Chemical Ecology and Pheromone Evolution in *Leptopilina*, a Genus of Parasitoid Wasps

DISSERTATION

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Fakultät für Biologie, Chemie und Geowissenschaften
der Universität Bayreuth

vorgelegt von

Lea Clara Böttinger
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Content

Summary

Chemical communication is ubiquitous and considered the oldest form of communication in nature. The way in which the remarkable diversity of information-transmitting pheromone compounds evolved is still a major topic in chemical ecology. To date, studies concerning differences in pheromone compositions between closely related species have so far focused on phylogenetic comparisons and genetic analyses with explanations such as large, saltational shifts in pheromone evolution during speciation or differential gene regulation leading to changes between species in enzymatic pathways generating the pheromone. Another explanation for the chemical composition of pheromones is the precursor hypothesis, which states that pheromones evolve from compounds formerly used for non-communicative purposes in the same organism. Finding case studies in which one can elucidate this mode of evolution is difficult, as one would need to find a species in which both functions of the same compound are still active, but mostly only the derived function of a pheromone is present and its former function lost. In a species of parasitoid wasps of *Drosophila* larvae, *Leptopilina heterotoma*, a rare example of a threefold semiochemical parsimony was found, as the compound (–)-iridomyrmecin serves as defensive secretion, as a cue for female competition avoidance and as part of the female sex pheromone to attract mates from a distance and to elicit the courtship behavior of males in close proximity. This way, an evolutionary route from the non-communicative defensive secretion over a cue to a sex pheromone can be assumed. Iridoids were also found in three other species of the same genus, and while the usage as defensive secretion was found in all species of *Leptopilina* wasps, the iridoid compounds do not in all species serve as cue for female competition avoidance. Only in *L. heterotoma* the females' iridoids evolved as sex pheromones, whereas other species use a combination of cuticular hydrocarbons (CHCs) and iridoids or only CHCs as sex pheromones. To shed light on the pheromone evolution and the way in which the different usages of iridoids evolved in the genus *Leptopilina*, I studied the chemical ecology of the three species *L. japonica, L. pacifica,* and *L. ryukyuensis*. I found that all *Leptopilina* species studied here produce iridoid compounds apart from CHCs, and I proved that these iridoids are emitted for defense in *L. japonica*, *L. pacifica*, and *L. ryukyuensis*. Therefore, the allomone function can be assumed to be the primary function of iridoids in *Leptopilina* wasps. Conspecific female competitors were not avoided by female *L. japonica* wasps, but *L. ryukyuensis* females avoid host patches already exploited by other conspecific females. Living female conspecifics of *L. pacifica* were not avoided by female wasps, however, the odor of female conspecifics' extracts was avoided. While *L. ryukyuensis* and *L. pacifica* use CHCs as female sex pheromones, in the species *L. japonica* the defensive iridoid compounds evolved a second function as sex pheromones. Interestingly, I found in all species, also in those that use CHCs as sex pheromones, that iridoids evolved as cues for males to locate female conspecifics.

However, research is limited regarding the degree to which the evolution of the impressive variety of sex pheromone compositions is driven by ecological or life-history traits and behavior. Here, I

propose the dispersal behavior of organisms for mate-finding as a process driving pheromone evolution, as it is restricted by and hence presumably correlated with the volatility of pheromonal compounds. Therefore, I studied the dispersal behavior of male and female wasps after emergence in the four species of the genus *Leptopilina, L. heterotoma* and *L. japonica*, which have highly volatile iridoids as sex pheromones, and *L. ryukyuensis* and *L. pacifica,* whose sex pheromones are composed of CHCs, which can only be perceived in close vicinity of the releasing individual. My analyses revealed that the dispersal behavior of *Leptopilina* males and females after emergence does significantly differ between species with differently volatile sex pheromones. Specifically, males of species with volatile iridoid sex pheromones start to disperse immediately before their females' emergence from the natal patch, whereas males of species with low volatile CHC-based sex pheromones delay dispersal until their conspecific females emerge. While the differences in female dispersal behavior turned out to be species-specific, differences in male dispersal correlated with the volatility of female-produced sex pheromones of each species. Probably, a tradeoff between the volatility of the sex pheromone for long range attraction and the information content for inbreeding avoidance plays an important role. My studies significantly contribute to the understanding of the evolutionary forces shaping the composition of sex pheromones.

German Summary − Zusammenfassung

Chemische Kommunikation ist ubiquitär und die älteste Kommunikationsart der Natur. Wie sich die beeindruckende Vielfalt an informationsübermittelnden Pheromonsubstanzen entwickelt hat ist immer noch ein großes Thema der Chemischen Ökologie. Bis heute haben sich Studien der Unterschiede in Pheromonkompositionen zwischen nahverwandten Arten hauptsächlich mit phylogenetischen Vergleichen und genetischen Analysen beschäftigt, mit Erklärungen wie großen Sprüngen in der Pheromonevolution während der Artbildung, oder unterschiedlichen Genregulierungen, die zu Unterschieden in den Enzympfaden der Pheromonbildung zwischen Arten geführt haben. Eine andere Erklärung ist die Vorläuferhypothese, die besagt, dass Pheromone aus Substanzen entstehen, die vorher in demselben Organismus für nichtkommunikative Zwecke genutzt wurden. Belege für diese Art der Evolution zu finden ist schwierig, da man Arten finden müsste, in denen beide Funktionen derselben Substanz immer noch aktiv sind, meistens ist jedoch nur noch die Pheromonfunktion vorhanden und die vorherige Funktion verschwunden. In einer Art parasitoider Wespen von *Drosophila*-Larven, *Leptopilina heterotoma*, wurde ein seltenes Beispiel für eine dreifache semiochemische Parsimonie gefunden, da die Substanz (–)-Iridomyrmecin als Abwehrsubstanz, als Reiz (engl. „cue“) für Weibchen zur Konkurrenzvermeidung und als Teil des weiblichen Sexpheromones dient, um Männchen aus der Entfernung anzulocken und deren Balzverhalten in der Nähe auszulösen. Somit kann eine evolutionäre Route von der nichtkommunikativen Abwehrfunktion über den Reiz zu dem Sexpheromon angenommen werden. In drei anderen Arten derselben Gattung wurden auch Iridoide gefunden, und während die Nutzung als Abwehrsubstanzen bei allen Arten der *Leptopilina*-Wespen gefunden wurde, dienen die Iridoide nicht in allen Arten als Reiz zur Konkurrenzvermeidung unter Weibchen. Nur in *L. heterotoma* entwickelten sich die Iridoide der Weibchen als Sexpheromone, während in anderen Arten Kombinationen von kutikulären Kohlenwasserstoffen (CHCs) und Iridoiden oder nur CHCs als Sexpheromone genutzt werden. Um die Pheromonevolution und die Art und Weise, wie sich die unterschiedliche Nutzung von Iridoiden in der Gattung *Leptopilina* entwickelt hat, aufzuklären, untersuchte ich die chemische Ökologie der drei Arten *L. japonica, L. pacifica* und *L. ryukyuensis*. Ich fand heraus, dass alle hier untersuchten *Leptopilina*-Arten neben CHCs auch Iridoide produzieren, und konnte beweisen, dass Iridoide in den Arten *L. japonica*, *L. pacifica* and *L. ryukyuensis* zur Abwehr abgegeben wurden. Deshalb kann angenommen werden, dass die Abwehrfunktion die ursprüngliche Funktion der Iridoide in *Leptopilina*-Wespen ist. Konkurrenz durch Artgenossinnen wurde von *L. japonica* nicht vermieden, jedoch vermieden Weibchen von *L. ryukyuensis* das Wirtsmedium (Fliegenfutter mit Fliegenlarven) mit anderen Weibchen. Obwohl lebende Artgenossinnen nicht von Weibchen von *L. pacifica* vermieden wurden, wurde der Geruch des Extrakts von Artgenossinnen vermieden. Während *L. ryukyuensis* und *L. pacifica* CHCs als weibliche Sexpheromone nutzen, entwickelten die iridoiden Abwehrsubstanzen in der Art *L. japonica* eine zweite Funktion als

Sexpheromone. Interessanterweise fand ich in allen Arten, auch in denen, die CHCs als Sexpheromone nutzen, dass sich die Iridoide als Reiz für Männchen zur Auffindung von Weibchen entwickelt haben.

Bisher weiß man noch nicht, inwiefern die Evolution der beeindruckenden Vielfalt von Sexpheromonkompositionen von ökologischen oder Lebenszyklus-Merkmalen und Verhalten bestimmt wird. Hier schlage ich das Ausbreitungsverhalten von Organismen zur Partnerfindung als einen Prozess vor, der als Treiber für die Pheromonevolution fungieren könnte, da das Ausbreitungsverhalten von der Volatilität der Pheromonsubstanzen abhängt und daher vermutlich mit diesem korreliert. Deshalb studierte ich das Ausbreitungsverhalten von männlichen und weiblichen Wespen nach deren Schlupf in vier Arten der Gattung *Leptopilina,* in *L. heterotoma* und *L. japonica*, die hochvolatile Iridoide als Sexpheromone nutzen, und in *L. ryukyuensis* und *L. pacifica,* deren Sexpheromone aus CHCs bestehen, die nur in naher Umgebung des abgebenden Individuums wahrgenommen werden können. Meine Analysen ergaben, dass sich das Ausbreitungsverhalten von Männchen und Weibchen von *Leptopilina*-Wespen nach dem Schlupf signifikant zwischen den Arten unterscheidet, die unterschiedlich volatile Sexpheromone nutzen. Genauer gesagt beginnen sich die Männchen der Arten mit volatilen iridoiden Sexpheromonen direkt vor dem Schlupf ihrer Artgenossinnen von ihrem Schlupfort auszubreiten, während die Männchen der Arten mit wenig volatilen CHCs als Sexpheromonen ihre Ausbreitung verzögern bis ihre Artgenossinnen schlüpfen. Die Ausbreitungsunterschiede der Weibchen stellten sich als artspezifisch heraus, doch die Unterschiede im Ausbreitungsverhalten der Männchen korrelierten mit der Volatilität der von den Weibchen produzierten Sexpheromone jeder Art. Sehr wahrscheinlich spielt ein Trade-off der Volatilität der Sexpheromone zur Langstreckenanlockung und deren Informationsgehalt zur Inzuchtvermeidung eine wichtige Rolle. Meine Studien tragen signifikant zum Verständnis der evolutionären Kräfte bei, die die Komposition von Sexpheromonen beeinflussen.

1. General Introduction

1.1 Chemical Communication

Communication, the transmission of information via evolved signals eliciting a response in the receiving organism, can be either visual, acoustic, tactile, electric, or chemical. While the acoustic and visual communication are predominant in humans, especially the chemical form of communication is largely unknown to us. Odors of illness, stress and fear may be perceivable by other humans (Ackerl et al. 2002; Shirasu and Touhara 2011; de Groot et al. 2020) as well as the attractiveness of body odors (i.e., Thornhill et al. 2003), but no species-wide chemical signals were found yet with which humans could purposefully communicate (reviewed by Wyatt 2020). In contrast, nearly all other organisms, from bacteria, protists, fungi, plants, fishes, reptiles, birds to mammals, in all habitats and all contexts, rely on chemical compounds to find prey or food, or use semiochemicals (Greek *semeon,* sign or signal; Law and Regnier 1971) to convey a rich variety of messages to other individuals. This widespread chemical form of communication is believed to be the oldest form of communication in nature (Wyatt 2014). If these semiochemical compounds convey incidental information to receiving organisms without having evolved for that function, they are considered cues, such as these former mentioned human odors or the compounds emitted by organisms which are used by predators for prey-detection. These compounds were not intended to and did not evolve as signals to attract predators, but the predator has evolved to respond to these cues. Signals, in contrast, evolved to transmit information which affect the behavior of other, intended organisms and evolved because of the adapted, beneficial response of the receiver (Maynard Smith and Harper 2003; Scott-Phillips 2008).

Depending on whether semiochemicals are used for interspecific or intraspecific information transfer they are classified into allelochemicals or pheromones. Allelochemicals (Whittaker 1970), the semiochemicals mediating interactions between organisms of different species, are further divided according to the benefits the information provides to the releasing (sender/emitter) or receiving organism (receiver). If only the receiver benefits from the emitted compounds of an individual of another species, these allelochemicals are called kairomones (Greek *kairos*, opportunistic) and are for example compounds such as the former mentioned prey-detection cues, attractant odors or aggregation stimulants (Brown et al. 1970). Allomones (Greek *allos*, other) are in contrast released compounds which are beneficiary to the emitter (Brown 1968; Brown et al. 1970), such as e.g. defensive secretions, whereas synomones (Greek *syn*, with) are mutually advantageous to both sender and releaser (Nordlund and Lewis 1976), such as e.g. flower odors that attract pollinators, which are in turn rewarded with nectar or pollen. Pheromones (Greek *pherein*, to carry and *horman*, to excite) are signals that are exchanged within members of a species and cause specific reactions adaptive to that species, for example a stereotyped behavior or a developmental process (Karlson and Butenandt 1959; Karlson and Lüscher 1959; Karlson 1960). Primer pheromones are compounds that are released by one individual to trigger

specific physiological or developmental responses in other individuals of the same species, for example the regulation of reproduction in social insect colonies, whereby queens suppress the ovarian development of workers (e.g., Hoover et al. 2003; Dietemann et al. 2005). In contrast, releaser pheromones orchestrate a wide variety of immediate behaviors and can be grouped based on the nature of the behavioral interaction: For example, sex pheromones are released to attract and locate mates and to induce courtship behavior, aggregation pheromones attract conspecifics, marking pheromones help to avoid conspecifics and potential competition imposed by them, appeasing pheromones calm down, alarm pheromones alert conspecifics and lead to escape or defense, dispersion pheromones disassemble groups, and other pheromones are used to mark trails and guide to resources (Wyatt 2014).

The first pheromone was identified from the silkworm moth *Bombyx mori* (L.), the sex pheromone bombykol, (*E,Z*)-10,12-hexadecadien-1-ol (Butenandt et al. 1959), which was thought to be a single sex pheromone compound as it elicits the whole mating behavior of male moths, but turned out to be accompanied by the compound bombycal, (*E,Z*)-10,12-hexadecadienal, at a rate of 11:1 (Kaissling et al. 1978). However, most pheromones consist of certain combinations of several compounds instead of just one or two molecules. The compounds of those multicomponent pheromones need to be in defined species-specific ratios to elicit the typical, stereotyped behavior (Wyatt 2014; Wyatt 2017). The composition of pheromones determines the range of communication, i.e. the distance of pheromonal activity. Small, light and volatile molecules are utilized when rapid dispersal of compounds is required and for long-range interactions, for example were males of the emperor moths and Chinese silkworm moths found to be attracted to the pheromones of female conspecifics about 11 km away (Mell 1922; Butenandt 1963), whereas larger, heavier and non-volatile compounds mediate more persistent communication upon close range or contact, such as hydrocarbons on the cuticle of arthropods (Nelson and Blomquist 1995). The variations in double-bond positions as well as in shape, size, isomeric form, functional groups, saturation of molecules as well as their ratios hence do not only enable complicated species-specific pheromone mixtures but also determine the pheromone's volatility and range of action.

Accordingly, there is a myriad of chemical molecules used for communication and each compound that was originally selected to serve as pheromone or allelochemical in one species can have evolved to have secondary functions in the same or in other species. Sex pheromones for instance are often also aggregation pheromones in a species (therefore sometimes labeled aggregation-sex pheromones, Cardé 2014), as following the attractant odors of conspecifics enables easier mate-finding (e.g. in longhorn beetles: Žunič-Kosi et al. 2019). Compounds emitted as defensive repellents (= allomones) act additionally often as alarm pheromones, informing conspecifics about aggressors (Cavill and Robertson 1965; Wilson 1965; Regnier and Wilson 1968), but can also serve in lower concentrations as attractants and recruitment stimuli (Ghent 1961; Blum 1996). This use of a chemical compound for more than one purpose is referred to as semiochemical parsimony (Blum 1996). Pheromones, allomones, or synomones can act additionally as kairomones attracting other organisms such as herbivores, predators, or

parasitoids, such as in the bark beetle *Ips*, whose sex pheromone also attracts other beetle species that prey on *Ips* (Wood et al. 1968).

Insects have compared to vertebrates a more profound use of chemical communication, possibly because their small body size constraints the physical abilities of acoustic and visual signal production and signal perception (Greenfield 2002). To date, more than 3000 pheromone components are known to be used as communication signals by well over 7000 insect species (Symonds and Elgar 2008; see http://www.pherobase.com, El-Sayed 2020). Especially for mate-finding in insects chemical communication is necessary. Potential mates can be found from a distance with chemical cues such as host-derived volatiles, which lead to encounter places on the respective hosts or host-plants (e.g. larval hosts, Hanks 1999; e.g. herbivore-induced plant volatiles, Xu et al. 2017; Xu and Turlings 2018). Alternatively, some species rely on the emission of volatile aggregation pheromones to find conspecifics of the opposite sex (e.g., in longhorn beetles, Žunič-Kosi et al. 2019; e.g., in parasitic wasps, Mohamed and Coppel 1987). However, species-specific volatile sex pheromones are the predominantly used attraction signals that enable the sexes to locate each other for mating over long distances (Greenfield 1981). Long-distance mate-finding was already well established ~92–108 million years ago in the Lower Cretaceous, as biflabellate male antennae of a fossil hymenopteran species were found, which carry olfactory receptor neurons and evolved for the reception of sex pheromones of conspecific females (Schmidt et al. 2006; Krogmann et al. 2013). While in most acoustic and visual sexual displays the male individual produces the attracting signals and the female individual decides on whether to respond or not, in most insects the sex pheromones are emitted by females (Quicke 1997; Johansson and Jones 2007; Ruther 2013) and only rarely by males (but see Fletcher 1969; Landolt 1997). Due to the typically higher investment of females in reproduction compared to males, the sexual selection theory suggests that females should minimize their risk of getting detected by predators during signaling (Andersson 1994). As the production and emission of sex pheromones might be less susceptible to detection by predators than the visual and acoustic cues produced by moving mate-searching individuals, it is assumed that females are the chemically signaling sex (Alexander and Borgia 1979). Sex pheromones do not only operate over long distances, but also at short range or only upon direct physical contact. Once potential mating partners are found, courtship is initiated by the same volatile sex pheromones which attracted the mate, or by more stable, low to non-volatile compounds on the partner's cuticle. These courtship sex pheromones are often female-derived cuticular hydrocarbons (CHCs; Singer 1998; Howard and Blomquist 2005). CHCs can be normal or branched, saturated or unsaturated, and are embedded in the cuticular lipid layer of all insects. The cuticular lipid layers can also be comprised of esters, fatty acids, alcohols, alkyl esters, glycerides, ketones, sterols and aldehydes (Hackman 1984; Lockey 1988), and convey a wealth of information (see Stökl and Steiger 2017). CHCs enable the recognition of species, sex, kin and fertility, and in social insects also the identification of nestmates or caste (Blomquist and Bagnères 2010b). Consequently, there is a huge diversity of CHCs, as the CHC layers normally consist of more than 20 but can consist of more than 100 different compounds, which

differ both quantitatively and qualitatively between but also within species (Kather and Martin 2015). This astonishing diversity of CHC structures is generated by the insertion of double bonds or addition of methyl groups along the carbon chain, causing the CHC molecule to bend (Van der Waals forces), which creates a different molecule shape (conformation). Additionally, each change in configuration of the molecule around its chiral centers leads to a completely different compound with a discriminately different enantiomeric odor (Lord Kelvin 1904; Bentley 2006). The perception of insects is so finetuned that they are able to discriminate between these structural differences, e.g., enantioselectively between the chirality of compounds (Mori 2007; Mori 2011) or between the position, presence or absence of double bonds (Dani et al. 2005), or methyl groups (Châline et al. 2005). Mostly however, the complexity of CHC compositions does not derive from several complex compounds, but through mixtures of structurally simple compounds or changes in their quantitative composition (van Zweden and d'Ettorre 2010; Leonhardt et al. 2016). How this usage of different compounds evolved for communication in different species is still unknown.

