1]	1491	1673	M^+ 168, 109, 150	4.91±1.18	2.99±0.68	1.46±1.16

Table B.3.: Cuticular hydrocarbons and amounts thereof found in females of *L. heterotoma*, *L. boulardi*, and *L. victoriae*

Compound	RI	Diagnostic ions	Diagnostic ions DMDS	Mean amount (ng ± SD) per female		
				L. heterotoma	*L. boulardi*	*L. victoriae*
4-methylhexacosane	2661	71, 337, 365 (M-15)		15.55±11.53	6.06±7.55	6.89±3.42
9-heptacosene	2677	M^+ 378, 97	M^+ 472, 173, 299	—	35.88±52.72	—
7-heptacosene	2685	M^+ 378, 97	M^+ 472, 145, 327	—	7.49±10.98	—

Continued on next page.

[1]Iridomyrmecin of unkown absolute configuration.

Continued from previous page.

Compound	RI	Diagnostic ions	Diagnostic ions DMDS	Mean amount (ng ± SD) per female		
				L. heterotoma	*L. boulardi*	*L. victoriae*
Octacosane	2799	M^+ 394		10.29±18.19	5.41±3.08	—
4-methyloctacosane	2863	71, 365, 393 (M-15)		141.81±36.87	72.14±30.56	40.88±8.21
Nonacosadiene; 9-nonacosene	2876	M^+ 406, 97, M^+ 404, 96	M^+ 500, 173, 327	—	—	12.38±16.03
9-nonacosene	2877	M^+ 406, 97	M^+ 500, 173, 327	53.36±46.92	23.75±25.34	—
7-nonacosene	2885	M^+ 406, 97	M^+ 500, 145, 355	11.52±11.09	54.49±51.09	22.53±19.68
Nonacosane; 5-nonacosene	2899	M^+ 406,M^+ 408	M^+ 500, 117, 383	—	—	7.21±2.35
Nonacosane	2899	M^+ 408		10.08±5.15	13.40±6.27	—
9/11/13/15-methylnonacosane	2925	169, 196, 224, 252, 281, 407 (M-15)		8.58±4.33	—	5.14±1.23
4-methylnonacosane	2959	71, 379, 407 (M-15)		8.57±2.73	19.54±8.64	8.38±1.31
5,17-dimethylnonacosane	2973	85, 196, 267, 379, 421 (M-15)		—	—	6.62±2.57
5,11-dimethylnonacosane	2975	183, 281, 379, 421 (M-15)		—	12.68±6.87	—
Dimethylnonacosane	3004	421 (M-15)		—	21.73±8.65	—
10/13-methyltriacontane	3030	155, 197, 267, 309, 421 (M-15)		—	22.84±9.99	—
Hentriacontadiene	3046	M^+ 432, 96		10.69±10.25	—	—
Hentriacontadiene	3051	M^+ 432, 96		150.07±63.67	—	—
Hentriaconta-7,17-diene	3052	M^+ 432, 96	145, 243, 283, 329, 381, 427, 479 (M-141), 526 (M-94)	—	—	43.86±75.21
4-methyltriacontane	3061	71, 393, 407 (M-15)		53.37±103.61	207.20±59.32	168.15±93.82
9-hentriacontene	3077	M^+ 434, 97	M^+ 528, 173, 355	37.64±37.91	34.07±28.75	81.26±68.25
7-hentriacontene	3086	M^+ 434, 97	M^+ 528, 145, 383	7.59±3.38	36.79±24.09	74.32±52.80
Hentriacontane	3098	M^+ 436		—	10.39±3.01	8.98±2.89
Unidentified CHC	3105			43.05±11.12	—	—
13/15-methylhentriacontane	3123	196, 224, 252, 281, 435 (M-15)		—	—	30.92±7.26
13-methylhentriacontane	3127	196, 281, 435 (M-15)		—	41.99±15.27	—
7-methylhentriacontane	3134	112, 365, 435 (M-15)		12.58±6.39	—	—
Unidentified CHC	3143			—	—	7.13±2.11
Unidentified CHC	3153			13.19±2.62	—	—
7,11-dimethylhentriacontane	3158	113, 183, 309, 379, 449 (M-15)		—	—	11.31±2.61
9,12-dimethylhentriacontane	3159	140, 196, 295, 351, 449 (M-15)		—	51.12±16.90	—
4-methylhentriacontane	3159	71, 407, 435 (M-15)		9.22±2.13	—	—
5,11-dimethylhentriacontane	3170	85, 183, 309, 407, 449 (M-15)		—	—	17.71±3.69
Unidentified CHC	3173			—	13.75±4.04	—
3,15-dimethylhentriacontane; unknown	3197	57, 183, 309, 435, 449 (M-15)		—	—	6.43±2.62
Dotriacontane	3199	M^+ 450		12.04±3.62	—	—
Unidentified CHC	3202			—	15.84±5.48	—
13-methyldotriacontane	3224	449 (M-15), 197		—	—	11.14±4.30

Continued on next page.