1.2 Pheromone Evolution

The diversity of the several thousand pheromones known to date (El-Sayed 2020) varying in compound structures, sizes, functional groups, and compositions from single substances to complex odor compound combinations is enormous. Theoretically every compound can sooner or later arise as pheromone component. But how can and could this communication system evolve? Why did certain compounds evolve as pheromones and others not, although similarly well suited as messenger compounds? How can small changes in composition of compounds lead to different communication tools or the emergence of a new communication system? It is difficult to reconstruct the evolution of signal compounds, as not only the compound emittance of the sending individual has to evolve without losing the integrity and functionality of the compound, but at the same time also the perception and the sensory apparatus of the receiving individual must co-evolve (Phelan 1992).

Albeit the evolution of pheromones is not as obviously comprehensible as for example the evolution of skeletons, where geologic records make the gradual change of fossil structures over time plausible through visible transitional features, intermediate steps are also assumed to play a role in the evolution of chemical communication. One major hypothesis proposed for the evolution of pheromones is the sender-precursor hypothesis which predicts that compounds used for chemical communication arose from compounds (precursors) originally fulfilling other non-communicative functions in the same organism (Otte 1974; Haynes and Potter 1995; Steiger et al. 2011; Wyatt 2014), over intermediate steps through a ritualization process. Hence, the signaling compound was ancestrally produced by the sender before the receptor in the receiver evolved (Stökl and Steiger 2017) and before it was used for communication. In each organism many compounds are unintentionally emitted for non-communicative

purposes, such as waste products or physiologic by-products, and all these are potential precursors and hence provide starting points for pheromone evolution (Stökl and Steiger 2017). If another individual of the same species evolves the ability to perceive the unintentionally emitted chemical compound, e.g. a generalist or not specialized receptor, this compound might provide the receiver with beneficial information about the quality of the emitting individual, in just indicating its presence, or information about its health, fitness, sex or fertility status, the chemical becomes a cue. Receivers can respond to these cues, which can in turn influence the emitter's condition and provide it with reciprocal information about the receiver. If the reaction of the receiver to the cue is beneficial for the emitting individual, the emitter becomes a sender and an evolution of the cue towards a communicative compound via refining chemical ritualization can be expected (Wyatt 2014). Examples for unintentionally emitted precursor compounds that contain inadvertent information about the emitting individual are defensive compounds (such as formic acid in some ants), that were often co-opted as alarm pheromones, as conspecifics can perceive them as reliable information about danger (Löfqvist 1976; Blum 1996), but defensive secretions also evolved as sex pheromones (Boppré 1986; Ruther et al. 2001; Geiselhardt et al. 2008b) or aggregation pheromones (Wheeler and Cardé 2013). Also excretions of hormones, metabolic waste products or physiological by-products can evolve into pheromones, for example can hormones emitted with the urine inform about the physiological state of an organism and turn into marking or sex pheromones, e.g. in house mice and brown rats (Takács et al. 2017) or sex pheromones as in females of the Atlantic Salmon and Goldfish (Stacey and Sorensen 2009). In several cases the CHC constituents on the waxy lipid layer on the cuticle of insects, which function originally as hydrophobic desiccation barrier (Lockey 1988; Gibbs 1998; Gibbs and Rajpurohit 2010) or as protection against microbes, were co-opted as pheromones mediating species recognition, mate finding, and courtship (Greenspan and Ferveur 2000; Howard and Blomquist 2005; Blomquist and Bagnères 2010a; Mori et al. 2010; Kühbandner et al. 2012; Chung and Carroll 2015; Menzel et al. 2019). All potential precursors can via functional shifts (exaptations) turn into pheromones (Steiger et al. 2011), but only a few really evolved a communicative function (Stökl and Steiger 2017). If both functions of a chemical as cue and as signal can be found in an organism, it is an example of semiochemical parsimony (Blum 1996). Although this semiochemical parsimony and the evolution from a cue to a pheromone have been reported in quite a number of species, in most studies only the pheromone function has been experimentally tested, while the primary non-communicative function is assumed. Furthermore, the patterns and mechanisms underlying the evolution from primary to secondary function of the compounds are often not addressed. The original functions of the pheromones might eventually be lost when the cue turns into a signal, therefore the sender-precursor hypothesis is difficult to prove.

Another hypothesis regarding the evolution of pheromones is the sensory exploitation hypothesis, according to which a chemical can evolve into a signal because the sending individual exploits a pre-existing sensory bias in the receiving individual for detecting this compound (Bradbury and Vehrencamp 2011; Steiger et al. 2011; Ryan and Cummings 2013; Wyatt 2014). The chemosensory requisites for the

perception of this pheromone evolved in the receiver due to a different function, e.g. to detect resources, before the sender evolved to emit this compound for communication. An example for this process is the male sex pheromone of the European bee-wolf *Philanthus triangulum*, (*Z*)-11-eicosen-1-ol, which is the same compound used by females to detect their honeybee prey (Herzner et al. 2005). Females of the bee-wolf evolved a high sensory bias to respond to this alarm pheromone compound of honeybees to locate them, which is exploited by males to attract females.

These two hypotheses explain how a pheromone compound can evolve, but not how the impressive diversity of species-specific pheromones evolved. Speciation, the formation of new species, can, however not alone, play an important role in the diversification of chemical communication (Symonds and Elgar 2008; Smadja and Butlin 2009). Stabilizing selection should prevent changes in the pheromone composition of a species by strong selection pressure against all deviations from either the sender's signaling system or the corresponding perception of the receiver to maintain species integrity (Paterson 1980; Butlin and Trickett 1997; see also Allison and Cardé 2016 and references therein). Nevertheless, dynamic variation in selection pressures could lead to changes in pheromone compositions, and any divergence from the pheromones important for species recognition and mate finding between populations of a species can lead to changed preferences in mating by receivers and hence to non-random mating. Divergent coevolution of mating signals and preferences therefore reduces the exchange of genetic material and leads to reproductive isolation and ultimately to the speciation of a population into a new species (Panhuis et al. 2001; Coyne and Orr 2004; Ritchie 2007; Nosil et al. 2017). As most pheromones are not single compounds but complex multicomponent mixtures, already small quantitative changes in the species-specific sex pheromone ratio of these compounds or a qualitative change, i.e. a loss or addition of molecules, will alter the communication and lead to reproductive barriers and pre-mating isolation. Thereby, a population may diverge from its parent population and become a new species. Hence, speciation can lead to changing sex pheromones and therefore also accounts for the diversification of pheromonal compounds (Symonds and Elgar 2008; Smadja and Butlin 2009).

Hereby it has to be differentiated between the reproductive barriers leading to a divergence between populations of a species. If the populations remain in the same geographical range, behavioral, physiological or ecological reproductive barriers can arise and change the communication of these two populations of a species (sympatric speciation), however, if environmental barriers separated the populations (allopatric speciation), the communication between the populations can also change (Hoskin et al. 2005; Bolnick and Fitzpatrick 2007; Fitzpatrick et al. 2008; Kopp et al. 2018).

If pheromones of sympatric diverging populations would evolve only with small changes gradually over time, the very similar pheromones would lead to mismating and hybridization of individuals and hence to fitness loss (Gröning and Hochkirch 2008; Mendelson and Shaw 2012; Nosil et al. 2017; Chouvenc et al. 2020). Because of this risk of interspecific interference there should be strong selection

against any small divergence from the species-specific sex pheromones (Paterson 1985). Hence, the general changes of mate recognition pheromones during sympatric speciation of one species into sibling species should occur via sudden large, saltational shifts (Löfstedt 1993; Baker 2002; Symonds and Wertheim 2005; Symonds and Elgar 2008; Symonds et al. 2009). Indeed, the compositions of pheromones can vary enormously between closely related species (Schulz 2004; Schulz 2005). For example, it is assumed that the highly divergent pheromones of several closely related bark beetles (Symonds and Elgar 2004), of sympatric moth species (Löfstedt et al. 1991), and of fruit flies (Ferveur 2005) have evolved through saltational changes in pheromone compositions (Symonds and Elgar 2008). Another example for a pheromone-induced reproductive barrier in sympatric populations of a species is found in the European corn borer moth (*Ostrinia nubilalis*), where two strains of this species (called *E*- and *Z*-strain) produce different mixtures of *E*-11-14-acetate and *Z*-11-14-acetate as female sex pheromones, with opposite isomeric mixture ratios (for the *E*-strain, the mixture ratio *E*:*Z* is 98:2, whereas for the *Z*-strain it is 3:97; Lassance 2010 and references therein). The changing of mate recognition pheromones of senders as well as the respective modifications of the pheromone detection systems in receivers provides a simple but powerful tool during speciation (Smadja and Butlin 2009). Therefore, identifying the sex pheromones of closely related species is necessary to understanding sympatric speciation.

However, if the speciation occurs due to geographic barriers between populations within a species and due to adaptations of these separated populations to different environments or hosts, selective forces driving the evolution of changes in sex pheromones are predicted to be low (Symonds et al. 2009). Therefore, the pheromones of allopatric populations of a species can stay the same, but over time small changes might accumulate and hence, their communication diverges in a fine gradual mode of evolutionary change (Roelofs and Brown 1982). The ecological divergence between the isolated populations will together with natural selection and random drift cause the populations to differentiate genetically and lead to reproductive isolation (Mayr 1963). Alternatively, the sexual isolation of allopatric populations can be a simple by-product of local adaptation to different environments (Schluter 2001). As long as there is a reproductive isolation due to any ecological, behavioral or geographical separation, the selection on pheromone changes should be weak and similar pheromone compositions used by closely related species are expected (Symonds et al. 2009).

Indeed, closely related species often share the same major compounds as sex pheromones, although in different ratios or different combinations with minor compounds. Also when sibling species have strikingly different pheromones, their pheromonal compounds are often biosynthetically related. This similarity is due to phylogenetically shared biosynthetic pathways (Tillman et al. 1999; Symonds and Elgar 2008). The pre-existence of major biosynthetic pathways that were already present in the early evolutionary history of Hymenopteran species (Kather and Martin 2015) is assumed to be able to explain the great diversity of pheromone compounds found today, as already small changes in the biosynthetic pathways can have led to new, but structurally related compounds (Baker 2002; Symonds and Elgar

2008). Also outside of the Hymenoptera examples for structural similarities between pheromone compounds of closely related species can be found. The species-specific female pheromone signals of ten corn borer moths belonging to the genus *Ostrinia* consist of different combinations and concentrations of the same six compounds (Kochansky et al. 1975; Cheng et al. 1981; Ishikawa et al. 1999; Tabata et al. 2008; Lassance 2010 and references therein). Closely related species of cockroaches also share pheromone compounds as components of their long-range sex pheromones, although each of the genera of cockroaches utilizes sex pheromone compounds belonging to completely different chemical classes (Gemeno and Schal 2004; Eliyahu et al. 2012).

One other (so far underappreciated) evolutionary trajectory that might have led to the diversification of pheromones is the trade-off between the non-communicative function of a cue and the modification of this cue to become a more informative signal, which could lead to a reduction of the effectiveness of the original compound (Steiger et al. 2011). Any change in the composition of CHC compounds, for example, affects the functionality of the biophysical properties such as waterproofing or the protection against microbes. In turn, the stabilizing selective pressure to preserve the species-specificity of pheromone compounds restricts flexible adaptations of the original cue precursor of the pheromone to changing environmental conditions, as is the case with CHC compounds that serve various communicative roles, but also serve as desiccation prevention in insects (Lockey 1988; Gibbs 1998; Gibbs and Rajpurohit 2010). However, the main signaling compounds can be evolutionary conserved, while others can adapt according to changing climatic conditions.

In contrast to species-specific mating signals, pheromones not involved in species recognition or mate finding are assumed to evolve gradually, because no selective force drives saltational changes among them as they are not responsible for creating a reproductive isolation between species (Symonds and Wertheim 2005; Symonds et al. 2009). For example, aggregation pheromones may be under a gradual mode of evolution and hence be shared by different species, as heterospecific attraction can provide benefits, e.g. result in better exploitation of resources. For example, in the genus *Drosophila*, several closely related species produce species-specific, dissimilar sex pheromones (Ferveur 2005) but share similar aggregation pheromones (Symonds and Wertheim 2005), as communal oviposition could facilitate larval development (Wertheim et al. 2002). Similarly, also alarm pheromones are often shared by different species, as there should be only advantages for individuals to respond to compounds alerting for the presence of a predator. Therefore it is beneficial for individuals to react to conspecific as well as to heterospecific alarm signals or defensive substances (Regnier and Law 1968; Blum 1969). For example, many aphid species in over 30 genera share the sesquiterpene, (*E*)-β-farnesene, as alarm pheromone compound (Byers 2005), while their specific sex pheromones are very multicomponent and diverse (Dewhirst et al. 2010). Iridoid compounds are another example of compounds being used as defensive allomones and alarm compounds by a diverse array of insect species (Pasteels et al. 1982; Dinda 2019), and as insecticides and secondary metabolites in plants (Inouye and Uesato 1986; Dinda 2019). Iridomyrmecin was originally identified in the argentine ant *Linepithema humile* (formerly

Iridomyrmex humilis) (Pavan 1949), and is released in large quantities together with another iridoid compound, dolichodial, from the pygidial gland of the ants when they are engaged in aggressive interactions with other ants (Welzel et al. 2018). In parasitoid wasps iridoids have only been found in the genera *Alloxysta* and *Leptopilina*, which use iridomyrmecin and other iridoids for defense (Völkl et al. 1994; Hübner and Dettner 2000; Hübner et al. 2002; Stökl et al. 2012; Weiss et al. 2013; Weiss et al. 2015a; Pfeiffer et al. 2018). Additionally, several species of ants and beetles use iridoid compounds as part of their defensive secretions as repellents against predators such as ants and spiders (Huth and Dettner 1990; e.g. Do Nascimento et al. 1998; Welzel et al. 2018).

1.3 The Study System

Parasitoids (termed by Reuter 1913) have a non-mutualistic association with their host, ultimately killing their respective host during their successful development (Eggleton and Belshaw 1992; Godfray 1994; Fleury et al. 2009). Most of them are parasitoid wasps belonging to the order Hymenoptera, which comprise over 200,000 species of ants, bees, wasps, and sawflies (LaSalle and Gauld 1991; Godfray 1994), but there are also parasitoid species of the orders Diptera, Coleoptera, Lepidoptera, Neuroptera, Strepsiptera, and Trichoptera (LaSalle and Gauld 1991; Eggleton and Belshaw 1992; Heraty 2009). Parasitoid wasps (Hymenoptera: Apocrita) develop in or on arthropod hosts (Quicke 1997; Ruther 2013) and account with about 50,000 described species, but probably many more yet undescribed species, for approximately 10–20% of all insect species (LaSalle and Gauld 1991). In regulating the population densities of their hosts and maintaining species diversity, parasitoid wasps are of great ecological importance (Godfray 1994; Quicke 1997; Macfadyen et al. 2011). Despite being so rich in species and tremendously important for ecosystems, the sexual communication of parasitic wasps was not studied much to date and our knowledge lags far behind that of other taxa (Ruther 2013). For example, while there were already 20 years ago more than 1000 sex pheromones of moths identified (Arn et al. 1992), it is only for little more than 30 parasitic wasp species known which pheromones they use to communicate with conspecifics (Ruther 2013).

The here studied species of *Leptopilina* FÖRSTER, 1869 (Cynipoidea: Figitidae: Eucoilinae) provide a perfect model system to investigate the evolution of pheromones. Species of these parasitoid wasps are studied since over five decades and some are well known model organisms for the host-parasitoid interactions with their *Drosophila* hosts. Some of the 29 *Leptopilina* species that have been described from Europe, Africa, North America and Asia (Nordlander 1980; Beardsley 1988; Quinlan 1988; Nordlander and Grijpma 1991; van Alphen et al. 1991; Allemand et al. 2002; Novković et al. 2011; Wachi et al. 2015; Lue et al. 2016) have a worldwide distribution (Fontal-Cazalla et al. 1997; Allemand et al. 2002; Buffington pers. obsv.). *Leptopilina* are small wasps of less than 2 mm length, with males having longer antennae than females (compare **Fig. 1a, b**). In this genus the females produce the sex

pheromones which attract the male wasps and elicit stereotypic pre-copulatory courtship behavior in them. Courting males respond with fast fanning of their wings upon perception of a conspecific female (wing fanning), touch the females' antennae, mount the females and show antennal movements by stroking the females' antennae with their own (Jenni 1951; van den Assem 1968; Isidoro et al. 1999), whereby they presumably transfer aphrodisiac compounds from their antennal glands to the female's antennae to elicit mating (van den Assem et al. 1980; Weiss et al. 2015b). Female wasps are koinobiont solitary larval-pupal endoparasitoids, as they oviposit single eggs in the hemocoel of larvae or pupae of *Drosophila* (see **Fig. 1c**). The wasp larvae then feed and develop solitarily within the drosophilid host without impeding its growth (but inevitably killing it in later stages of its development) and adult wasps emerge about three weeks later from the hosts puparium (Carton et al. 1986; Godfray 1994, see **Fig. 1d**). However, as *Drosophila* are mostly clumped together, *Leptopilina* wasps are considered as quasi-gregarious (van den Assem et al. 1980). All hymenopteran species have a haplodiploid sex determination with males having a haploid and females having a diploid chromosome set. Female *Leptopilina* wasps are arrhenotokous, i.e. have control over fertilization of an egg with sperm during oviposition. This means they can lay unfertilized eggs before mating, which will develop into males, whereas they can decide after mating whether to lay unfertilized or fertilized eggs, with the latter developing into female wasps.

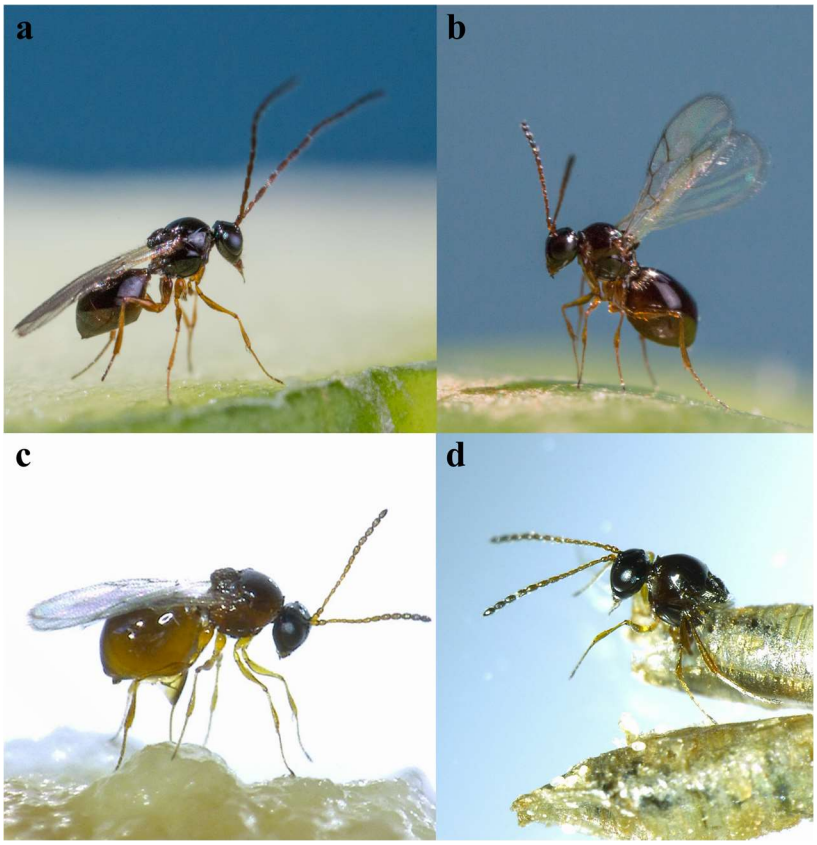

Figure 1 Photographs showing **a)** a male and **b)** a female wasp of *Leptopilina boulardi*. **c)** A female *L. boulardi* wasp inserting her ovipositor into substrate to search for a host (a larva of e.g. *Drosophila melanogaster*) to lay an egg into. The wasp larva then develops within the fly larva and pupa. **d)** An adult female *L. boulardi* wasp emerging from a fly pupa. Below a parasitized pupa from which soon a wasp will emerge. © Johannes Stökl

However, knowledge of their communication remains scarce, as so far only the compounds produced by four species have been studied: *L. heterotoma* THOMSON (Stökl et al. 2012; Weiss et al. 2013; Stökl et al. 2015; Weiss et al. 2015b; Weiss et al. 2015a), *L. boulardi* BARBOTIN, CARTON & KELNER-PILLAULT (Weiss et al. 2015b; Weiss et al. 2015a), *L. victoriae* NORDLANDER (Weiss et al. 2015b; Weiss et al. 2015a), and *L. clavipes* HARTIG (Pfeiffer et al. 2018). Nevertheless, the species *L. heterotoma* serves as a prime example for the comprehension of the sex pheromone evolution, which presumably followed the route of the precursor hypothesis in this species. The compound (−)-iridomyrmecin (see **Fig. 2c**) is produced in the mandibular glands of male and female *L. heterotoma* wasps (Stökl and Herzner 2016) and used (together with minor concentrations of its stereoisomer (+)-isoiridomyrmecin, see **Fig. 2d**) as their defensive allomones deterring natural enemies (Stökl et al. 2012), as well as a cue for females to avoid competition with con- and heterospecific females, and most interestingly, as main compound of the females' sex pheromone, initiating mate finding, recognition and courtship (Weiss et al. 2013). However, while for defense and the avoidance of exploited host patches, (−)-iridomyrmecin alone is sufficient, only the mix of (−)-iridomyrmecin and minor concentrations of (+)-isoiridomyrmecin, another iridomyrmecin compound and two iridodial compounds can trigger courtship behavior in males (Weiss et al. 2013). An evolution from the precursor defensive substance to a sex pheromone function can be assumed. This is a rare case of evidence for the evolution of pheromones, as primary functions of those compounds used for specific intraspecific communication across the whole animal kingdom, but also in bacteria, fungi and plants, often have been lost. However, the phenomenon of a parsimonious use of the same substance being non-communicative *and* having communicative functions within the same species, i.e. semiochemical parsimony, enables us to understand the evolution from a non-communicative compound gaining communicative means.

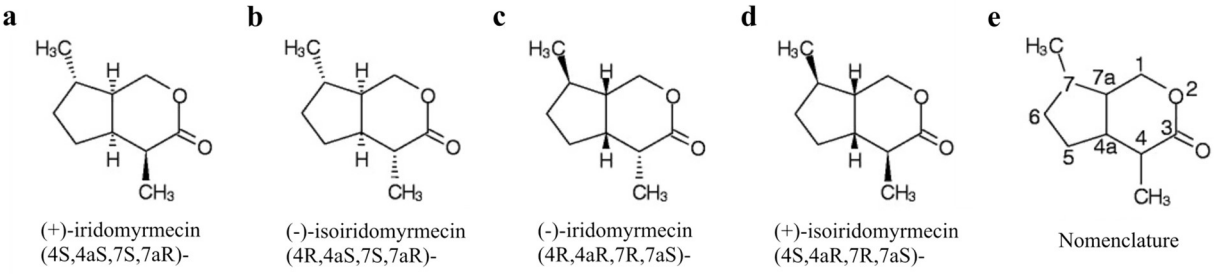

Figure 2 a)-d) Molecular structure of four stereoisomers of iridomyrmecin. **e)** Numbering of carbon atoms in the molecule of iridomyrmecin. Numbers 4, 4a, 7, and 7a represent the chiral centers. Modified from Stökl et al. 2012

1.4 Aims of this Thesis

The aim of this thesis was to the expansion of the knowledge about different pheromonal usages of several species of *Leptopilina* wasps, thereby elucidating the mode of evolution that has taken place in the chemical communication of the genus *Leptopilina* and contributing to the general understanding of pheromone evolution. As mentioned above, in the species *L. heterotoma* the compound (−)-iridomyrmecin fulfils the purpose to deter predators and competitors, but also evolved to attract male conspecifics and elicits their courtship behavior (Stökl et al. 2012; Weiss et al. 2013). Three other species of *Leptopilina* have been studied, and as *L. heterotoma*, they all produce cuticular hydrocarbons (CHCs) as well as iridoid compounds in species-specific combinations (albeit not always with the same stereoisomer of iridomyrmecin as in *L. heterotoma*). However, the usage of iridoid compounds and the compounds used for the sexual communication differed drastically between the species *L. boulardi, L. victoriae,* and *L. clavipes* (see **Fig. 3**). Whereas females of *L. boulardi* use combinations of iridoids and CHCs as their sex pheromones (Weiss et al. 2015a), the female sex pheromone of *L. victoriae* consists only of the species-specific CHCs (Weiss et al. 2015a), and in *L. clavipes* the females' iridoids even deter conspecific males and only their CHCs elicit the stereotypic mating behavior (Pfeiffer et al. 2018). In *Leptopilina*, it has been already proven that females of *L. heterotoma* release increased amounts of (−)-iridomyrmecin and its naturally occurring epimer (+)-isoiridomyrmecin from a cephalic gland as a defensive allomone when encountering natural enemies, in this case different species of ants (Stökl et al. 2012; Stökl et al. 2015). Also in *L. boulardi* and *L. victoriae* iridoid compounds were released when wasps were attacked by aggressive ants (Machacek 2015). As (−)-iridomyrmecin and three of its stereoisomers were shown to repel ants effectively, with (−)-iridomyrmecin having the strongest deterrent effect (Stökl et al. 2012), and because of the usage as defensive allomone in *L. heterotoma* and several other species (see **1.2 Pheromone Evolution**), we can assume that also other species of *Leptopilina*, such as *L. clavipes,* use (−)-iridomyrmecin as defensive chemical, although it was not experimentally tested (Pfeiffer et al. 2018; see **Fig. 3**). The usage of iridoid compounds for competition avoidance of females has been tested in *L. heterotoma* (Weiss et al. 2013), *L. boulardi* (unpublished), and *L. clavipes* (Pfeiffer et al. 2018), but not in *L. victoriae* (see **Fig. 3**). The differential use of iridoid compound for defense, competition avoidance and as attractants for mating partners and courtship stimulants in this genus of parasitoid wasps proposes an evolutionary conundrum, which I want to approach in this work.

To understand this intriguing example of an evolution of a pheromonal compound found in *L. heterotoma* better, I aimed to analyze the use of iridomyrmecins in more species of the genus *Leptopilina* and to disentangle the mode of evolution that could have taken place during evolutionary development of the communication in these species. Therefore, I investigated in this thesis the chemical communication of *L. japonica* NOVKOVIĆ & KIMURA*, L. ryukyuensis* NOVKOVIĆ & KIMURA, and *L. pacifica* NOVKOVIĆ & KIMURA. Nothing is known to date about the produced chemical compounds

and of the pheromone usage in these closely related species. I analyzed and identified the compounds of male and female wasps of these three species, analyzed the defensive secretions emitted by female wasps upon attack or disturbance, analyzed the competition avoidance behavior of female wasps during host-finding, and the components of the sex pheromones of female wasps of all species by analyzing the courtship behavior exhibited by males in response to specific female compounds (*L. japonica* and *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1** and *L. pacifica*: Böttinger et al. 2020, see **publication 2**). Furthermore, I investigated whether iridoids evolved in these species as cues for males to find female wasps over larger distances, and whether their dispersal behavior after emergence is correlated with the volatility of the females' sex pheromones (Böttinger and Stökl 2020, see **publication 3**). Elucidating the communication system of these species, specifically, their reliance on iridoids, should yield important insights into the evolutionary trajectories promoting variation in sexual signaling within the genus *Leptopilina*.

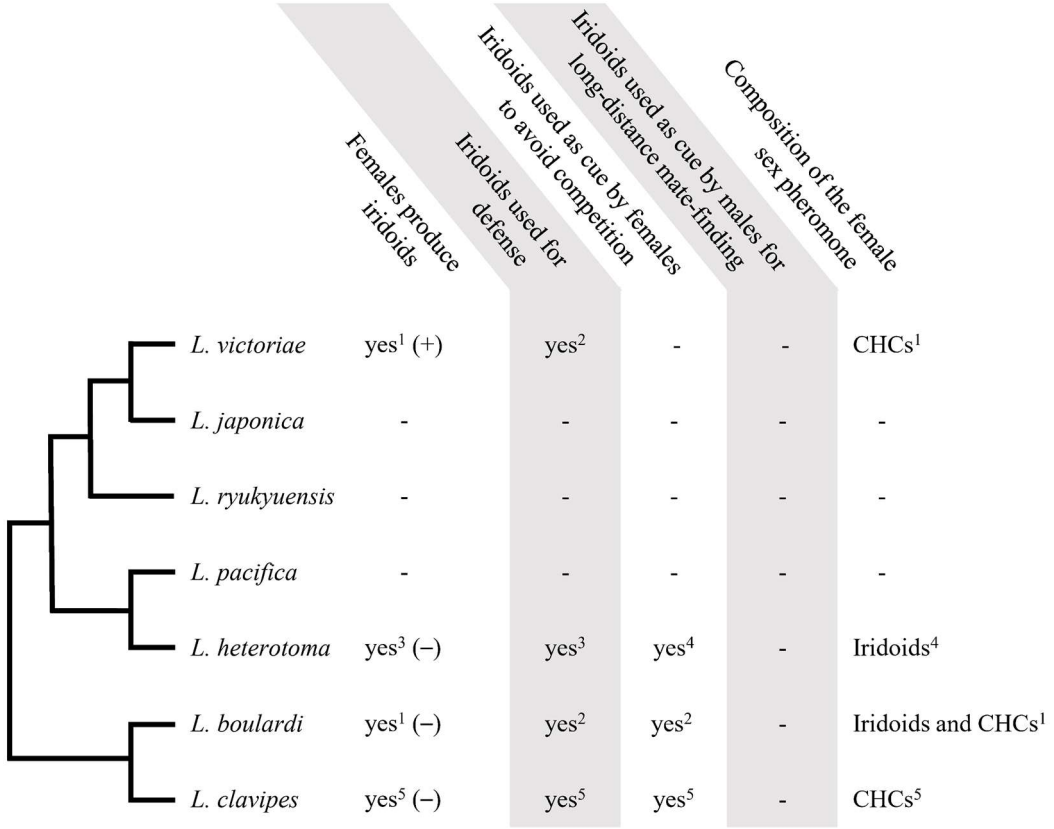

Figure 3 Phylogenetic relationship and current knowledge on the production and use of iridoids by the four species of *Leptopilina* studied so far. (+) indicates that (+)-iridomyrmecin is produced (among other iridoid compounds), and (–), that (–)-iridomyrmecin is produced. Hyphens indicate that this function has not been studied. Phylogenetic tree based on ITS2, modified from Wachi et al. 2015. [1]Weiss et al. 2015a; [2]unpublished; [3]Stökl et al. 2012; [4]Weiss et al. 2013; [5]Pfeiffer et al. 2018, here, the defensive function of iridoids is assumed, but has not been experimentally tested.

2. Synopsis: An Overview of the Publications of this Thesis

Publication 1:

Semiochemicals Mediating Defense, Intraspecific Competition, and Mate Finding in *Leptopilina ryukyuensis* and *L. japonica* (Hymenoptera: Figitidae), Parasitoids of *Drosophila*

Lea C. Böttinger, John Hofferberth, Joachim Ruther, and Johannes Stökl

Published in the Journal of Chemical Ecology (2019) 45: 241–252

As outlined above (see **1.2 Pheromone Evolution**) the evolution of pheromone communication is a topic far from resolved. Two hypotheses explain how changes in pheromone compositions between closely related species could have evolved: in absence of other means of reproductive isolation between potentially diverging populations, large, saltational evolutionary changes in pheromones important for species recognition and mate finding are assumed to happen (Löfstedt 1993; Baker 2002; Symonds and Wertheim 2005; Symonds and Elgar 2008; Symonds et al. 2009), because if pheromones would stay similar, mismating and hybridization between individuals of the two populations could lead to fitness losses (Gröning and Hochkirch 2008; Mendelson and Shaw 2012; Nosil et al. 2017; Chouvenc et al. 2020). If there are however reproductive barriers isolating the population, which could be, for example, geographical or behavioral, pheromones of the two populations are under low selection pressures to differentiate and only small, gradual, "darwinian" changes are assumed to accumulate in their compositions over evolutionary time (Roelofs and Brown 1982; Symonds et al. 2009).

How pheromones evolved in the first place is again discussed by two hypotheses. The sensory exploitation hypothesis states that pheromones evolved because of a preexisting sensory bias in the receiving organism that the sending organism exploits (Bradbury and Vehrencamp 2011; Steiger et al. 2011; Ryan and Cummings 2013; Wyatt 2014). The precursor hypothesis states that the compounds used for communication evolved from precursor compounds fulfilling non-communicative purposes before in the same organism (Otte 1974; Haynes and Potter 1995; Steiger et al. 2011; Wyatt 2014). For the latter hypothesis a rare example of one compound being used as non-communicative defensive allomone, as cue for female competition avoidance, and as sex pheromone in the parasitoid wasp species *Leptopilina heterotoma* was found (Weiss et al. 2013). This interesting case of a threefold parsimony enables the comprehension of an evolutionary route of a compound emitted for defensive purposes, which presumably then informed conspecific receivers about the presence of a sending individual, which

led to the usage of the compound as a cue to avoid competitors during host search, presumably to avoid superparasitization, and lastly to the usage of the compound to locate and recognize mating partners.

To find out whether this evolutionary route can be found in related species of *L. heterotoma*, in follow-up studies the chemical communication of three other species of the genus *Leptopilina* was investigated, *L. boulardi* (Weiss et al. 2015a), *L. victoriae* (Weiss et al. 2015a), and *L. clavipes* (Pfeiffer et al. 2018). In these previous studies it was found that all of the species produce iridoid compounds apart from CHCs and while it was found for *L. heterotoma* that iridoids were emitted for defense, the allomone function was also assumed for the other species. Iridoids were also used by females of *L. heterotoma*, *L. boulardi* and *L. clavipes* to avoid conspecific females (Weiss et al. 2013; unpublished; Pfeiffer et al. 2018) and evolved additionally in *L. heterotoma* and *L. boulardi* (together with CHCs) as sex pheromones, attracting males and eliciting courtship behavior in them (Weiss et al. 2013; Weiss et al. 2015a). In contrast, females of *L. clavipes* and *L. victoriae* use only CHCs as sex pheromones and surprisingly, in *L. clavipes* the iridoid compounds alone and in the females' whole body extract were deterrent to conspecific males (Pfeiffer et al. 2018). The evolution of a defensive secretion to a cue therefore happened in three species, while the communicative function evolved only completely in *L. heterotoma*. The pheromones in *L. clavipes* and *L. victoriae* could have evolved in a gradual mode of evolutionary change, while the difference between the groups using iridoids or CHCs as sex pheromones is so large, that presumably saltational shifts led to these changes. The difference in the pure iridoid sex pheromones in *L. heterotoma* and the combination of CHCs and iridoid compounds as sex pheromones in *L. boulardi* are not easy to explain. Further knowledge of sex pheromone compositions of closely related species could help shed light on whether this sex pheromone shift from only iridoids to iridoids with CHCs or vice versa happened more often in this genus, whether the evolution from a non-communicative to a communicative function of iridoids happened also in other species and how the evolution of pheromones could have happened in the genus *Leptopilina*.

Therefore, I analyzed in this study (**publication 1**) the compounds produced by and used for communication by males and females of *L. japonica* and *L. ryukyuensis*. These two species were only recently described on the Japanese Ryukyu archipelago and in Taiwan (Novković et al. 2011). The aim of this study was to investigate whether these species also produce iridoid compounds and CHCs in a similar composition as in *L. heterotoma*, *L. boulardi*, *L. victoriae* or *L. clavipes*, which could help elucidating the mode of evolution that has taken place in the speciation of these closely related species. If the compounds differ drastically, saltational selection events could have led to the separation of species, whereas large similarities could indicate behavioral or physiological adaptations to different environments in which case the differentiation of pheromones would not have been necessary to isolate populations and lead to speciation. Additionally, we wanted to investigate whether these species also use their potential iridoid compounds for defense, as in *L. heterotoma* (Stökl et al. 2012), for female competition avoidance, as in *L. heterotoma* (Weiss et al. 2013), *L. boulardi* (unpublished), and *L. clavipes* (Pfeiffer et al. 2018; not tested in *L. victoriae*), and as sex pheromone components, as in

L. heterotoma (Weiss et al. 2013) and partly in *L. boulardi* (Weiss et al. 2015a), which use a combination of CHC and iridoid compounds as female sex pheromones.

Here, we could indeed show in chemical analyses of specimens of *L. japonica* and *L. ryukyuensis* that these two species, as all species of this genus studied to date, produce CHCs and iridoid compounds. In contrast to males and females of *L. heterotoma* (Stökl et al. 2012), *L. boulardi* (Weiss et al. 2015a), and *L. clavipes* (Pfeiffer et al. 2018), which produce (−)-iridomyrmecin, we found that females of *L. ryukyuensis* produce a combination of the iridoid epimers (+)- and (−)-iridomyrmecin, whereas their males produce only (−)-iridomyrmecin. In contrast, males and females of *L. japonica* produce only (+)-iridomyrmecin (see **Fig. 2a**), which has before only been found in the species *L. victoriae* (Weiss et al. 2015a). When looking at the phylogeny of the *Leptopilina* species studied to date (**Fig. 4**; see also **Fig. 5** in **publication 1**; Wachi et al. 2015), species producing (−)-iridomyrmecin are more closely related with each other than with the species producing (+)-iridomyrmecin, and *L. ryukyuensis* (with females producing both epimers (+)- and (−)-iridomyrmecin) represents the species in between those two groups. Hence, these differentially produced stereoisomers within the genus *Leptopilina* could indicate a shift from (−)- to (+)-iridomyrmecin from (−)-iridomyrmecin-producing species of *Leptopilina* over *L. ryukyuensis*, whose males produce (−)-iridomyrmecin, while their females produce both stereoisomers, to the (+)-iridomyrmecin producing species *L. japonica* and *L. victoriae*.

Additionally, we investigated in these species whether the emit iridoids as deterrent substances during encounters with predators. Thereby we found upon encounters of them with predatory insects and also upon irritating female wasps with a magnetic stirring bar that females of both species emitted higher amounts of iridoid compounds than when left undisturbed. Females of *L. ryukyuensis* emit iridoids such as (+)- and (–)-iridomyrmecin upon being teased whereas *L. japonica* females emit several different iridoids during the artificial attacks and those of predators. The comparison of compounds emitted by female wasps of *L. japonica* and *L. ryukyuensis* during artificial attacks with a magnetic stirring bar and of compounds emitted by them in undisturbed situations were done for the first time with specimens of *Leptopilina*. This analysis method proved to be more consistent and reliable than analysis of compounds emitted during attacks of natural predators (lacewing larvae), as these were not always with the same intensity or duration and emitted compounds of females consequently highly variable. We furthermore found out in behavioral analyses, that females of *L. ryukyuensis* also use their defensive compounds to avoid host patches already exploited by other conspecific females. In contrast, female *L. japonica* wasps did not avoid other conspecific females or their defensive compounds during host search. We also show that the sex pheromone of female *L. ryukyuensis* consists of CHCs, as males showed strong courtship behavior (wing fanning) towards these compounds. Males of *L. japonica* use their females' iridoids to recognize and court them but might need the combination of CHCs and iridoids to elicit the full courtship behavior.

We conclude that the compounds produced by both species are qualitatively and quantitatively relatively similar, except for the structural differences in the stereochemistry of the iridomyrmecin compounds, as the compound (+)-iridomyrmecin gets produced by males and females of *L. japonica*, whereas male wasps of *L. ryukyuensis* produce (–)-iridomyrmecin instead, and females of *L. ryukyuensis* produce both stereoisomers. Both species use their iridoid compounds as defensive allomones, which evolved in the perception of female wasps of *L. ryukyuensis* to be perceived as female competition avoidance cues during host search, but not in females of *L. japonica*. In the latter species, however, the defensive allomones evolved additionally as female sex pheromones. Further comparative studies on the pheromone production, the mating system of wasp species and a more detailed phylogenetic approach are necessary to understand the evolution of the chemical communication within the genus *Leptopilina*.