Continued from previous page.

Compound	RI	Diagnostic ions	Diagnostic ions DMDS	Mean amount (ng ± SD) per female		
				L. heterotoma	*L. boulardi*	*L. victoriae*
Unidentified CHC	3224			14.90±3.96	—	—
Unidentified CHC	3227			—	12.47±5.36	—
Unidentified CHC	3231			—	13.01±5.69	—
Tritriacontadiene	3244	M^+ 460, 96		16.59±12.12	—	13.32±18.04
Tritriaconta-7,17-diene	3251	M^+ 460, 96	145, 271, 283, 329, 409, 455, 507 (M-141), 554 (M-94)	—	—	41.39±57.35
Tritriaconta-7,21-diene	3251	M^+ 460, 96	145, 341, 507 (M-141)	—	32.16±25.93	—
Tritriacontadiene	3253	M^+ 460, 96		5.57±7.53	—	—
4-methyldotriacontane; tritriacontene	3258	71, 421, 449 (M-15); M^+ 462, 97		—	—	36.60±15.15
4-methyldotriacontane	3258	71, 421, 449 (M-15)		23.90±12.00	—	—
4-methyldotriacontane; tritriacontadiene	3259	71, 421, 449 (M-15); M^+ 460,96		—	71.80±41.98	—
Tritriacontene	3263	M^+ 462, 97		7.49±8.77	—	—
Tritriaconta-6,26-diene	3278	M^+ 460, 96	131, 145, 409, 423, 455, 470, 507 (M-141), 554 (M-94)	—	—	20.45±15.46
11/13/15-methyltritriacontane	3322	196, 224, 280, 309, 337, 463 (M-15)		50.57±12.93	—	—
13/15/17-methyltritriacontane	3322	197, 224, 252, 281, 309, 463 (M-15)		—	—	22.60±6.12
13,15/15,17-dimethyltritriacontane	3344	197, 239, 224, 253, 267, 281, 295, 323, 478 (M-15)		8.85±1.76	—	—
Unidentified CHC	3352			6.63±1.46	—	—
Unidentified CHC	3358			9.05±2.11	—	—
5,11/5,15-dimethyltritriacontane	3369	85, 183, 239, 281, 337, 435, 477 (M-15)		—	—	7.22±1.97
Pentatriacontatriene	3443	M^+ 486, 96		—	—	75.79±99.50
Pentatriacontadiene	3443	M^+ 489, 96		11.82±6.77	—	—
Pentatriacontadiene	3448	M^+ 488, 96		—	42.94±26.80	—
Pentatriaconta-9,19-diene	3451	M^+ 488, 96	173, 271, 311, 357, 409, 455, 535 (M-141), 582 (M-94)	—	—	25.11±30.93
Pentatriacontadiene	3455	M^+ 488, 96		—	22.47±13.47	—
Pentatriacontene	3461	M^+ 490, 97		—	—	6.29±4.79
Pentatriaconta-7,17-diene	3470	M^+ 488, 96	145, 299, 283, 329, 437, 483, 535 (M-141), 582 (M-94)	—	—	13.70±14.67
13/15/17-methylpentatriacontane	3521	197, 224, 252, 280, 309, 337, 491 (M-15)		15.63±4.97	—	—
15/17-methylpentatriacontane	3521	224, 252, 280, 308, 491 (M-15)		—	—	11.48±4.44
Heptatriacontadiene	3642	M^+ 516, 96		38.70±23.93	—	—

Table B.4.: Statistical details on the pairwise comparisons of courtship duration towards extract, fractions, and control for *L. heterotoma*. Data were compared using the *Mann-Whitney* U-test test with *Bonferroni-Holm* correction. All *p*-values are rounded to the third digit, uncorrected p-values are given in parantheses.