Publication 2:

Mate Attraction, Chemical Defense, and Competition Avoidance in the Parasitoid Wasp *Leptopilina pacifica*

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In the parasitoid wasp *Leptopilina heterotoma*, a rare example of a threefold semiochemical parsimony of a single compound, (−)-iridomyrmecin, has been found (see **1.3 The Study System**). This substance is emitted by wasps of this species for defense (Stökl et al. 2012; Stökl et al. 2015), it is used by female wasps to detect conspecifics and to avoid their competition (Weiss et al. 2013), and male wasps use it to find and recognize female mating partners and elicit the male courtship behavior (Weiss et al. 2013). This iridoid therefore presumably evolved from a non-communicative defensive allomone to a female competition avoidance cue and into a sex pheromone compound (Weiss et al. 2013). To find out whether also other species of the same genus of parasitoid wasps show this route of pheromone evolution from a non-communicative to a communicative function of a compound, the compounds produced by and used in the communication of six other species have been analyzed (*L. heterotoma*: Stökl et al. 2012; Weiss et al. 2013; Stökl et al. 2015; Weiss et al. 2015b; Weiss et al. 2015a; *L. boulardi*, *L. victoriae*: Weiss et al. 2015a; *L. clavipes*: Pfeiffer et al. 2018; *L. japonica*, *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**).

All studied species produce apart from cuticular lipids (cuticular hydrocarbons; CHCs) different combinations of iridoids and different stereoisomers of the compound iridomyrmecin. The usage of iridoid compounds seems to be the ancestral usage of the compounds, as is has been experimentally found in three of these species (*L. heterotoma*: Stökl et al. 2012; Stökl et al. 2015; *L. japonica*, *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**) and can be assumed for the others. Also the usage of iridoids as competition avoidance cue was found in some species (*L. heterotoma*, *L. boulardi*: Weiss et al. 2013; Weiss et al. 2015a; *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**). However, the sex pheromones were surprisingly different between the studied species. Whereas some species use solely long-chained CHCs as female sex pheromones (*L. victoriae*: Weiss et al. 2015a; *L. clavipes*: Pfeiffer et al. 2018; *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**), others rely solely on volatile iridoid compounds as sex pheromones (*L. heterotoma*: Weiss et al. 2015a; *L. japonica*: Böttinger et al. 2019, see **publication 1**) and one species uses a combination of both, iridoids and CHCs (*L. boulardi*, Weiss et al. 2015a).

As strong stabilizing selection pressures should work against changes in species-specific pheromones used for mate-finding and mate recognition, the diversity of the production and use of iridomyrmecins as well as of sex pheromones found until now within the genus *Leptopilina* is remarkable. This diversity of the produced compounds and different sex pheromones can either be explained by adaptations of the closely-related species to different environments, which could have changed the metabolism or physiological pathways generating the pheromones of these species. Alternatively, if the species are sympatric, i.e. occur in the same or in overlapping geographical areas, the differences in mating signals could be due to saltational evolutionary changes in pheromone composition during speciation.

To investigate the evolutionary scenario which could have taken place in the speciation of *Leptopilina* wasps it is necessary to investigate the chemical communication of more closely-related species of this genus. Therefore, we elucidated in **publication 2** the chemical communication of the species *L. pacifica* NOVKOVIĆ & KIMURA 2011, which is the sister species of *L. heterotoma*, the model organism for the evolution of sex pheromones. By this, we also wanted to investigate whether *L. pacifica* produces and uses similar compounds for communication as *L. heterotoma*, and, to better understand the evolution of sex pheromones from defensive compounds, whether its sex pheromone also evolved from a formerly non-communicative iridoid compound. If the mating signals of *L. pacifica* and *L. heterotoma* would be strikingly different, the speciation of an ancestral parasitoid wasp species into these two sibling species can be assumed to have taken place because of selective forces driving large, saltational shifts in their mating signals, leading to reproductive isolation. As the two species have at least partially overlapping geographic distribution areas (both species have been found in Japan; Novković et al. 2011), the saltational changes in pheromone compositions are highly probably in the absence of other means of reproductive isolation. However, their pheromones could also be just slightly different and could have only gradually evolved over time, if the wasp species for example diverged due to adaptations to different drosophilid host species. Indeed, whereas *L. heterotoma* uses various drosophilid species such as *Drosophila melanogaster* or *D. simulans* as host species, *L. pacifica* was not able to reproduce on these species but can parasitize larvae of the *D. immigrans* group (Kimura and Suwito 2012), e.g. *D. virilis*. However, if both species share the same sex pheromones and this threefold semiochemical parsimony found in *L. heterotoma* would also to be found in *L. pacifica,* we expect that the isolation of these species happened after (−)-iridomyrmecin evolved from a non-communicative defensive allomone to a female competition avoidance cue and into a sex pheromone compound. Alternatively, both closely-related species could have the same sex pheromones but have diverged because of adaptations to different environments or because they are allochronic (i.e. do not mate at the same time). To test these assumptions and to solve these hypotheses and expectations, this study wants to ask the following question: Did the evolution from a single non-communicative chemical compound to a compound fulfilling three different functions, also as mating signal, only take place in *L. heterotoma* or also in its sibling species *L. pacifica*?

To answer this question, we report here on the production and composition of chemical compounds in *L. pacifica*, showing that *L. pacifica* produces a species-specific mix of several different iridoid compounds, more complex and with differing stereochemistry than found in *L. heterotoma,* among which are (+)-iridomyrmecin and in males also (+)-isoiridomyrmecin (see Table 1 in **publication 2**). For the first time in analyses of *Leptopilina*, we also found Citral ((*Z*)-3,7-dimethyl-2,6-Octadienal (Neral) and (*E*)-3,7-dimethyl-2,6-Octadienal (Geranial)) in the volatile compounds of the *L. pacifica* wasps. We demonstrate that *L. pacifica* wasps release these iridoid and Citral compounds upon attack and irritation as defensive secretion, indicating that the production of iridoids evolved in the whole genus *Leptopilina* for the purpose of defense. The compounds emitted from attacked or teased *L. pacifica* females were the same volatiles found in total body extracts of the wasps, namely iridoids such as iridodial, actinidine, nepetalactone and (+)-iridomyrmecin, and citral components. In contrast, on average only very little amounts of volatiles at all were detected in the headspace of the control wasps. Therefore, we show here that the iridoid and citral compounds produced by *L. pacifica* females are used as defensive allomones to deter natural enemies in case of an attack. Females of *L. pacifica* use iridoids for competition avoidance during host finding. However, in contrast to *L. heterotoma*, we found no avoidance of live females by conspecific females of *L. pacifica*, but an avoidance of their extracts, possibly due to higher iridoid concentrations than emitted by live females. In the sex pheromone iridoids can be neglected, as the females' cuticular hydrocarbons (CHCs) and pure female extracts resulted in the elicitation of courtship of mating partners.

We show here that the use of iridomyrmecins as defensive compounds or alarm pheromones in *Leptopilina* is common. However, although closely related, the two sister species show substantial differences in the use of the defensive secretion for communicative purposes. The evolution of a non-communicative compound for defense into a cue for competition avoidance and into a sex pheromone did take place only in one species, *L. heterotoma*, studied so far. Also in *L. japonica* iridoid compounds serve for defense and as sex pheromone, but not as competition avoidance cue (Böttinger et al. 2019, see **publication 1**). We assume a speciation of the sibling species *L. heterotoma* and the here studied *L. pacifica* because of a host shift and an adaptation of the two wasp species to different environments and hosts. Alternatively, other selection pressures, such as the mating system or the dispersal behavior, could have resulted in these strikingly different mating signals (Böttinger and Stökl 2020, see **publication 3**). Saltational shifts in the sex pheromone composition and also in the production of the different stereoisomers (+)- and (–)-iridomyrmecin might have led to the speciation of the sister species. Variation in pheromone usage in this genus still presents a conundrum, highlighting the need for additional studies to understand the selective forces shaping the evolution of pheromone composition.

Publication 3:

Dispersal from Natal Patch Correlates with the Volatility of Female Sex Pheromones in Parasitoid Wasps

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Chemicals are used by insects as cues to find oviposition sites, prey or hosts, or as signals to locate and identify mating partners or aggregations with conspecifics (Greenfield 1981; Cardé and Baker 1984; Wyatt 2014). These compounds can either be volatile, transmitting information over large distances, or non-volatile, only informing receivers in the close surrounding or upon contact with the emitting/sending individual. If animals disperse after birth or emergence, they can locate prey, hosts, conspecifics or mating partners with long-range volatiles, and then recognize the prey or host species or conspecific individuals with non-volatile compounds upon finding them. Finding mating partners by using chemical communication over larger distances is therefore only possible, if these emit highly volatile compounds as sex pheromones. However, several species only use long-chained CHC compounds as sex pheromones (Blomquist and Bagnères 2010a), which are non- to semi-volatile and therefore only perceivable upon close range of the emitting individual (e.g., Schiestl and Ayasse 2000; Blomquist and Bagnères 2010a; Ruther et al. 2011; Kühbandner et al. 2012; Ruther 2013).

The question arises, how dispersal and mate-finding is possible for these species if they cannot locate mating partners over large distances. If dispersal is not necessary for these species, they could have evolved to avoid producing volatile compounds which could potentially attract predators and just use species-specific combinations of non-volatile sex pheromone compounds only perceivable upon close range. In contrast, if species need to disperse to avoid for example inbreeding or to find new breeding grounds or hosts, they could in order to find mating partners either follow species-specific volatile sex pheromones or, if no volatile sex pheromones exist in the respective species, locate conspecific mating partners at hosts or prey, which they can again locate via long-range volatile compounds (e.g., Xu and Turlings 2018). We therefore wanted to investigate, whether the dispersal behavior could have shaped the sex pheromone composition of species. Do species which do not disperse after birth or emergence not need long-range compounds and therefore only use non-volatile sex pheromones, as these are sufficient for mate finding and recognition upon close distance? Do species which disperse use long-range volatile sex pheromones?

To test this, one would need to find closely-related species with known sex pheromones of different volatility. This can be found in the genus *Leptopilina*, in which the chemical ecology of several

parasitoid wasp species has been analyzed. The sex pheromones in these wasps range from highly volatile iridoid compounds in *L. heterotoma* (Weiss et al. 2013) and *L. japonica* (Böttinger et al. 2019, see **publication 1**) to combinations of iridoids with non-volatile long-chained CHCs in *L. boulardi* (Weiss et al. 2015a), to only non-volatile long-chained CHCs in *L. victoriae* (Weiss et al. 2015a), *L. clavipes* (Pfeiffer et al. 2018), *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1**), and *L. pacifica* (Böttinger et al. 2020, see **publication 2**). Therefore, in **publication 3** I investigated the correlation of the dispersal behavior of male and female wasps of four species of *Leptopilina* with differently volatile sex pheromones. More specifically, I investigated the dispersal of the two species *L. heterotoma* and *L. japonica* with volatile iridoid sex pheromones, and of the two species *L. ryukyuensis* and *L. pacifica* with non-volatile long-chained CHC compounds as sex pheromones, to see whether males and females of the species with volatile sex pheromones, which should be capable of long-distance mate-finding and could disperse to avoid inbreeding, are dispersing earlier after emergence or with a stronger rate than species with non-volatile sex pheromones that can only find mating partners in the near surrounding. As the species with non-volatile sex pheromones should not be able to find mates over longer distances by use of their sex pheromones, they might need other means of inbreeding avoidance or long-distance mate-finding. To find their mating partners after leaving their natal host patch, wasps could either follow the long-range pheromones of their prospective partner or use host-patch-derived volatiles as attractants, because host patches are probably optimal places for mate encounters. They can subsequently use short-range pheromones for mate recognition. I therefore additionally analyzed the attraction of males to conspecific females and vice versa, and additionally analyzed the attraction of the species to host-patch derived volatiles.

Life-history traits such as dispersal and mate-finding have so far been neglected in the investigation of pheromone evolution. Our analyses revealed, that males of those species that use volatile iridoid compounds as sex pheromones (*L. heterotoma* and *L. japonica*) disperse earlier and at a higher rate, i.e. with a higher proportion of dispersed individuals, after emergence than males of species with non-volatile sex pheromones (*L. ryukyuensis* and *L. pacifica*), which delay dispersal until their conspecific females start to emerge, which is generally two days after male emergence (Carton et al. 1986). Hence, male dispersal before the time point of female emergence correlates with the volatility of female sex pheromones. In contrast, the dispersal of female wasps was not dependent on sex pheromone volatility, although the dispersal was continuously more pronounced in females with volatile sex pheromones than in females with non-volatile sex pheromones when species were pooled according to sex pheromone volatility. Instead, as the proportions of dispersed *L. heterotoma* females were always higher than that of females of the other species, female dispersal was species-specific. Therefore, the evolution of the female sex pheromone diversity might have been shaped by the different dispersal patterns of male wasps in the genus *Leptopilina*. We found in olfactometer experiments that male wasps of all species were attracted to conspecific female wasps, irrespective of their sex pheromone volatility. This is surprising for species with non-volatile CHC-based sex pheromones mediating mate recognition and

courtship, which consequently must also use the iridoid compounds of their females for long-range attraction and mate-finding. Whereas male wasps were not attracted to the odor of host patches, female wasps of all species were irrespective of their mating status attracted to host patch-derived volatiles. We can conclude that male wasps disperse to find mating partners. In contrast, female wasps were not attracted to male wasps, but to host-patch-derived volatiles, and hence disperse to find oviposition sites.

In this study we provide to our knowledge the first evidence for the correlation of the dispersal behavior of male wasps and the sex pheromone volatility of the conspecific female wasps. This study significantly contributes to our understanding of the evolution of sex pheromones by differences in dispersal behavior.

3. Conclusion

In this thesis, I contributed to the knowledge about the sex pheromone diversity in parasitoid wasps of the genus *Leptopilina* (Hymenoptera: Figitidae), as I studied the chemical composition of the compounds produced by three species of this genus (*L. japonica* and *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**; *L. pacifica*: Böttinger et al. 2020, see **publication 2**). I furthermore analyzed which compounds are emitted by female wasps of these species when being attacked, and which of the female-produced compounds elicit the mate-finding and courtship behavior of male wasps in the respective species. The results of my experiments show, that all species of *Leptopilina* produce volatile iridoid compounds apart from non-volatile long-chained cuticular hydrocarbons (CHCs; Böttinger et al. 2019, see **publication 1**, Böttinger et al. 2020, see **publication 2**). In contrast, the species *Ganaspis xanthopoda* of the closely related genus *Ganaspis* does not produce iridoid compounds (unpublished/ in preparation), which therefore seem to be typical for the genus *Leptopilina* (see also **Fig. 3, 4**). Our results show that iridoids are used as female sex pheromones in the species *L. japonica* (Böttinger et al. 2019, see **publication 1**), as has been found before only in the species *L. heterotoma* (Weiss et al. 2013), and in the species *L. boulardi* together with CHC compounds (Weiss et al. 2015a). All other studied species, the species *L. clavipes* (Pfeiffer et al. 2018), *L. victoriae* (Weiss et al. 2015a), *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1)**, and *L. pacifica* (Böttinger et al. 2020, see **publication 2**), use CHCs of females as sex pheromones (**Fig. 4**).

Current evolutionary theory predicts that compounds used for communication evolved from precursor compounds previously used for non-communicative purposes in the same organism (Otte 1974; Haynes and Potter 1995; Steiger et al. 2011; Wyatt 2014). It has been found in *L. heterotoma*, that the mating signals evolved from compounds used for defense (Weiss et al. 2013). Therefore, to elucidate whether the mate-finding compounds in *L. japonica* evolved also from non-communicative defensive compounds in the same species, and to find out whether defense is the primary function of iridoids in *Leptopilina*, I analyzed which compounds get emitted for defense in three species (*L. japonica* and *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**; *L. pacifica*: Böttinger et al. 2020, see **publication 2**). The results of these experiments show that iridoids are emitted by all species of *Leptopilina* for defensive purposes, and indicate that defense is the primary function of iridoids in this parasitoid wasp genus (Böttinger et al. 2019, see **publication 1**; Böttinger et al. 2020, see **publication 2**; see **Fig. 4**). Iridoids are also used for defense in several other insect species (Pasteels et al. 1982; Dinda 2019), and as insecticides and secondary metabolites in plants (Inouye and Uesato 1986; Dinda 2019), therefore their usage for defense in *Leptopilina* wasps is not surprising. However, it is very rare that the primary functions of pheromone compounds can be found, as in *Leptopilina* wasps (Weiss et al. 2013; Böttinger et al. 2019, see **publication 1**; see also **Fig. 4**).

As iridoid compounds were found to serve as competition avoidance cue for female wasps of *L. heterotoma*, I investigated in this thesis whether the defensive compounds are also used as cues by

female wasps of other species of this genus to recognize conspecifics and potentially avoid their competition during host search. Therefore, I also analyzed the competition avoidance behavior in female wasps of *L. japonica*, *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1)**, and *L. pacifica* (Böttinger et al. 2020, see **publication 2**). Iridoid compounds evolved only in some species to be perceived as cues by females in order to avoid conspecific female competitors: in females of *L. heterotoma* (Weiss et al. 2013), *L. boulardi* (unpublished), *L. clavipes* (Pfeiffer et al. 2018), and *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1**), but not in female wasps of *L. japonica* (Böttinger et al. 2019, see **publication 1**) and *L. pacifica* (Böttinger et al. 2020, see **publication 2**), which did not avoid live conspecific females, but avoided the extracts of conspecifics.

As the defensive iridoid compounds of female wasps were shown to have evolved to sex pheromones in some species (*L. japonica*: Böttinger et al. 2019, see **publication 1**, *L. heterotoma*: Weiss et al. 2013, *L. boulardi* together with CHC compounds: Weiss et al. 2015a), the perception of male wasps of these species must have before evolved to perceive the iridoid compounds as cues, so they were able to recognize conspecific females. I therefore analyzed whether males of several species of *Leptopilina* are attracted to conspecific females or their extracts in olfactometer experiments over a distance of ca. 30 cm, in which the CHC compounds cannot be attractive anymore, as long-chained CHCs can only be perceived over very short distances from the emitting individual (e.g., Schiestl and Ayasse 2000; Blomquist and Bagnères 2010a; Ruther et al. 2011; Kühbandner et al. 2012; Ruther 2013). I found that males of all studied species were attracted over distances of about 30 cm to conspecific females, which can only be mediated by the volatile iridoid compounds of females (*L. japonica*, *L. ryukyuensis*, *L. heterotoma, L. pacifica*: Böttinger and Stökl 2020, see **publication 3**). This suggests, that iridoids evolved in all studied species from non-communicative defensive compounds to being perceived by males as cues for long-range attraction to female wasps or, alternatively, as long-range sex pheromones (*L. japonica*, *L. ryukyuensis*, *L. heterotoma, L. pacifica*: Böttinger and Stökl 2020, see **publication 3**). Therefore, iridoids either evolved from female defensive allomones to being perceived as cues by males to locate females and further as sex pheromones in the species *L. heterotoma* (Weiss et al. 2013) and *L. japonica* (Böttinger et al. 2019, see **publication 1**), or they evolved in these species from allomones to both, long-range and short-range sex pheromones. However, although the iridoid compounds evolved from defensive compounds to being perceived as cues or long-range sex pheromones by male wasps in *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1**) and *L. pacifica* (Böttinger et al. 2020, see **publication 2**; Böttinger and Stökl 2020, see **publication 3**), they did not evolve as close-range sex pheromones in these species, but instead CHCs are used as contact sex pheromones.

The diversity of compound combinations as sex pheromones between closely related wasps of the genus *Leptopilina* is remarkable: either only CHCs are used as sex pheromones (*L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**; *L. pacifica*: Böttinger et al. 2020, see **publication 2**; *L. victoriae*: Weiss et al. 2015a; *L. clavipes*: Pfeiffer et al. 2018), or combinations of iridoids with CHCs

(*L. boulardi*: Weiss et al. 2015a), or only iridoid compounds serve as mate-finding cues and courtship initiating signals (*L. heterotoma*: Weiss et al. 2013, *L. japonica*: Böttinger et al. 2019, see **publication 1**).

Taken together, our results suggest that the remarkable diversity of sex pheromone compositions in the genus *Leptopilina* cannot be explained by the phylogeny, as closely related species have not more similar or dissimilar pheromones than more related species (see **Fig. 4**). A phylogenetic pattern can be seen, however, regarding the different stereoisomers of the compound iridomyrmecin produced by the studied species of *Leptopilina* (**Fig. 4**). The sister species *L. victoriae* (Weiss et al. 2015a) and *L. japonica* (Böttinger et al. 2019, see **publication 1**) both produce the compound (+)-iridomyrmecin, as well as the species *L. pacifica* (Böttinger et al. 2020, see **publication 2**), whereas females of the species *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1**), which is closely related to these species, produces both stereoisomers, (+)- and (–)-iridomyrmecin (**Fig. 4**). The species *L. heterotoma* (Stökl et al. 2012; Weiss et al. 2013), *L. boulardi* (Weiss et al. 2015a), *L. clavipes* (Pfeiffer et al. 2018), and *L. guineaensis* (unpublished) produce the compound (−)-iridomyrmecin.

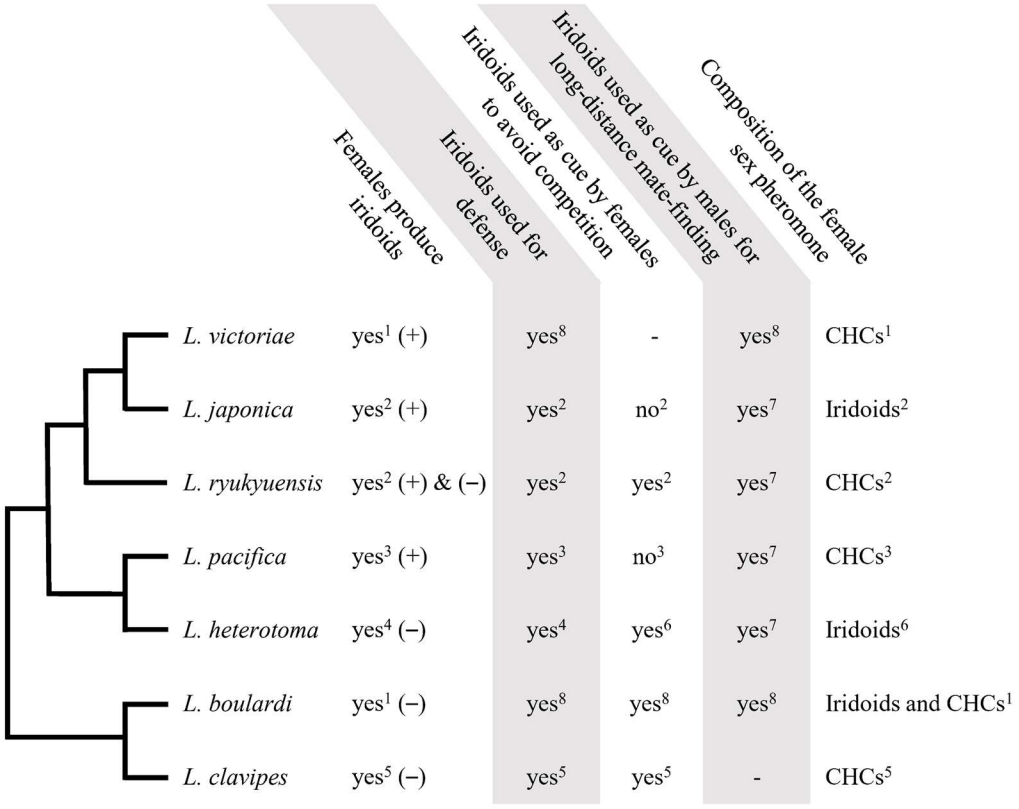

Figure 4 Phylogenetic relationship and current knowledge on the production and use of iridoids by the seven species of *Leptopilina* studied so far. (+) indicates that (+)-iridomyrmecin is produced (among other iridoid compounds), and (–), that (–)-iridomyrmecin is produced. Hyphens indicate that this function has not been studied. Phylogenetic tree based on ITS2, modified from Wachi et al. 2015. [1]Weiss et al. 2015a; [2]Böttinger et al. 2019 (**publication 1**); [3]Böttinger et al. 2020 (**publication 2**); [4]Stökl et al. 2012; [5]Pfeiffer et al. 2018, here, the defensive function of iridoids is assumed, but has not been experimentally tested; [6]Weiss et al. 2013; [7]Böttinger and Stökl 2020 (**publication 3**); [8]unpublished.

The phylogeny (Wachi et al. 2015) suggests that the production of (−)-iridomyrmecin represents the ancestral state in the genus *Leptopilina* and therefore supports the hypothesis of a switch to the production of (+)-iridomyrmecin in *L. japonica* and *L. victoriae* via *L. ryukyuensis* as intermediate step (**Fig. 4**). A shift from (−)- to (+)-iridomyrmecin must have also occurred between the (+)-iridomyrmecin-producing species *L. pacifica* and the (−)-iridomyrmecin-producing species *L. heterotoma* (**Fig. 4**).

We know from behavioral experiments that male wasps of *L. heterotoma* are able to discriminate between the compounds (–)-iridomyrmecin and (+)-iridomyrmecin (see Fig. 3g and 3h in Weiss et al. 2013) and that four different stereoisomers of iridomyrmecin have different repellent effects on ants (Stökl et al. 2012). However, 16 different stereoisomers of iridomyrmecin exist, as a single molecule has four chiral centers (**Fig. 2**), and so far we have found 5 different stereoisomers in the different species of *Leptopilina*: (–)- and (+)-iridomyrmecin, (–)- and (+)-isoiridomyrmecin (**Fig. 2 a-d**), as well as one iridomyrmecin of unknown configuration. In the iridoid profiles of female wasps the iridomyrmecin compounds are accompanied by several other iridoid compounds. This diversity of the produced compounds in the iridoid profiles of the studied *Leptopilina* wasps is remarkable and could indicate the need for species-specific diversification of these compounds as well as of a higher complexity of the compound combinations the more they became involved in the communication of this genus. This can be seen in the here studied species, as the iridoid profile of *L. japonica* is more complex than that of *L. ryukyuensis* (compare Table 1 in Böttinger et al. 2019, **publication 1**). However, the iridoid profile of females of *L. pacifica* is as complex as that of *L. japonica* females (see Table 1 in Böttinger et al. 2020, **publication 2**), and even more complex than that of its sister species *L. heterotoma* (Stökl et al. 2012; Weiss et al. 2015a), indicating a saltational shift in pheromone usage between *L. pacifica* (CHCs) and *L. heterotoma* (iridoids; see Böttinger et al. 2020, **publication 2**). Nevertheless, we expect species which use iridoid compounds not only for defense, but also for communication purposes, to have greater quantities of iridoids. Indeed, this can be seen in *L. victoriae*, which produces lower amounts of iridoids as *L. heterotoma*, and in which iridoids are just emitted for defense (unpublished) and not involved in the sexual communication as in other species (Weiss et al. 2015a). Also the lower quantities and less diverse combinations of iridoid compounds of male wasps compared to female wasps support the hypothesis of the evolution of the defensive compounds for a usage in communication in female wasps of the genus *Leptopilina* (Weiss et al. 2015a; Pfeiffer et al. 2018; Böttinger et al. 2019, see **publication 1**; Böttinger et al. 2020, see **publication 2**).

This thesis contributes to the understanding of the evolutionary trajectories that have led to the diversification of pheromones in nature. I provide here evidence for the correlation of the dispersal behavior of male wasps with the sex pheromone compositions of conspecific female wasps (Böttinger and Stökl 2020, see **publication 3**). We could show, that males of the parasitoid wasp species with volatile female sex pheromones, *Leptopilina heterotoma* (Weiss et al. 2013) and *L. japonica* (Böttinger et al. 2019, see **publication 1**), disperse earlier and at a higher rate than males of the species with non-

volatile sex pheromones, *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1**) and *L. pacifica* (Böttinger et al. 2020, see **publication 2**). This indicates, that the dispersal behavior is a life-history trait which is linked with the sex pheromone composition of a species. If individuals stay at their place of emergence and no dispersal occurs in a species, no long-range pheromones are necessary, as low volatile compounds are sufficient to find mates in close vicinity. In non-dispersing species mating between relatives is likely and either inbreeding causes no negative effects in individuals of these species, or other means of inbreeding avoidance must exist. We assume that the CHC compounds used as non-volatile sex pheromones in the non-dispersing species serve this function of kinship information transmission, as CHCs are known to contain rich information about species-specificity, sex, and health of insects (reviewed by Steiger and Stökl 2014).

In contrast, if species disperse after emergence, individuals of these species have to find mating partners either by the use of volatile long-range sex pheromones or by other volatile cues of potential mate encounter places, such as volatiles emitted by prey species or host patch-derived volatiles. We found in all studied species of *Leptopilina* that male wasps were attracted to conspecific females and not to host patch derived volatiles, which means that only volatile female sex pheromones are important for male mate-finding. Hence, in our study, mate-finding is the reason for male dispersal, indicating the need of long-range female sex pheromones. This need for volatile sex pheromones is higher in species in which dispersal is more pronounced, and indeed we found higher proportions of dispersed males in species with volatile sex pheromones. In contrast, female wasps dispersed to find host patches, as all of them were attracted to host patch-derived odors, and not in search for mates, and their dispersal behavior was species-specific and not dependent on the volatility of their sex pheromones. Therefore, we suggest that the dispersal behavior of male wasps is the driver of pheromone compositions, making the volatility of sex pheromone compounds necessary if dispersal occurs in a species, or making the usage of non-volatile sex pheromones sufficient in species in which no dispersal occurs.

Interestingly, even in species in which long-chained non-volatile CHC compounds are used as female sex pheromones, long-range attraction via female iridoids must exist, as these males were also attracted to conspecific females and to their extracts in our olfactometer analyses over distances in which the females' CHCs could not be perceivable. Additionally, also males of these species disperse and need to find mating partners over larger distances, albeit male dispersal after emergence in species with non-volatile sex pheromones was low, but proportions of dispersed males increased after the emergence of conspecific females (whereas proportions of males of species with volatile sex pheromones were already high directly after male emergence and did not change with female emergence), presumably to increase mating chances or to find less related conspecific females farther away. This was the first time we found that species of the genus *Leptopilina* use different compounds for courtship initiation and for long-range mate-finding. We therefore suggest, that males of *L. ryukyuensis* and *L. pacifica* use their females' iridoid compounds as mate-finding cues or long-range sex pheromones, whereas their females' CHCs elicit the male courtship behavior upon close range. In *L. heterotoma* and *L. japonica* iridoids evolved

from defensive allomones to cues for male attraction to females, and then as sex pheromones to elicit courtship (Stökl et al. 2012; Weiss et al. 2013; Böttinger et al. 2019, see **publication 1;** Böttinger and Stökl 2020, see **publication 3**; and unpublished).

To summarize, we can conclude from this thesis, that iridoids evolved in all studied species of *Leptopilina* for defense, as we found iridoid compounds emitted for defense in the species *L. heterotoma* (Stökl et al. 2012; Stökl et al. 2015), in *L. japonica* and *L. ryukyuensis* (Böttinger et al. 2019, see **publication 1**), in *L. pacifica* (Böttinger et al. 2020, see **publication 2**). Our studies also indicate that iridoid compounds evolved in some species as cues for females for avoidance of conspecific female competitors (*L. heterotoma*: Weiss et al. 2013; *L. boulardi*: unpublished; *L. clavipes*: Pfeiffer et al. 2018; and *L. ryukyuensis*: Böttinger et al. 2019, see **publication 1**), but not in female wasps of *L. japonica* (Böttinger et al. 2019, see **publication 1**) and *L. pacifica* (Böttinger et al. 2020, see **publication 2**; **Fig. 4**). Iridoid compounds only evolved in two species as cues for males during mate-finding and further as female sex pheromones (*L. heterotoma*: Weiss et al. 2013; *L. japonica*: Böttinger et al. 2019, see **publication 1**). In these species, iridoids serve either as cues for long-distance mate-finding and then upon close range as courtship initiating sex pheromones together with the CHC compounds, or the iridoids serve both as short-range and long-range female sex pheromones. In contrast, the species *L. victoriae*, *L. clavipes*, *L. ryukyuensis*, and *L. pacifica* use solely CHCs as female sex pheromone signals (Weiss et al. 2015a; Pfeiffer et al. 2018; Böttinger et al. 2019, see **publication 1**; Böttinger et al. 2020, see **publication 2**; **Fig. 4**). However, the iridoid compounds evolved also in species as long-range mate attractants, in which they are not used for courtship initiation, but instead CHCs are used as sex pheromones: in *L. ryukyuensis*, and *L. pacifica* (Böttinger and Stökl 2020, see **publication 3**). Taken together, iridoids evolved in one species, *L. heterotoma*, from defensive allomones as cues for female competition avoidance, and as cue for male mate-finding from a distance, into a sex pheromone signal (Stökl et al. 2012; Weiss et al. 2013; Böttinger and Stökl 2020, see **publication 3**). In the species *L. japonica*, iridoids evolved from defensive allomones as cues for male mate-finding from a distance, into a sex pheromone signal (Böttinger et al. 2019, see **publication 1;** Böttinger and Stökl 2020, see **publication 3**). However, in this species iridoids did not evolve as a cue for females to avoid conspecific females, presumably because females in this species suffer less from competition than other species (Böttinger et al. 2019, see **publication 1**). In both of these species the route of the iridoid pheromone signal evolution is a perfect example of the precursor hypothesis and classic communication evolution hypotheses, that chemical communication evolves from non-communicative compounds over a function as a cue to a signaling compound. In all other studied species, iridoids evolved for defensive purposes and in two of these species (*L. ryukyuensis*, *L. pacifica*) as cue for males for mate-finding, although the final switch to the mating signal did not evolve. However, we assume that iridoid compounds serve also in the other species which use non-volatile CHCs as sex pheromones as long-range attractants. The species *L. clavipes*, however, could present an exception, as males were repelled from the iridoid compounds of conspecific females (Pfeiffer et al. 2018), therefore a long-range attraction of males to

their females' iridoids is not assumed. This species could present the ancestral state in the genus *Leptopilina*: *L. clavipes* females produce (–)-iridomyrmecin, presumably for defense, as the defensive function has been found in all other species studied so far, and these defensive compounds get avoided by male and female conspecific wasps, if emitted by females, and males use the females CHCs as sex pheromones (Pfeiffer et al. 2018; see also **Fig. 4**). The evolution of iridoids as cues for males can have started in speciation events after *L. clavipes* (see **Fig. 4**). The evolution of the CHC-based sex pheromones of *Leptopilina* wasps started presumably from the primary non-communicative desiccation barrier function, as CHCs can be found on the cuticle of almost every insect (Gibbs 1998) and achieve waterproofing (Gibbs 1998; Blomquist and Bagnères 2010b; Gibbs and Rajpurohit 2010). However, although in many species CHCs function as semiochemical cues or evolved into chemical signals (Howard and Blomquist 2005), their primary role as desiccation barrier is difficult to prove.

We propose a saltational mode of pheromone evolution between the *Leptopilina* wasp species using iridoids as sex pheromones and the species using CHC-based sex pheromones. This means, that between *L. ryukyuensis* (CHCs) and *L. japonica* (iridoids) (see Böttinger et al. 2019, **publication 1**), but also between *L. victoriae* (CHCs) and *L. japonica*, between *L. heterotoma* (iridoids) and *L. pacifica* (CHCs) (see Böttinger et al. 2020, **publication 2**), as well as between *L. boulardi* (iridoids and CHCs) and *L. clavipes* (CHCs) saltational changes in the composition of sex pheromones can be expected (**Fig. 4**). A gradual mode of evolution can be assumed for the pheromones of species using CHC compounds as sex pheromones (between *L. ryukyuensis* and *L. victoriae*, between *L. pacifica* and *L. ryukyuensis*, and between *L. clavipes* and *L. pacifica*, see **Fig. 4**), as the compositions of CHCs vary only slightly between these species. However, also the different combinations of iridoid compounds found in the profiles of female *Leptopilina* wasps using CHCs as sex pheromones could serve as distinct conspecific and mate recognition compounds upon finding another wasp. Saltational evolutionary changes could have occurred between the iridoid compounds of species using CHCs as sex pheromones, to enable a clear chemical profile of each species and to avoid mismatings and hybridizations between individuals of either diverging populations in speciation events, or between individuals of different species. As we could show in Böttinger and Stökl 2020, see **publication 3**, the dispersal behavior of male wasps correlates with the volatility of female sex pheromones in four species of *Leptopilina*. We therefore suggest that this life-history trait is an important factor which has shaped the pheromone evolution in this genus.

Collectively, our results provide evidence for a combination of impacts of dispersal behavior differences and saltational modes of evolution on the mating signal evolution and evolution of iridoid usage of *Leptopilina* wasps, and therefore contribute to the further understanding of pheromone evolution and the causation of divergence in mating signals. Future phylogenetic comparative approaches could clarify the mode of evolution of the different stereoisomers of iridomyrmecin in that genus.

4. References

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5. Publications and Manuscripts

5.1 Publication 1

Semiochemicals Mediating Defense, Intraspecific Competition, and Mate Finding in *Leptopilina ryukyuensis* and *L. japonica* (Hymenoptera: Figitidae), Parasitoids of *Drosophila*

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The study was initiated and designed by JS and LB. Chemical synthesis of iridomyrmecins was carried out by JH. Chemical sample preparation, analysis and identification was done by LB with support from JS. Results were interpreted and discussed by LB, JS and JR. Figures and tables were created by LB. LB wrote the first draft of the manuscript. Revision and rewriting of the manuscript were done by all authors.

Own contribution: Concept and study design 75%, data acquisition 85%, data analysis and figures 90%, interpretation of results 90%, manuscript writing 90%.

SEMIOCHEMICALS MEDIATING DEFENSE, INTRASPECIFIC COMPETITION, AND MATE FINDING IN *Leptopilina ryukyuensis* AND *L. japonica* (HYMENOPTERA: FIGITIDAE), PARASITOIDS OF *Drosophila*

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Abstract
Deciphering the processes driving the evolution of the diverse pheromone-mediated chemical communication system of insects is a fascinating and challenging task. Understanding how pheromones have arisen has been supported by studies with the model organism *Leptopilina heterotoma*, a parasitoid wasp whose defensive compound (–)-iridomyrmecin also evolved as a component of the female sex pheromone and as a cue to avoid competition with other females during host search. To understand how compounds can evolve from being non-communicative to having a communicative function and to shed light on the evolution of the multi-functional use of iridomyrmecin in the genus *Leptopilina*, the chemical communication of two additional species, *L. ryukyuensis* and *L. japonica*, was studied. We demonstrate that in both species a species-specific mixture of iridoids is produced and emitted by wasps upon being attacked, consistent with their putative role as defensive compounds. In *L. ryukyuensis* these iridoids are also used by females to avoid host patches already exploited by other conspecific females. However, females of *L. japonica* do not avoid the odor of conspecific females during host search. We also show that the sex pheromone of female *L. ryukyuensis* consists of cuticular hydrocarbons (CHCs), as males showed strong courtship behavior (wing fanning) towards these compounds, but not towards the iridoid compounds. In contrast, males of *L. japonica* prefer their females' iridoids but CHCs also elicit some courtship behavior. The use of iridoid compounds as defensive allomones seems to be common in the genus *Leptopilina*, while their communicative functions appear to have evolved in a species-specific manner.