	Extract	Iridoids	CHCs	Control
Extract	-	$U = 226$	$U = 122$	$U = 48$
Iridoids	$p = 0.095$ ($p = 0.095$)	-	$U = 195$	$U = 101$
CHCs	$p < 0.001$ ($p < 0.001$)	$p = 0.043$ ($p = 0.021$)	-	$U = 192$
Control	$p < 0.001$ ($p < 0.001$)	$p < 0.001$ ($p < 0.001$)	$p = 0.038$ ($p = 0.013$)	-

Table B.5.: Statistical details on the pairwise comparisons of courtship duration towards extract, fractions, combined fractions, and control for *L. boulardi*. Data were compared using the *Mann-Whitney* U-test test with *Bonferroni-Holm* correction. All *p*-values are rounded to the third digit, uncorrected *p*-values are given in parantheses.

	Extract	Iridoids	CHCs	Combined fr.	Control
Extract	-	$U = 57$	$U = 86$	$U = 169$	$U = 0$
Iridoids	$p < 0.001$ ($p < 0.001$)	-	$U = 161.5$	$U = 74$	$U = 10$
CHCs	$p = 0.006$ ($p = 0.002$)	$p = 0.608$ ($p = 0.304$)	-	$U = 106$	$U = 10$
Combined fr.	$p = 0.608$ ($p = 0.414$)	$p = 0.002$ ($p < 0.001$)	$p = 0.031$ ($p = 0.010$)	-	$U = 0$
Control	$p < 0.001$ ($p < 0.001$)	$p < 0.001$ ($p < 0.001$)	$p < 0.001$ ($p < 0.001$)	$p < 0.001$ ($p < 0.001$)	-

Table B.6.: Statistical details on the pairwise comparisons of courtship duration towards extract, fractions, and control for *L. victoriae*. Data were compared using the *Mann-Whitney* U-test test with *Bonferroni-Holm* correction. All *p*-values are rounded to the third digit, uncorrected *p*-values are given in parantheses.

	Extract	Iridoids	CHCs	Control
Extract	-	$U = 72$	$U = 157.5$	$U = 21$
Iridoids	$p = 0.013$ ($p = 0.004$)	-	$U = 72$	$U = 54$
CHCs	$p = 0.899$ ($p = 0.899$)	$p = 0.013$ ($p = 0.004$)	-	$U = 27$
Control	$p < 0.001$ ($p < 0.001$)	$p < 0.001$ ($p < 0.001$)	$p < 0.001$ ($p < 0.001$)	-

C. Experimental parameters for the investigation of mate attraction in *Leptopilina heterotoma*

The investigation of behaviour requires proper experimental setups to gather reliable behavioural data. A variety of experimental setups has been described in the literature and many of these have proven to reproducibly generate reliable results. The reproducability and reliability, however, depend upon the proper choice of experimental parameters, such as the experiment's duration or the time of the day at which the experiment is conducted.
In this chapter, the suitability of the classic y-tube bioassay for the investigation of mate attraction in *Leptopilina heterotoma* is studied. The main experimental parameter investigated is the time of the day at which bioassays are conducted. The response of *L. heterotoma* males to a known attractive stimulus is studied at different times of the photoperiod.
Leptopilina heterotoma males are found to respond uniformly to female-derived extracts throughout the photoperiod in terms of both attraction frequency and decision times. The y-tube bioassay is thus well suited to investigate mate attraction in *L. heterotoma*, as behavioural data can be quickly acquired throughout the day.

Introduction

Behaviour and especially communication are phenomena that inspire research in many different taxa. As behavioural research covers a wide range of behaviours, a large number of experimental setups has been designed for the investigation of behaviour. The general specifics of these setups depend on the studied behaviour, the individuals observed, and, especially in the case of communication, the involved sensory modalities—be it visual, acoustic, or chemical stimuli.

In insects, communication often involves (volatile) chemicals that transport information. To investigate such odour-mediated communication, many different experimental setups are available; one of them is the classic 'y-tube' olfactometer (Hare, 1998).

No matter what experimental setup is employed, however, experimental parameters have to be chosen. These parameters greatly influence the reliability of an experimental setup and the reproducibility of the results obtained from it. Experimental parameters can be divided into abiotic and biotic parameters. Abiotic parameters are general physical qualities, such as e. g. temperature, relative humidity, and the time of the photoperiod at which the experiment is conducted. Biotic parameters, on the other hand, are qualities inherent to the individual specimen used in an experiment. These include e. g. sex, age, and previous experience. These parameters of course greatly depend on the question that should be answered, and often require *a priori* knowledge. For example, the investigation of a certain behaviour should be restricted to times of the day at which the behaviour is known to be displayed.

Calling behaviour e. g. is known to be shown in a specific temporal pattern—or periodicity—in many insects. The periodicity of the sex pheromone release is especially well known from Lepidoptera and the rhythmicity is often circadian (e. g. Cardé et al. 1974; Castrovillo and Cardé 1979). The rhythmicity of the female calling is often reflected in the periodicity of the male response (e. g. Castrovillo and Cardé 1979), and both calling and response periodicity are often modified by ambient temperature (Cardé et al., 1975). Other taxa in which temporal patterns of calling and response behaviour have been found include Coleoptera (e. g. Ma and Burkholder 1978; Hammack 1995) and Hymenoptera (e. g. McNeil and Brodeur 1995). Such periodicity restricts experimental investigation to certain times of the day.

One way to increase the reliability and reproducibility of experimental results is to conduct experiments with known behaviourally active stimuli to obtain suitable experimental parameters. If behavioural periodicity is suspected, such experiments can be conducted to identify times of high responsiveness. After identifying putative periodicities in the studied behaviour, experiments can then be reliably conducted during periods of high behavioural acitivity.

In a recent study (Weiss et al., 2013), we identified

the composition of the female sex pheromone of *Leptopilina heterotoma*, a larval parasitoid of *Drosophila* species. To identify the sex pheromone, we used a y-tube bioassay in which we let *L. heterotoma* males chose between an odour sample and a solvent control. Males 'decided' for either the sample or the control by entering the respective arm of the y-tube within the alloted time. The y-tube bioassay had been used in preliminary experiments with *L. heterotoma* and males had shown a strong attraction towards female-derived extract. While males had shown a high responsiveness in the preliminary experiments, a potential periodicity had not been investigated, and experiments had thus always been conducted at the same time of the photoperiod.

To analyse whether male responsiveness changes during the photoperiod, we investigate the attractiveness of female-derived extracts during different times of the photoperiod. The goal is to identify the optimal time of the photoperiod to conduct the y-tube bioassays. Specifically, we ask the following questions:

1. Does the proportion of males attracted to the female pheromone change during the photoperiod?

2. Do males decide within the same time span throughout the photoperiod?

Material & methods

Insects. We reared *L. heterotoma* using *D. melanogaster* as host species. *Drosophila melanogaster* was reared on a corn-based diet (504 ml water, 66 g sugar, 6 g baker's yeast, 2.3 g agar, 52 g cornmeal, 1.3 ml propanoic acid, 0.8 g nipagin) and kept at 25 °C, roughly 75 % humidity, and a 16:8 h L:D cycle. For each rearing, about 30 flies (mixed sexes) were placed into a jar containing fresh fly food. After 48 h, the flies were removed and about 10 *L. heterotoma* (both sexes) were put into the jar. Parasitized fly pupae were removed from the jars before emergence and put singly into 1.5 ml microcentrifuge tubes to obtain naive and virgin wasps of known age.

Extraction. We extracted virgin 1-d-old female *L. heterotoma* for 10 min in 10 µl dichloromethane (DCM) per female. After extraction, the extract was diluted with DCM by a factor 2, so the final concetration equalled 1 female per 20 µl DCM.