Key Words Iridoids, Iridomyrmecin, Cuticular hydrocarbons, Sex pheromone, Chemical defense, Allomone

INTRODUCTION

Several thousand chemical compounds used for communication among organisms of the same species, i.e. pheromones (Karlson and Lüscher 1959), have been identified (El-Sayed 2020). For example, pheromones can 1) help individuals locate and identify conspecifics and mating partners; 2) convey information about sender condition; 3) demarcate trails and territories; and 4) serve as warning signals to conspecifics (Wyatt 2014). Pheromones can consist of single components, but can also be complex combinations of many compounds, resulting in an enormous diversity of potential and species-specific signals. Deciphering the processes driving the evolution of this chemical language devoid of visible, tactile, or audible signals is a fascinating and challenging field of science. Hence, although having been thoroughly studied and being considered the oldest and almost ubiquitous form of communication in nature (Wyatt 2014), our understanding of how this diverse chemical communication system has arisen still lacks experimental evidence. The precursor hypothesis states that pheromone-based

communication can evolve from chemical compounds produced by an organism, which were already in use for non-communicative purposes (Bradbury and Vehrencamp 2011; Steiger et al. 2011; Wyatt 2014; Stökl and Steiger 2017). Hormones excreted with urine, cuticular constituents used as a desiccation barrier and defensive secretions to ward off predators are examples of non-communicative compounds that have been co-opted to serve as intraspecific olfactory communication signals. For example, in females of the goldfish and the Atlantic salmon, hormones excreted with urine evolved into female sex pheromones (Stacey and Sorensen 2009). In many insect species cuticular lipids serve as a desiccation barrier (Lockey 1988; Gibbs and Rajpurohit 2010), but additionally evolved to mediate species recognition, mate finding, and courtship (Greenspan and Ferveur 2000; Mori et al. 2010; Kühbandner et al. 2012). Defensive compounds can also evolve a communicative function and serve as alarm pheromones (Löfqvist 1976), sex pheromones (Boppré 1986; Ruther et al. 2001; Geiselhardt et al. 2008a) or aggregation pheromones (Wheeler and Cardé 2013). This is an example of semiochemical parsimony, which refers to situations in which a chemical has several different functions (Blum 1996). Although it has been reported in quite a number of species, in most studies only the pheromone function has been experimentally tested, while the primary non-communicative function is assumed. Furthermore, the patterns and mechanisms underlying the evolution from primary to secondary function of the compounds are often not addressed.

Over the past several years the parasitoid wasp species *Leptopilina heterotoma*, a solitary parasitoid of *Drosophila* larvae (Carton et al. 1986; Fleury et al. 2009), has become a model organism to study the evolution of chemical communication according to the precursor hypothesis (Stökl et al. 2012; Weiss et al. 2013; Weiss et al. 2015a). Females of *L. heterotoma* emit a defensive secretion containing mainly (–)-iridomyrmecin and four additional minor iridoids upon predator attacks (Stökl et al. 2012). Females are able to adjust the released amount of this mandibular secretion (Stökl and Herzner 2016) depending on the size of the predators (Stökl et al. 2015). As females also release very small amounts of their defensive allomone when not under attack, they can recognize and avoid host patches already exploited by conspecific competitors by detecting the (–)-iridomyrmecin released by the female on the host patch (Weiss et al. 2013). Additionally, males of *L. heterotoma* are attracted to conspecific females by the same compounds, which thereby serve as female sex pheromones and elicit male courtship behavior (Weiss et al. 2013). However, while (–)-iridomyrmecin alone is sufficient for defense and the avoidance of exploited host patches, only the mixture of (–)-iridomyrmecin and minor iridoid components can trigger courtship behavior in males. This threefold semiochemical parsimony of (–)-iridomyrmecin in *L. heterotoma* females suggests an evolutionary route from a defensive compound into a competition avoidance cue and a species specific sex pheromone (Weiss et al. 2013). The closely related species *L. boulardi, L. victoriae,* and *L. clavipes* also produce a defensive secretion containing iridomyrmecin (Weiss et al. 2015a; Pfeiffer et al. 2018). But only in *L. heterotoma* and *L. boulardi* has iridomyrmecin been incorporated into the female sex pheromone, while the sex pheromones of *L. victoriae* and *L. clavipes* consist solely of cuticular hydrocarbons (CHCs) (Weiss et al. 2015a; Pfeiffer et al. 2018). Moreover, in contrast to males of *L. heterotoma* and *L. boulardi,* which are attracted to their females' iridoids, males of *L. clavipes* avoided the iridoids from the females' defensive secretion (Pfeiffer et al. 2018).

The comparison of the chemical communication of more *Leptopilina* species will help elucidate how this diversity in *Leptopilina* has arisen. Here we studied the chemical communication of two additional species, *L. ryukyuensis* and *L. japonica*, to shed light on the evolution of the multi-functional use of iridomyrmecin in the genus *Leptopilina*. We analyzed the composition of wasp-derived volatiles, studied the use of iridoids as defensive compounds and competition avoidance cues, and disentangled the role of iridoids and CHCs as the female sex pheromone.

METHODS AND MATERIALS

Insects. We reared *Leptopilina japonica* and *L. ryukyuensis* wasps using *Drosophila simulans* as host species. For each rearing, about 30 *D. simulans* flies of both sexes were placed into a jar containing fresh corn-based diet (ingredients: 1 l water, 50 g cornmeal, 50 g wheat germ, 50 g sugar, 40 g baker's yeast, 8 g agar, 5 ml propanoic acid) and kept in a climate- and light-controlled environment at 25 °C, ~60 % humidity, and a 16:8 h L:D cycle. After 48 h, the flies were removed and ~10 mated females of either *L. japonica* or *L. ryukyuensis* were put into the jar to parasitize the fly larvae. A few days before emergence the parasitized pupae were removed from the jar and put singly into 1.5 ml microcentrifuge tubes to obtain unmated and naïve wasps of known age. Once emerged, wasps were fed ad libitum with diluted honey and kept individually until used in an experiment.

Chemical Analysis. Male and female wasps of either species were extracted in batches of 30 to 50 individuals for 10 min in 10 µl dichloromethane (DCM) per wasp in

order to obtain extracts for behavioral experiments and chemical analyses. To disentangle the contribution of iridoids and CHCs to the female sex pheromone, iridoids and CHCs were isolated from the extracts by solid-phase extraction. For this purpose, raw extracts were dried under a gentle stream of nitrogen, and the residues were re-dissolved in 50 µl hexane. Cyanopropyl-modified silica gel columns (100 mg, CN, Chromabond, Macherey-Nagel, Germany) were pre-conditioned by rinsing them with 2 ml of DCM and hexane; subsequently, the samples were applied to the columns and eluted successively with 300 µl each of hexane and DCM. Raw extracts fractions were analyzed by GC/MS and stored at -20 °C until being used for behavioral tests. The hexane fractions contained the CHCs while the iridoids were present in the DCM fractions. Prior to the behavioral bioassays, the concentration of the fractions was determined by GC/MS and re-adjusted to the concentration of respective compounds found in the raw extracts.

To determine the quantity of chemical components produced by the wasps, single male and female wasps of *L. japonica* or *L. ryukyuensis* were extracted for 10 min in 20 µl DCM containing 20 ng μl^{-1} methyl undecanoate as an internal standard and extracts were analyzed by GC/MS. The total amounts of individual components were calculated using a standard linear calibration curve obtained by analyzing known amounts of (+)-iridomyrmecin (1 ng μl^{-1}, 5 ng μl^{-1}, 10 ng μl^{-1}, 25 ng μl^{-1}, 50 ng μl^{-1}). We analyzed 13-15 individuals of each sex and each species.

Extracts, fractions and headspace samples (see below) were analyzed by splitless injection on an Agilent 7890B gas chromatograph (GC; Agilent Technologies, Germany) with a non-polar capillary column (DB-5, 30 m length, 0.25 mm inner diameter, 0.25 µm film thickness; Agilent Technologies, Germany) combined with an Agilent 5977A mass selective detector (Agilent Technologies, Germany). The injector temperature was set to 280 °C and helium was used as carrier gas at 50 cm s^{-1} constant linear velocity. The GC oven temperature started at 80 °C, was raised with 5 °C min^{-1} to 280 °C and held at that temperature for 20 min. The mass spectrometer scanned masses between 35 and 550 m z^{-1}. Additional analyses with reference compounds to identify the iridoid compounds were performed on a polar capillary column (Rtx-Wax, 30 m length, 0.25 mm inner diameter, 0.25 µm film thickness; Restek Corporation). Here, the temperature of the GC started at 80 °C, held for 4 min and then raised at 3 °C min^{-1} to 230 °C. Helium was used as carrier gas with 50 cm s^{-1} linear velocity and samples were injected in splitless mode.

To separate iridoid compounds enantioselectively, we used a Shimadzu GC2010 gas chromatograph (GC), equipped with a chiral BetaDEX 225 column (=25 % 2,3-di-*O*-acetyl-6-*O*-tert-butyldimethylsi-lyl-ß-cyclo-dextrin in polydimethylsiloxane, 30 m, 0.25 mm i.d., 0.25 µm film thickness; Sigma-Aldrich, Germany). The GC was coupled to a QP2010 plus mass spectrometer (MS; Shimadzu, Duisburg, Germany). A 1 µl aliquot of each of the DCM fractions of extracts of male and female *L. ryukyuensis* and *L. japonica* wasps was injected in splitless mode. Helium was used as carrier gas at 50 cm s^{-1} linear velocity and the injector temperature was set to 250 °C. The oven program started at 80 °C, the temperature was held for 4 min and then raised at a rate of 3 °C min^{-1} to 230 °C. Compounds were identified by comparison of their mass spectra and linear retention indices with those of authentic reference compounds (synthesized as described in Stökl et al. 2012) on the different columns (non-polar, polar, chiral). Saturated *n*-alkanes were identified by comparison of mass spectra and retention indices/times with those of a reference mix of alkanes (Sigma-Aldrich) and with those of previously analyzed compounds from other *Leptopilina* species (Stökl et al. 2012; Weiss et al. 2013; Weiss et al. 2015a; Pfeiffer et al. 2018). Double-bond positions of unsaturated CHC compounds were identified by the diagnostic ions of compounds after derivatization with dimethyl disulfide (Carlson et al. 1989). Derivatized samples were analyzed on the non-polar capillary column with the above-mentioned method, but the final oven temperature was set to 310 °C (instead of 280 °C) and the mass range was 35 to 800 mz^{-1}. Methyl-branched CHCs were determined by comparison of linear retention indices with literature data (Carlson et al. 1998) and by interpretation of diagnostic ions.

Iridoids for Defense. We used dynamic headspace collection to measure the amount of iridoids released by females of *L. ryukyuensis* or *L. japonica*. To this end, two conspecific female wasps were carefully placed together with a small magnetic stirring bar (8 x 3 mm, BRAND, Sigma-Aldrich, Germany) in a 1 ml glass vial. A headspace collection needle trap device (packed with 3 cm Tenax TA, NeedlEx, Shinwa Chemical Industries LTD, Japan) and an activated charcoal filter (6 mm x 75 mm, 100/50 mg, ORBO, Sigma-Aldrich) for clean air input were inserted through the septum in the lid of the vial. Instead of using living predators like ants or lacewing larvae, we used a magnetic stirring bar to standardize the amount of teasing between experiments. To imitate the attack of a predator, the wasps were briefly teased every 30 s by moving the magnetic stirring bar in the vial with a magnet on the outside of

the vial. The emitted volatiles were drawn out of the vial through the needle by a sampling system (PAS Technology, Germany) with an air flow of 6 ml min^{-1} for 5 min. The trapped volatiles were thermally desorbed by injecting the needle into the hot injector of the Agilent system followed by analysis by GC/MS on the non-polar capillary column (see above). The magnetic stirrer was washed twice with DCM after each experiment and experiments were conducted 15 times per species.

Competition Avoidance of Females. To test whether female wasps of *L. ryukyuensis* or *L. japonica* use iridoids to avoid competition of conspecific females during host search, we used Y-tube olfactometer experiments. The Y-tube was made of glass (length of base 6 cm, length of arms 9 cm, inner diameter of 1.5 cm) and positioned at a 30° slope with the arms (divided by a 45° angle) pointing upwards. The olfactometer was illuminated from above by two LED tubes (350 lm, 5 W). Both ends of the arm tubes were connected to separate Erlenmeyer flasks (50 ml) via plastic tubes. Humidified air was pumped through the flasks into the Y-tube arms with an air flow rate of 30 ml min^{-1}. Flasks contained artificial host patches consisting of 5 g of *Drosophila* rearing substrate, on which 10 *D. simulans* females were allowed to oviposit for 48 h. As cues we used either living females put directly on the artificial host patch in the jar or extracts of females (equivalent to one 5^{th} of a female) that were applied on filter paper disks (5 mm diameter) and positioned into the end of one arm. As a control, 2 µl of DCM was applied to a filter paper disk and positioned in the other arm of the Y-tube. Impregnated filter paper disks were left to evaporate for 30 s before being used for the experiment. For each run of the experiments, one female was carefully put into the entrance of the base of the Y-tube olfactometer by using an aspirator. Subsequently, the female was allowed, for a maximum of 10 min, to choose between the control odor (host patch) and the treatment odor (host patch with 20 conspecific females or host patch with purified iridoids from conspecific females). The experiment was stopped after the females had either crossed a decision line, which was marked at each Y-tube arm 2 cm beyond the branching point, or after 10 min (no choice) and decisions were recorded. After each run, the Y-tube was turned and the control and treatment odor were swapped, after every second run the Y-tube was cleaned with ethanol and hot water.

To prepare female wasps for the Y-tube olfactometer experiments, freshly hatched to 1-day-old females of either species were left to mate with a conspecific male for 12 h in 1.5 ml microcentrifuge tubes. Subsequently, to increase the responsiveness of female wasps towards the host odor, mated females were put together for 1 h in groups of 10-20 on a host patch with *D. simulans* larvae for parasitization. Subsequently, they were isolated again in microcentrifuge tubes and provided with diluted honey ad libitum and left there for at least one hour before they were used for the experiments. Each female was only used once and the experiment repeated until 30 females (for each species and treatment) had made a choice.

In both species we tested two sets of cues: 1) odor of host patch with 20 conspecific females vs. odor host patch without any females; 2) odor of host patch and the DCM fraction of the extract of females (the DCM fraction contains only iridoid compounds) vs. odor of host patch and DCM.

Female Sex Pheromone. To identify the compounds triggering the courtship behavior in males we measured the duration of wing fanning shown by males during courtship in an arena bioassay. For this purpose, 2 µl of the female extract (equivalent to one 5^{th} of a female), fractions thereof, or the pure solvent (control) were applied to small disks of filter paper (5 mm diameter), which were let dry for approximately 30 s. For *L. japonica*, naïve males (2 to 5 days old) were put singly in glass petri dishes (55 mm diameter, 8.5 mm height) and left there for a few minutes to acclimatize, before the filter paper disk with the sample was introduced to the arena. The duration of the courtship behavior, i.e. high frequency movement of wings (wing fanning), and location of males were recorded for 3 min with a video camera (Canon EOS M with macro lens) and the videos were analyzed with the behavior coding software 'The Observer' (Noldus Information Technology, Netherlands). *L. ryukyuensis* males were observed similarly, but in a smaller arena with a size of 15 mm diameter and a height of 2 mm. Each treatment was repeated 17 to 20 times, and the arena was rinsed between replicates with ethanol and left to dry for 2 minutes.

Statistics. We compared the total duration of the male wasps' wing fanning behavior between treatments using a non-parametric Kruskal-Wallis ANOVA followed by pairwise Mann-Whitney *U*-tests with Bonferroni-Holm correction for multiple comparisons (Benjamini and Hochberg 1995). Differences of the emitted iridoid amounts of teased female wasps and unteased control wasps were compared by Mann-Whitney *U*-tests. The decisions of female wasps in the Y-tube olfactometer were analyzed using a two-sided binomial test. All statistics were done in R Version 3.3.0 (R Core Team 2017).

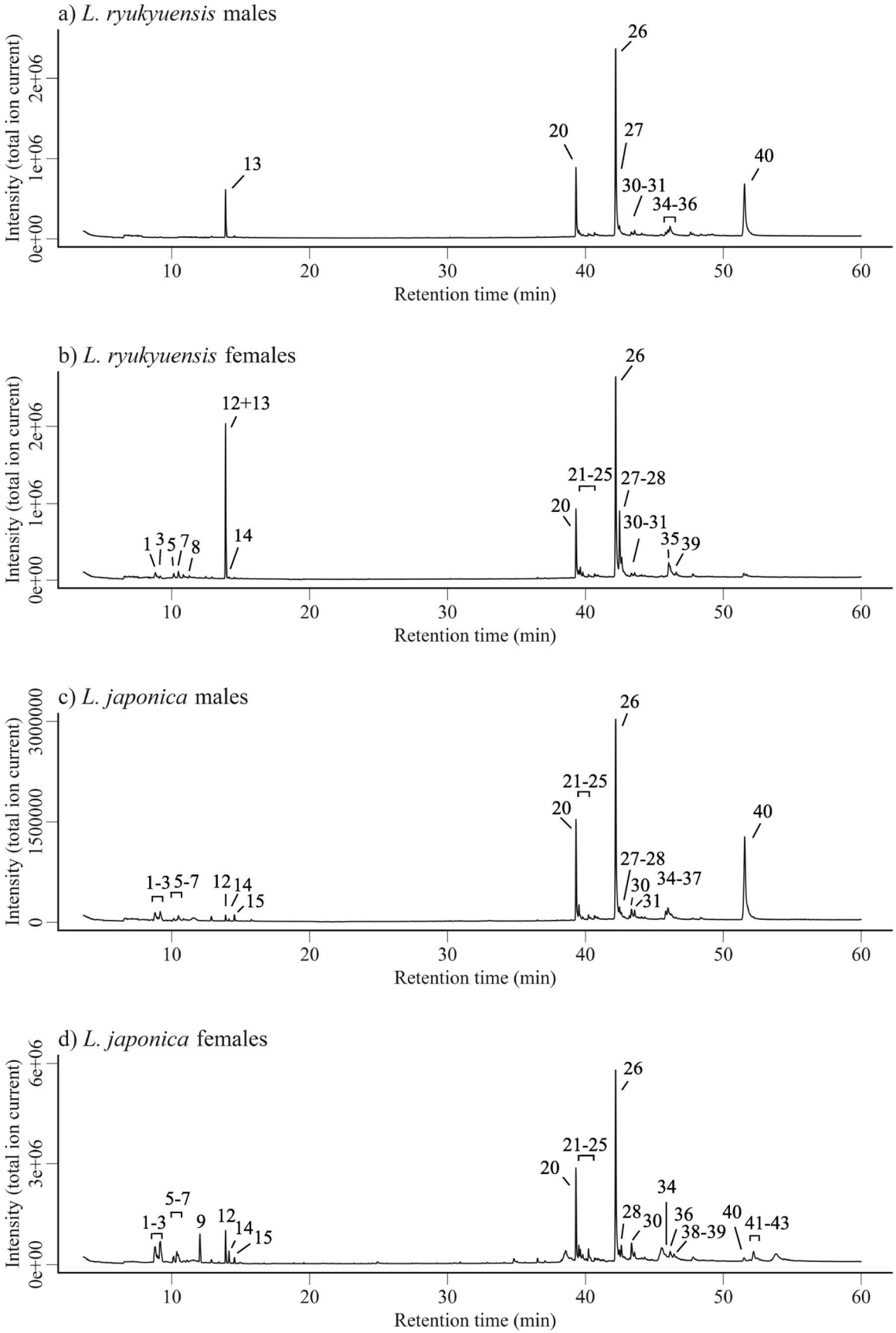

Figure 1. Chemical compounds produced by *L. ryukyuensis* and *L. japonica* wasps. Total ion current chromatograms of extracts of a) *L. ryukyuensis* males, b) *L. ryukyuensis* females, c) *L. japonica* males and d) *L. japonica* females, analyzed on a non-polar DB-5 GC column. Only peaks >1 % contribution are indicated with numbers. Peaks 1-15 are iridoids, peak 20-43 are CHCs. Peak numbers correspond to Table 1

Table 1 Compounds identified in whole body extracts from male and female wasps of *Leptopilina ryukyuensis* and *Leptopilina japonica*