Bioassays. We used a y-tube olfactometer to investigate the behaviour of naive male *L. heterotoma* towards extracts of virgin female *L. heterotoma*. The glass y-tube rested at an angle of 30°, the two arms were pointing up the slope. The arms and base were 90 mm and 60 mm long, respectively, and the tube had an inner diameter of 15 mm. The two arms diverged at an angle of 45°. Humidified air was pumped through the tube's arms at a total rate of 150 ml min^{-1} using an air pump (Fürguth, Tannheim, Germany). The experimental setup was illuminated with two neon tubes (8 W). For all tests, a control (consisting of only DCM) was applied to one arm, and the extract was applied to the other arm. Extract and control were applied by putting 2 µl of DCM or extract onto a small filter paper discs. The paper discs were left to dry for 1 min before they were put into the upper openings of the two arms. To control for potential side preferences, we switched the arms for solvent and extract every two replicates and turned the y-tube after every replicate. For each trial, we carefully transferred a single naive 1-d-old *L. heterotoma* male into the base of the y-tube. Trials lasted for at most 5 min and were prematurely terminated when the male passed a 'decision line', which was marked 20 mm into the tube's arms. The tube was rinsed with ethanol and water every other trial. Each male was used for only one replicate. For each trial, we recorded the male's decision—i. e. 'control' or 'extract'—and the time passed until the male had crossed the decision line. To investigate whether the males' behaviour changed over the course of the photoperiod, the bioassays were replicated during five different times of the day: 8:00 – 10:00 ('dawn'), 10:30 – 11:30 ('morning'), 12:00 – 13:00 ('noon'), 14:30 – 15:30 ('afternoon'), and 17:30 – 18:30 ('evening'). The photoperiod started at 7:00 and ended at 23:00. We sampled 30 replicates for each time of the day except for dawn, where we sampled only 29 replicates.

Statistical analysis. The number of males attracted towards extract and control, respectively, was analysed with the binomial test for each time of the day. To find differences between the decision frequencies for the different times of the day, decision frequencies were analysed with the chi-squared test. The decision times for control and extract, respectively, were tested for significant differences for each time of the day using the Mann-Whithney U test. As no significant differences were found, decision times for control and extract were pooled for each time of the day. The pooled decision times were then tested for significant differences between the different times of the day using the Kruskal-Wallis rank sum test. All statistical tests were performed using R version 3.1.1 (R Core Team, 2014).

Results

Attraction of males does not change during the photoperiod. Males were strongly attracted to fe-

male extract during each time of the day (binomial test: $p < 0.01$ for all times of the day; fig. C.1). We found no significant difference between the different times of the day (chi-squared test: $\chi^2 = 5.1923$, $df = 4$, $p = 0.2681$).

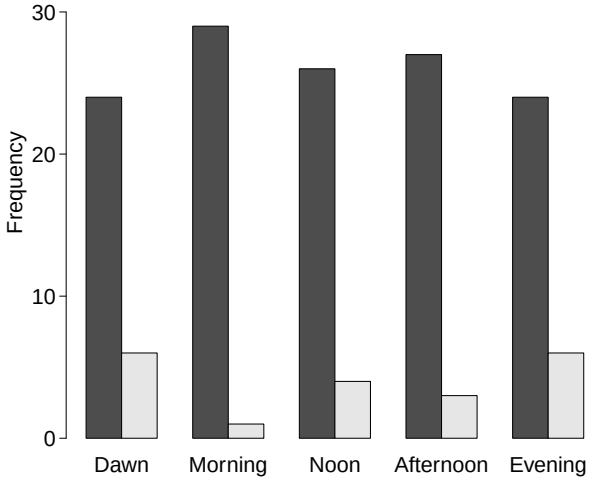

Figure C.1.: Number of *L. heterotoma* males that were attracted towards extracts of *L. heterotoma* females (dark bar) and control (light bar) during different times of the day; $n = 30$ for each time of the day, except for 'dawn', where $n = 29$. No significant differences were found between the different times of the day (chi-squared test: $p = 0.2681$).

Male decision times do not change during the photoperiod. On average, males took 49.5 s (SD 46.7 s, SE 3.8 s) to cross the decision line. We found no significant differences for the males' decision times between the different times of the day (Kruskal-Wallis rank sum test: $\chi^2 = 2.9185$, $df = 4$, $p = 0.5715$; pooled results for extract and control for each time of the day; fig. C.2).

Discussion

We found that males show great responsiveness to the female-derived extracts and thus the sex pheromone throughout the photoperiod. Thus, bioassays can be conducted at any time during the photoperiod. This also means that the results of bioassays conducted at different times during the photoperiod can be compared without having to treat 'time of day' as a confounder. Conducting experiments with a known positive stimuli can help to determine suitable experimental parameters. This can be especially helpful for behavioural experiments, in which the observed individual requires motivation to show the behaviour that is to be observed. In *L. heterotoma*, males are clearly motivated to respond to the female sex pheromone throughout the photoperiod.

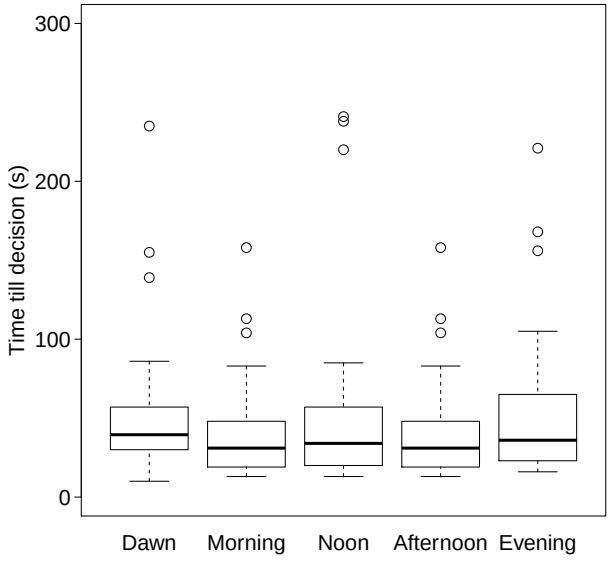

Figure C.2.: Time until *L. heterotoma* males crossed the decision line in the y-tube experiments during different times of the day when choosing between female-derived extracts and a control (pooled results for extract and control); $n = 30$ for each time of the day, except for 'dawn', where $n = 29$. No significant differences were found between the different times of the day (Kruskal-Wallis rank sum test: $p = 0.5715$; pooled results for extract and control).

It is, however, unclear whether the females release their sex pheromone throughout the photoperiod, i. e. whether their calling behaviour is restricted to certain times of the photoperiod. Such temporal patterns in calling behaviour are well known from several insect orders (e. g. Cardé et al. 1974; Castrovillo and Cardé 1979; Ma and Burkholder 1978; Hammack 1995; McNeil and Brodeur 1995). We investigated the female calling behaviour in *L. heterotoma* in parallel to the male response. Our experimental headspace setup, however, yielded so low amounts of female-borne chemical compounds, that a quantitative analysis could not be conducted reliably. Further experiments are thus required to identify a putative calling pattern and the ecological relevance of the male behaviour. Due to the uniform response of males throughout the photoperiod and the fact that we did not observe any calling behaviour in females, however, we expect that females show no specific calling pattern.

Distinct periodicities in calling or response behaviour can even establish species borders in species that utilize a common chemical communication system (Cardé et al., 1975). For example, in *Platyptilia carduidactyla* and *P. williamsi* (Lepidoptera: Pterophoridae), males respond to the same sex pheromone, (*Z*)-11-hexadecenal, but the release of pheromones by

females follows species-specific periodicities (Haynes and Birch, 1986). *Platypilia carduidactyla* females call during the first half of the night whereas *P. williamsi* females call during the second half of the night; this periodicity is reflected in the male response behaviour.

In parasitic Hymenoptera, however, little is known about diel calling and response periodicities and their role in reproductive isolation, especially when the specificity of pheromones does not prevent cross-attraction between species. Weiss et al. (2013) found that *L. heterotoma* males are also attracted towards females from the closely related species *L. boulardi*. As *L. heterotoma* male respond uniformly throughout the photoperiod, however, a presumed species-specific temporal calling pattern of females from both species can hardly mitigate this cross-attraction. Future research into diel periodicity in the chemical communication of parasitic Hymenoptera is required to understand whether diel periodicity is a rare phenomenon in parasitoids—and if so, to elucidate why this is the case.

Throughout the experiments, most decisions were made in less than 120 s (fig. C.2). Reducing the maximum amount of time for a decision from 300 s to 120 s, would thus only very mildly impair future investigations. Doing so would reduce the amount of time consumed by unsuccessful (i. e. males that do not decide within the alloted time) experiments. Identifying a reasonable maximum duration for the experiment is especially important for experiments in which the proportion of responding individuals is inherently low—which was not the case in the y-tube bioassays with *L. heterotoma*, but in the closely related species *L. boulardi* (Weiss et al., unpublished). In *L. boulardi*, males showed a much lower overall responsiveness in y-tube bioassays, thus a greater proportion (roughly 50 %) of the experiments lasted the maximum time without yielding a result. This shows, that even in closely related species, experimental setups can not be simply transferred from one species to another without exception.

In *L. heterotoma*, male responsiveness to female extracts shows no periodicity. Thus, the y-tube bioassay can be used to quickly acquire reliable behavioural data from *L. heterotoma* males throughout the photoperiod.