No.	Compound	RT	RI`	Diagnostic ions	Diagnostic ions DMDS	Mean amount (ng ± SD) per *L. ryukyuensis* female	Mean amount (ng ± SD) per *L. ryukyuensis* male	Mean amount (ng ± SD) per *L. japonica* female	Mean amount (ng ± SD) per *L. japonica* male
1	unidentified iridoid	8.822	1236	67, 81, 95, 109, 138, 159, 174		3.68 ± 1.86	0.34 ± 0.20	18.16 ± 7.42	6.90 ± 2.92
2	unidentified iridoid	9.016	1244	67, 81, 95, 109, 116, 138, 159		–	–	4.37 ± 2.03	1.18 ± 0.59
3	unidentified iridoid	9.175	1251	67, 81, 93, 111, 121, 138, 159		2.64 ± 1.35	0.98 ± 0.52	19.38 ± 8.03	7.19 ± 2.96
4	unknown compound	9.61	1270	60, 73, 84, 115, 129, 158		1.64 ± 0.91	2.69 ± 1.60	1.73 ± 1.27	5.33 ± 1.74
5	Iridodial	10.151	1293	111, 135, 168 (M+)		2.50 ± 1.06	0.20 ± 0.08	5.21 ± 2.08	1.62 ± 0.57
6	Iridodial 1	10.381	1303	111, 135, 168 (M+)		–	–	3.11 ± 1.85	0.49 ± 0.34
7	Iridodial 2	10.498	1308	109, 135, 168 (M+)		3.48 ± 1.49	0.22 ± 0.12	3.90 ± 1.90	2.69 ± 0.99
8	Actinidine	11.304	1341	117, 132, 147 (M+)		2.41 ± 1.13	0.24 ± 0.13	–	–
9	Nepetalactone	12.051	1372	69, 81, 123, 166 (M+)		–	–	13.42 ± 4.36	0.22 ± 0.07
10	unidentified iridoid	12.475	1390	67, 84, 95, 109, 123, 138, 151, 166 (M+)		1.28 ± 0.56	0.52 ± 0.22	0.29 ± 0.14	0.09 ± 0.03
11	unidentified iridoid	12.887	1407	56, 111, 166 (M+)		–	–	2.08 ± 0.92	1.90 ± 0.86
12	(+)-Iridomyrmecin	13.922	1451	95, 109, 168 (M+)		47.58[b] ± 16.59[b]	–	13.69 ± 4.38	2.02 ± 0.80
13	(−)-Iridomyrmecin	13.922	1451	95, 109, 168 (M+)			21.00 ± 7.83	–	–
14	(+)- & (−)-Isoiridomyrmecin	14.163	1461	95, 109, 168 (M+)		0.92 ± 0.42	0.20 ± 0.15	5.02 ± 1.89	0.81 ± 0.31
15	Iridomyrmecin X (= *L. heterotoma*)	14.557	1478	109, 168 (M+)		–	0.70 ± 0.26	2.75 ± 0.83	2.26 ± 0.76
16	unknown compound	15.692	1526	74, 87; 121, 138, 166 in *L. japonica* females		0.46 ± 0.08	0.47 ± 0.07	2.20 ± 1.43	3.89 ± 9.42
17	n-Hexadecanoic acid	24.856	1959	43, 60, 73, 256 (M+)		0.31 ± 0.43	0.26 ± 0.19	1.62 ± 1.96	2.53 ± 3.70
18	4-methyl hexacosane	36.509	2662	71, 337, 365 (M-15)		–	0.16 ± 0.09	1.30 ± 1.17	0.29 ± 0.24
19	unknown compound	38.262	2786	59, 72		1.08 ± 1.12	1.88 ± 1.47	2.03 ± 1.75	2.65 ± 3.76
20	4-methyl octacosane	39.309	2863	365, 393 (M-15), 408 (M+)		36.74 ± 20.50	31.74 ± 8.73	22.79 ± 10.80	52.82 ± 24.98
21	9-nonacosene & nonacosadiene[a]	39.503	2877	97, 406 (M+); 97, 404 (M+)	173, 327, 500 (M+)	4.42 ± 6.54	4.51 ± 1.97	18.76 ± 11.20	11.30 ± 6.69
22	7-nonacosene	39.62	2886	97, 406 (M+)	145, 355, 500 (M+)	7.16 ± 13.10	2.76 ± 1.30	17.23 ± 10.69	3.17 ± 1.86
23	Nonacosane	39.803	2899	408 (M+)		3.02 ± 2.17	1.03 ± 0.43	7.13 ± 3.90	1.05 ± 0.70
24	13-methyl nonacosane & 15-methyl nonacosane	40.208	2928	196/197, 252/253; 224/225		1.78 ± 2.17	0.87 ± 0.44	3.20 ± 2.21	2.60 ± 2.72
25	4-methyl nonacosane	40.638	2960	71, 379, 407 (M-15)		1.47 ± 1.04	1.09 ± 0.42	1.30 ± 0.85	1.75 ± 1.41
26	4-methyl triacontane	42.173	3060	393, 421 (M-15), 436 (M+)		118.31 ± 59.23	97.23 ± 17.95	120.00 ± 52.50	140.36 ± 56.75
27	9-hentriacontene	42.461	3078	97, 434 (M+)	173, 355, 528 (M+)	51.89 ± 72.68	10.14 ± 2.96	17.24 ± 9.68	11.58 ± 5.58
28	7-hentriacontene	42.597	3087	97, 434 (M+)	145, 383, 528 (M+)	20.98 ± 31.80	–	46.29 ± 29.25	5.65 ± 3.11
29	Hentriacontene[a]	42.732	3095	97, 434 (M+)		–	–	7.75 ± 3.93	0.84 ± 0.62
30	13-methyl hentriacontane & 15-methyl hentriacontane	43.326	3127	196/197, 280/281; 224/225, 252/253		2.70 ± 1.78	1.15 ± 0.74	3.60 ± 2.94	4.85 ± 3.92
31	unknown compound	43.532	3137	275, 301, 353, 368, 386		2.50 ± 2.11	3.35 ± 1.56	3.00 ± 2.30	5.61 ± 4.39

Table 1 (continued)

No.	Compound	RT	RI`	Diagnostic ions	Diagnostic ions DMDS	Mean amount (ng ± SD) per *L. ryukyuensis* female	Mean amount (ng ± SD) per *L. ryukyuensis* male	Mean amount (ng ± SD) per *L. japonica* female	Mean amount (ng ± SD) per *L. japonica* male
32	unknown CHC	44.085	3166	57, 71, 85		–	–	0.27 ± 0.16	0.61 ± 0.47
33	unknown CHC	44.291	3177	57, 71, 85		–	–	0.63 ± 0.49	0.75 ± 0.74
34	Tritriacontadiene[a]	45.826	3245	96, 460 (M+)	507 (M-141), 554 (M-94)	–	2.29 ± 0.82	0.97 ± 0.65	9.18 ± 8.56
35	Tritriacontadiene[a]	46.091	3258	96, 460 (M+)	507 (M-141), 554 (M-94)	25.15 ± 46.56	4.50 ± 1.68	0.30 ± 0.18	14.96 ± 14.48
36	4-methyl dotriacontane	46.132	3260	421, 449 (M-15)		–	4.41 ± 1.75	1.79 ± 1.14	5.38 ± 5.18
37	Tritriacontene[a]	46.22	3264	97, 462 (M+)	507 (M-141), 554 (M-94)	–	–	–	4.74 ± 4.75
38	Tritriacontadiene[a]	46.402	3271	96, 460 (M+)	507 (M-141), 554 (M-94)	–	–	8.80 ± 8.18	–
39	Tritriacontadiene[a]	46.561	3278	96, 460 (M+)	507 (M-141), 554 (M-94)	4.31 ± 8.77	–	4.76 ± 4.65	0.76 ± 0.68
40	9,19-Pentatriacontadiene	51.537	3447	96, 488 (M+)	173, 535 (M-141), 582 (M-94)	–	83.31 ± 23.47	2.61 ± 1.68	151.90 ± 108.61
41	Pentatriacontadiene[a]	52.173	3465	96, 488 (M+)		–	–	30.70 ± 28.46	–
42	Pentatriacontadiene[a]	52.449	3473	96, 489 (M+)		–	–	9.46 ± 8.81	–
43	Pentatriacontadiene[a]	52.684	3480	96, 490 (M+)		–	–	3.99 ± 4.13	–
	Total amount					349.02 ± 252.77	278.24 ± 56.92	432.85 ± 190.05	471.90 ± 260.19

RI = Retention index on a non-polar DB-5 GC column. Diagnostic ions DMDS: diagnostic ions of unsaturated compounds after derivatization with DMDS. Numbers of compounds correspond to Fig. 1. *N* = 13–15

[a] The position of the double bond(s) and/or the methyl group(s) could not be determined

[b] Amounts for (+)- and (−)-iridomyrmecin enantiomers in *L. ryukyuensis* females taken together

RESULTS

Chemical Analysis We identified two classes of chemical compounds, iridoids and CHCs, in extracts of male and female wasps of *L. ryukyuensis* and *L. japonica* (Fig. 1; Table 1). In both species, the qualitative chemical composition of whole body extracts within species is relatively similar, but there are prominent qualitative and quantitative differences between species (Table 1). Extracts of both species contained (+)-iridomyrmecin, (+)- and (–)-isoiridomyrmecin, two stereoisomers of iridodial (see Weiss et al. 2013; Weiss et al. 2015a), one iridomyrmecin of unknown absolute configuration (as in *L. heterotoma*), and three putative, yet unidentified iridoids. Additionally, *L. ryukyuensis* produce actinidine (unknown configuration) and (–)-iridomyrmecin, while extracts of *L. japonica* also contained nepetalactone, one more iridodial, and two more putative, unidentified iridoids (the exact configuration of all four compounds is unknown). In *L. ryukyuensis* females, (+)- and (–)-iridomyrmecin were predominant within the iridoids and contribute with on average 72% to their total iridoid profile, while males produce only (–)-iridomyrmecin, which makes up 80% in their total produced iridoids. In contrast, in *L. japonica* wasps nepetalactone, (+)-iridomyrmecin, and two unidentified putative iridoids were the major components in the iridoid profile. In both species, the CHC profiles consisted mainly of methyl-branched alkanes and mono- or di-unsaturated alkenes. Although extracts of *L. japonica* wasps contained more CHCs than those of *L. ryukyuensis*, some compounds such as 4-methyltriacontane and 4-methyloctacosane were highly abundant in both species, as well as several other minor compounds. However, apart from the relatively similar intraspecific qualitative composition, the quantitative composition of whole body extracts differs remarkably between males and females of both species. In both, *L. ryukyuensis* and *L. japonica,* female wasps produced on average about three times more iridoids than males (Table 1). Extracts of females contained on average approximately 20% iridoids (18.95% in *L. ryukyuensis* females and 21.51% in *L. japonica* females), while iridoids in male extracts make up only 7% (*L. japonica* males) to 9.5% (*L. ryukyuensis* males), respectively. Regarding the CHCs, females of both species produce more 7-nonacosene, 9- and 7-hentriacontene than the respective males, while male-derived extracts contained much higher amounts of 9,19-pentatriacontadiene than those from females.

Iridoids for Defense. Upon teasing by a small magnetic stirring bar, females of both *Leptopilina* species emitted significantly more iridoids than undisturbed females (Fig. 2). On average each teased individual female of *L. ryukyuensis* emitted 0.76±0.90 ng (mean±SD; control females: 0.03±0.01 ng), while females of *L. japonica* emitted 1.60±1.45 ng (control females: 0.05±0.02 ng) during this artificial predatory attack. Emitted iridoids were the same as in the whole body extracts of female wasps (Fig. 2, Table 1). The average total amount of iridoids found in the whole body extracts of *L. ryukyuensis* females were 66.13±23.60 ng, while those of *L. japonica* contained on average 93.12±33.78 ng (see Table 1). Hence, individual *L. ryukyuensis* females emitted on average only 1.15% of their total iridoids during artificial predatory attack by the small magnetic stirring bar, while *L. japonica* females emitted 1.72% of the total iridoid amounts found in their whole body extracts.

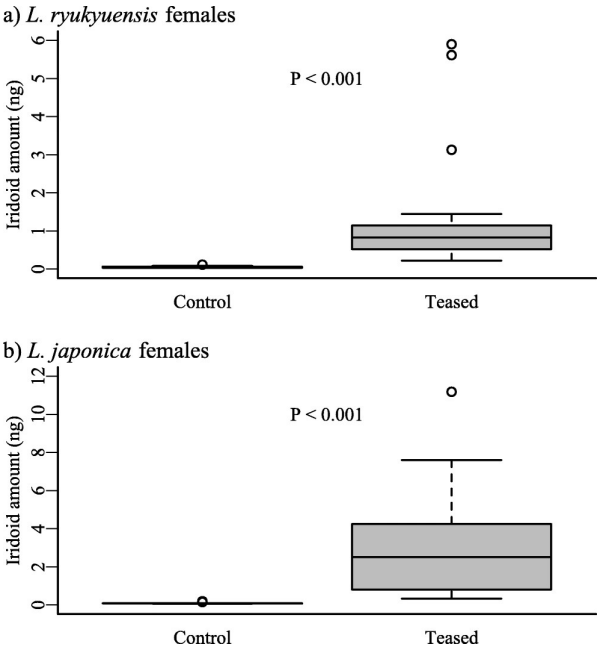

Figure 2. Iridoids for defense. Released iridoid amounts (ng) of two females of a) *L. ryukyuensis* or b) *L. japonica* in a small vial when left alone (Control) or when slightly teased 10 times in 5 min with a small magnetic stirring bar (Teased). P-values are given for Mann-Whitney *U*-tests ($N = 15$).

Competition Avoidance of Females. Females of *L. ryukyuensis* searching for hosts significantly preferred the odor of empty host patches over this of host patches occupied by other conspecific females (Fig. 3). Moreover, they also avoided the odor of host patches to which the odor of the iridoid containing DCM fraction of extracts of conspecific females was added and preferred the odor of host patches without the fractions (Fig. 3). In these experiments, 18 and 14 females, respectively, did not reach the decision line in either arm within 10 min. *L. japonica* females, however, did not show any preference for odors of empty host

patches or those of host patches occupied by conspecific females or the odor of host patches with added DCM fractions of extracts of female *L. japonica* wasps (Fig. 3). In the experiments with *L. japonica*, 9 and 10 females, respectively, did not reach a decision line within 10 min.

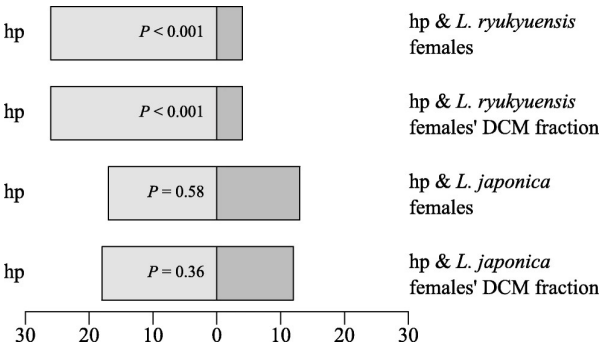

Figure 3. Competition avoidance of females. Frequency of decision for control (light grey bars) or sample (dark grey bars) of females of *L. ryukyuensis* or *L. japonica* in a Y-tube experiment when choosing between the odor of unexploited host patches (hp) or host patches with either 20 conspecific females or the (iridoid containing) DCM fraction of conspecific females. *P*-values are given for two sided binomial tests. Each experiment $N = 30$.

Female Sex Pheromone. Males of both species responded with high frequency wing fanning when exposed to disks of filter paper impregnated with whole body extracts from conspecific females (Fig. 4). *L. ryukyuensis* males showed a wing fanning duration of on average 12.72±12.28 s (mean±SD), while *L. japonica* males showed 7.7±9.96 s of wing fanning. The wing fanning response of *L. ryukyuensis* males to the CHC fraction of conspecific females (18.9±16.58 s) was not significantly different from the whole body extract. The iridoid fraction (DCM fraction) elicited a significant wing fanning response in *L. ryukyuensis* males when compared to the solvent control but wing fanning duration was significantly less when compared to whole body extract and CHC fraction, respectively. When *L. japonica* males were exposed to filter paper impregnated with the iridoid fraction of female-derived extracts, there was no significant difference between the duration of wing fanning when compared to the whole body extracts. When exposed to the CHC fraction, *L. japonica* males showed significantly less wing fanning than towards to whole female extracts and the response was not significantly different from the iridoid fraction and solvent control, respectively.

DISCUSSION

In this study, we demonstrate that wasps of *L. ryukyuensis* and *L. japonica* produce multi-component blends of several iridoid compounds, which

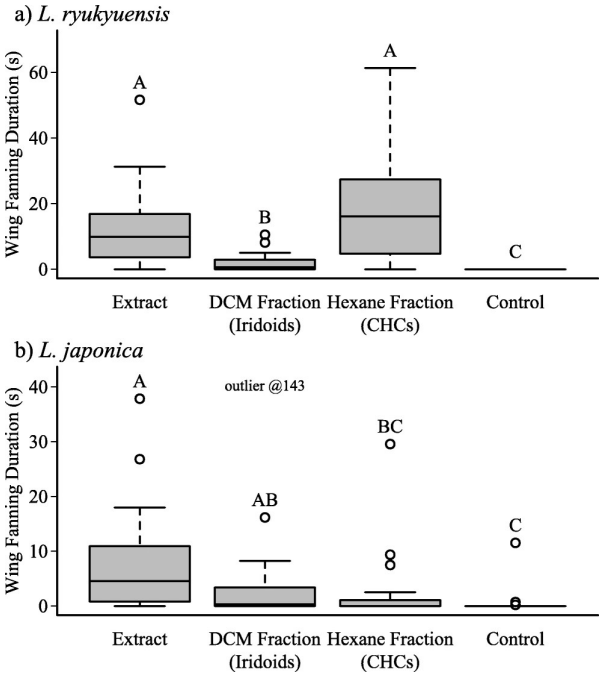

Figure 4. Female sex pheromone. Box-and-whisker plots showing median (horizontal line), inter-quartile range (box), outliers (unfilled circles), and maximum/minimum of the 1.5 * inter-quartile range (whiskers) of the duration of courtship behavior (wing fanning) displayed by males of a) *L. ryukyuensis* or b) *L. japonica* towards whole body extracts of conspecific females, as well as towards the iridoid containing DCM fractions and the CHC containing hexane fractions of the female extracts and the solvent control. Different letters indicate a significant difference (Kruskal-Wallis ANOVA followed by pairwise Mann-Whitney *U*-tests with Bonferroni-Holm correction, $P < 0.05$). Each experiment $N = 17$-20.

are used by both species as a defensive secretion. Females of *L. ryukyuensis* also use the iridoids to avoid competition during host search, but not as a female sex pheromone. In contrast, females of *L. japonica* use the iridoids as a female sex pheromone, but not to avoid other females during host search.

All previously studied species of the genus *Leptopilina* produce iridoid compounds, with either (–)- or (+)-iridomyrmecin being the major compound. *Leptopilina ryukyuensis* is the first species in which we identify both enantiomers of iridomyrmecin as the most abundant iridoids in extracts from females, while conspecific males produce only (–)-iridomyrmecin. In *L. japonica,* females and males produce four iridoids in nearly the same ratios: (+)-iridomyrmecin, nepetalactone, and two yet unidentified iridoids. Males, however, produce lower amounts of the iridoid compounds overall (Fig. 1, Table 1). Additionally, we found low amounts of several other iridoids, such as isoiridomyrmecin and iridodial (unknown configurations) in the extracts of *L. ryukyuensis* and

L. japonica. Those compounds have already been found in other species of *Leptopilina* (Stökl et al. 2012; Weiss et al. 2013; Weiss et al. 2015a; Pfeiffer et al. 2018). While most species of *Leptopilina* (5 out of 8) produce (–)-iridomyrmecin, only three species are known to produce (+)-iridomyrmecin: *L. japonica*, *L. victoriae* (Weiss et al. 2015a), and *L. ryukyuensis,* which produces (+)-iridomyrmecin and (–)-iridomyrmecin. *Leptopilina victoriae* and *L. japonica* are sister species and *L. ryukyuensis* is phylogenetically located between these two species and all other species producing (–)-iridomyrmecin. The phylogeny (Wachi et al. 2015) suggests that the production of (–)-iridomyrmecin represents the ancestral state in the genus *Leptopilina* and therefore supports the hypothesis of a switch to the production of (+)-iridomyrmecin in *L. japonica* and *L. victoriae* via *L. ryukyuensis* as intermediate step.

In total we found 19 cuticular hydrocarbon compounds in extracts of *L. ryukyuensis* and 27 in extracts of *L. japonica* (Table 1). Most CHCs produced by *L. ryukyuensis* and *L. japonica* were methyl-branched alkanes and mono-unsaturated alkenes (Table 1) and are also found in other species of *Leptopilina*. Nevertheless the relative composition of CHC profiles are species- and sex-specific (Stökl et al. 2012; Weiss et al. 2013; Weiss et al. 2015a; Pfeiffer et al. 2018). While females of *L. ryukyuensis* and *L. japonica* produce relatively large amounts of 4-methyltriacontane and unsaturated hydrocarbons (7-nonacosene, 9-hentriacontene, 7-hentriacontene), males of both species produce relatively large amounts of 9,19-pentatriacontadiene, which also occurs in large amounts in males of *L. clavipes* and *L. heterotoma* (Weiss et al. 2015b; Pfeiffer et al. 2018). The compound 9,19-pentatriacontadiene is assumed to be part of the antennal pheromones of males of *Leptopilina,* which is presumably transferred between male and female antennae during courtship and is necessary to elicit receptiveness in females (Isidoro et al. 1999; Weiss et al. 2015b).

All six species of *Leptopilina* tested so far use their iridoid secretion for defence (Fig. 5), which strongly supports the hypothesis that defence is the primary function of the iridoid compounds in *Leptopilina*. Our chemical analyses of the headspace experiments revealed that females of *L. japonica* and *L. ryukyuensis* released iridoids upon being gently teased with a small magnetic stirrer. In other species iridomyrmecins were released when confronted with ants (Stökl et al. 2012). We therefore conclude, that the teasing with the magnetic stirrer can be interpreted as an artificial predatory attack, to which females reacted with the release

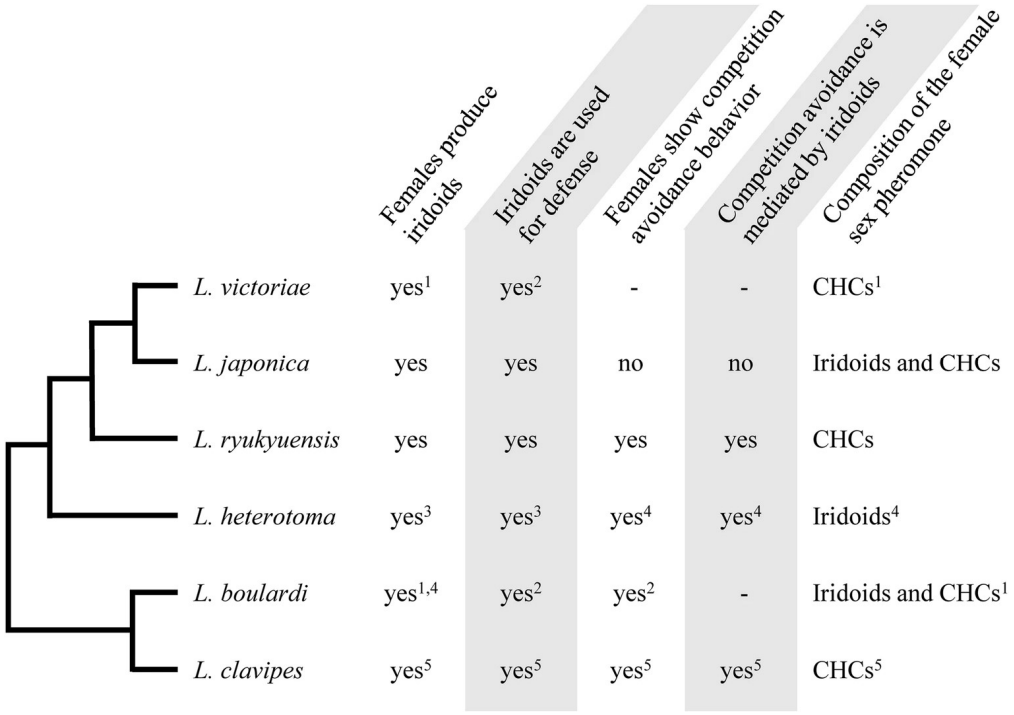

Figure 5. Phylogenetic relationship and current knowledge on the production and use of iridoids by the six species of *Leptopilina* studied so far. Hyphens indicate that this function has not been studied. Phylogenetic tree based on ITS2, modified from Wachi et al. 2015. [1]Weiss et al. 2015; [2] unpublished, [3]Stökl et al. 2012; [4]Weiss et al. 2013; [5]Pfeiffer et al. 2018.

of iridoids as defensive allomones. In parasitoid wasps, iridoids have hitherto been found only in the genera *Alloxysta* and *Leptopilina*, both of which use iridomyrmecins and other iridoids for defense (Völkl et al. 1994; Hübner and Dettner 2000; Hübner et al. 2002; Stökl et al. 2012; Weiss et al. 2013; Weiss et al. 2015a; Pfeiffer et al. 2018). Additionally, several species of ants and beetles use iridoids as part of their defensive secretions as repellents against predators such as ants and spiders (Huth and Dettner 1990; e.g. Do Nascimento et al. 1998; Welzel et al. 2018). As (−)-iridomyrmecin repels ants more effectively than (+)-iridomyrmecin (Stökl et al. 2012), this could explain why *L. ryukyuensis* females, which produce both stereoisomers, emitted smaller amounts of the iridoid secretion upon artificial attack than *L. japonica* females, which only produce (+)-iridomyrmecin.

The use of the iridoid compounds to avoid host patches with conspecific females is also widespread in the genus *Leptopilina* (Fig. 5) and has been demonstrated in *L. ryukyuensis* (this study) as well as in females of *L. heterotoma* (avoidance of con- and heterospecific females; Weiss et al. 2013), and *L. clavipes* (Pfeiffer et al. 2018). Females of *L. boulardi* avoid competition (unpublished), it is however unclear, whether iridoids elicit this behavior (Fig. 5). If presented without the background odor of the host patch, females of *L. heterotoma* and *L. clavipes* did not avoid the odor of conspecific females (Weiss et al. 2013; Pfeiffer et al. 2018). Therefore, we conclude that the avoidance behavior of other females is context-specific and not due to the deterrence of the defensive compounds. However, females of *L. japonica* are the first not to avoid conspecific females or their defensive compounds during host search (Fig. 5). This finding cannot be explained by a lack of odorous iridoid compounds, as females of *L. japonica* produce higher amounts of iridoids than females of *L. ryukyuensis*. Hence, our result suggests that females of *L. japonica* do not suffer from competition through conspecifics or the cost of ignoring a possible oviposition site is higher than the cost of competition through conspecific females. Alternatively, the females might gain advantages from lower individual predation pressure when many conspecifics are nearby.

There is much more variation in the use of the iridoids in the female sex pheromone than in their use for defense or competition avoidance. The degree of which iridoids are used in the female sex pheromone ranges from only iridoids in *L. heterotoma* to only CHCs in *L. clavipes* (Fig. 5). But we also find species like *L. boulardi* that use a combination of both, iridoids and CHCs, species in which males respond with courtship to CHCs and iridoids, but CHCs are sufficient to trigger full courtship (*L. victoriae* and *L. ryukyuensis*). However, while the production of iridomyrmecin enantiomers shows a phylogenetic pattern, current data suggest that the phylogeny will not be able to explain the differing contribution of iridoids and CHCs to female sex pheromones among *Leptopilina* species (Fig. 5). For example, a CHC-based sex pheromone is used by basal species like *L. clavipes* and derived species like *L. victoriae* or *L. ryukyuensis*. Also, sister species and phylogenetic subgroups in the genus are not similar in their sex pheromone composition (Fig. 5). Large differences in the pheromone composition of sister species could be explained by saltational shifts in the pheromone composition, which are one way to diversify sex pheromones during speciation despite the strong stabilizing selection pressure (Symonds and Elgar 2008). Male iridoids and CHCs are probably not under such strong stabilizing selection and could reflect the phylogenetic relationship of *Leptopilina* species. The mating system of *Leptopilina* wasp species could offer an alternative explanation of the chemical diversity of the sex pheromones in the genus. The dispersal behavior of male and female wasps after emerging from the *Drosophila* pupae, which usually occurs gregariously, could affect the usage and the composition of sex pheromones. If males and females disperse immediately after emerging, long-range mate attraction might become necessary. For this purpose, volatile substances like iridoids are better suited than CHCs. If dispersal is delayed and mating occurs directly on the host patch, short-range mate attraction by low volatility CHCs could be sufficient. Further studies on the mating and dispersal behavior of sexes in the different *Leptopilina* species could shed light on the evolution of pheromone use of these parasitoid wasps.

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5.2 Publication 2

Mate Attraction, Chemical Defense, and Competition Avoidance in the Parasitoid Wasp *Leptopilina pacifica*

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LB and JS conceived and designed the research plan. LB performed the quantitative and qualitative chemical analyses as well as the chemical defense experiment. FH conducted the behavioral analyses for the sex pheromones and the female competition avoidance. LB and FH analyzed the data. LB wrote the manuscript. JS edited and all authors approved the manuscript.

Own contribution: Concept and study design 75%, data acquisition 65%, data analysis and figures 80%, interpretation of results 90%, manuscript writing 90%.

Chemoecology
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CHEMOECOLOGY

ORIGINAL ARTICLE

Mate attraction, chemical defense, and competition avoidance in the parasitoid wasp *Leptopilina pacifica*

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Abstract
A major hypothesis for the evolution of chemical signals is that pheromones arise from non-communicative precursor compounds. However, data supporting this hypothesis are rare, primarily because the original functions of the antecedent compounds often have been lost. A notable exception, however, is the parasitoid wasp species *Leptopilina heterotoma*, whose compound (−)-iridomyrmecin is used as a defensive secretion, a cue for females to avoid competition with con- and heterospecific females, and as the primary component of the females' sex pheromone. To better understand the evolution of sex pheromones from defensive compounds, we examined the chemical ecology of *L. pacifica*, the sister species of *L. heterotoma*. Here, we show that *L. pacifica* also produces a defensive secretion containing a species-specific mixture of mostly iridoid compounds. However, the composition of the secretion is more complex than in *L. heterotoma*, and iridomyrmecin is only a minor component. Moreover, in contrast to *L. heterotoma*, conspecific female competitors were not avoided by female subjects, and a role of the iridoids in the female sex pheromone of *L. pacifica* can be excluded, as only the females' cuticular hydrocarbons (CHCs) resulted in the elicitation of courtship by males. Although closely related, the two sister species show substantial differences in the use of the defensive secretion for communicative purposes. Variation in pheromone usage in this genus still presents a conundrum, highlighting the need for additional studies to understand the selective forces shaping the evolution of pheromone composition.

Keywords Figitidae · Pheromone · Evolution · Iridomyrmecin · Citral · Cuticular hydrocarbons

Introduction

Chemical communication is believed to be the oldest form of communication and is widespread in the animal kingdom (Wyatt 2014). However, the origin and evolution of chemical communication remains a major question in chemical ecology. Several thousand chemical compounds used in chemical communication have been identified (El-Sayed 2020), and the diversity in chemical structures and relative amounts of substances allows an infinite number of complex odor compound combinations. The sender-precursor model posits that pheromone signals can arise via an evolutionary transition from precursor molecules that initially acted as chemical cues (Sorensen and Stacey 1999; Wyatt 2010; Bradbury and Vehrencamp 2011; Steiger et al. 2011; Stökl and Steiger 2017). Potentially, any chemical can evolve into a pheromone if it provides a selective advantage to both the sender and receiver (Wyatt 2014). A prerequisite for a compound to evolve into a pheromone is that it is produced and emitted by one individual in a non-communicative context and perceived by a second individual of the same species (Wyatt 2010). In this way, the compound acts as a chemical cue, transmitting information to the receiving individual without being selected for that function (Maynard Smith and Harper 2003). For example, a hormone released in urine, or alternatively, a defensive compound, can become a chemical cue. Selection can then act on the behavioral responses

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of the receiving individual, and ultimately ritualize and fine-tune the emission, sensitivity and specificity of the information transferred by this compound and its subsequent reception. Various fish pheromones have evolved via this mechanism, in which steroid hormones of females or their derivatives have been co-opted as sex pheromones over evolutionary time (Stacey and Sorensen 2011). If a pheromone compound is used for multiple functions, this is termed semiochemical parsimony and occurs in various species (Blum 1996; Bordereau and Pasteels 2011). Such parsimony corroborates the evolutionary transition that can ensue when the original function of a chemical compound is co-opted for its current use in pheromonal communication.

The iridoid compound (−)-iridomyrmecin functions as a defensive compound in the parasitic wasp *Leptopilina heterotoma* Thompson 1862 (Hymenoptera, Figitidae) to deter predators (Stökl et al. 2012). Females of this species also use (−)-iridomyrmecin as a cue to avoid competition with conspecific and heterospecific females during host search and egg-laying (Weiss et al. 2013). Interestingly, (−)-iridomyrmecin also serves as the main component of the females' sex pheromone, which initiates mate finding, species recognition, and courtship (Weiss et al. 2013). Therefore, we can infer a functional shift from the use of (−)-iridomyrmecin as a defensive compound to its utility as a sex pheromone in female *L. heterotoma*. In *L. heterotoma*, (−)-iridomyrmecin is an example of a threefold chemical parsimony, extending our understanding of the evolutionary transition from a non-communicative defensive compound to a species-specific mating signal.

The diversity of the production and use of iridomyrmecins, as well as of sex pheromones, within the genus *Leptopilina* is highly diverse. All studied species of *Leptopilina* produce iridoid compounds, but with different stereoisomers of iridomyrmecin and several additional species-specific iridoid substances (Stökl et al. 2012; Weiss et al. 2013, 2015a; Pfeiffer et al. 2018; Böttinger et al. 2019). While the use of iridoids as defensive allomones and cues for competition avoidance appears to be common in the genus *Leptopilina* (Weiss et al. 2013; Pfeiffer et al. 2018; Böttinger et al. 2019, unpublished), their function as a sex pheromone has not evolved in all species (Fig. 1). Two species (*Leptopilina heterotoma*: Weiss et al. 2013; *L. japonica*: Böttinger et al. 2019) rely on iridoids as female sex pheromones, whereas all other species either use CHCs (*L. victoriae*: Weiss et al. 2015a; *L. clavipes*: Pfeiffer et al. 2018; *L. ryukyuensis*: Böttinger et al. 2019) or a combination of iridoids and CHCs (*L. boulardi*: Weiss et al. 2015a) in mate attraction and courtship initiation.

To date, nothing is known about the chemical communication of *L. pacifica* Novković & Kimura 2011, the sister species of *L. heterotoma* (Novković et al. 2011; Wachi et al. 2015). Whereas *L. heterotoma* occurs in the Holarctic as well as in the Oriental region (Allemand et al. 2002) and parasitizes various drosophilid flies (Carton et al. 1986), *L.*

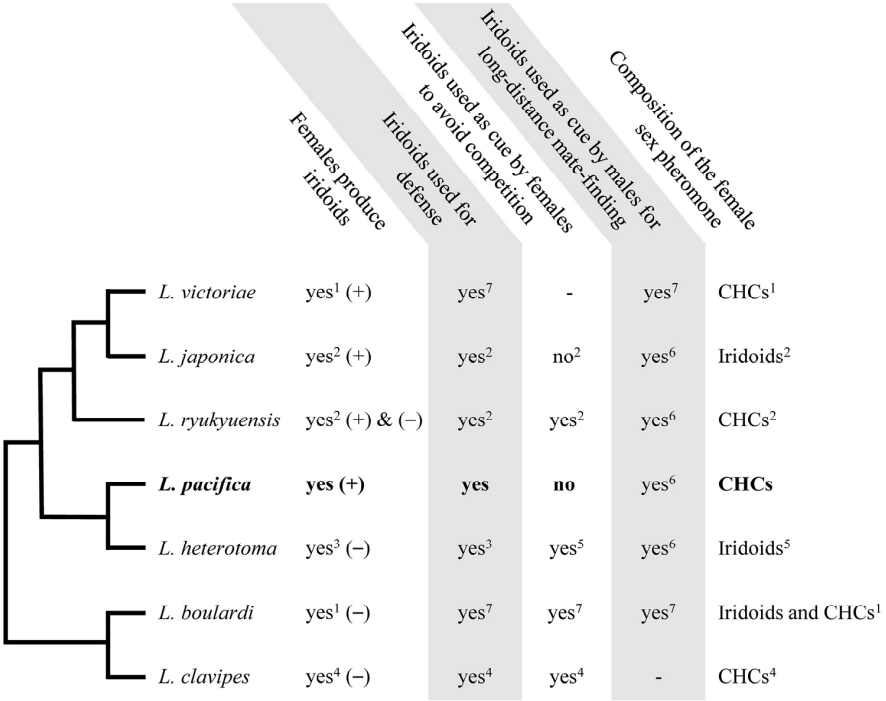

Fig. 1 Phylogenetic relationship and current knowledge on the production and use of iridoids by the nine species of *Leptopilina* studied so far. (+) indicates that (+)-iridomyrmecin is produced (among other iridoid compounds), and (−), that (−)-iridomyrmecin is produced. Hyphens indicate that this function has not been studied. In bold is the here studied species and the results presented in this study. Phylogenetic tree based on ITS2, modified from Wachi et al. (2015). [1]Weiss et al. (2015a), [2]Böttinger et al. (2019), [3]Stökl et al. (2012), [4]Pfeiffer et al. (2018), here, the defensive function of iridoids is assumed, but has not been experimentally tested; [5]Weiss et al. (2013); [6]Böttinger and Stökl (2020), [7]unpublished

pacifica is an oriental species occurring in subtropical and tropical regions of Asia (Novković et al. 2011) and develops on hosts of the *D. immigrans* group (Kimura and Suwito 2012). In this study, we ask: Does *L. pacifica*, as previously documented in *L. heterotoma,* (1) produce iridoid compounds, (2) emit these for defensive purposes, (3) use these as cues for females to avoid competition, and (4) as female sex pheromone signal for males to find females?

To address this question, we analyzed the production and use of chemical compounds in *L. pacifica*. We report on the compounds released as defensive secretions upon attack or disturbance of *L. pacifica*, and on the competition avoidance behavior of female wasps during host finding. We identify the components of the sex pheromone of female *L. pacifica* wasps by analyzing the courtship behavior exhibited by males in response to specific female compounds. For the evaluation of courtship behavior, the duration of male wing-fanning display is used, which *Leptopilina* males display upon perception of conspecific females and maintain during all stages of courtship. Elucidating the communication system of *L. pacifica*, specifically, their reliance on iridoids, should yield important insights into the evolutionary trajectories promoting variation in sexual signaling within the genus *Leptopilina*.

Material and methods

Experimental animals

A strain of the parasitoid wasp *Leptopilina pacifica* was collected by M. T. Kimura in June, 2011 on Irimote-jima, Japan and maintained in the laboratory using *Drosophila virilis* STURTEVANT as a host species. For each rearing bout, about 30 *D. virilis* flies of both sexes were placed into a jar containing fresh corn-based *Drosophila* medium (1 l water, 50 g cornmeal, 50 g wheat germ, 50 g sugar, 40 g baker's yeast, 8 g agar, 5 ml propionic acid). Four days later, when the flies had laid eggs, the flies were removed and approximately 15 female and male *L. pacifica* wasps were introduced into the jar. *Drosophila virilis* flies and *L. pacifica* wasps were kept in a climate and light controlled environment at 24 °C, 60% humidity and a 16:8 h L:D cycle. A few days before wasp emergence (approximately three weeks after oviposition), the parasitized fly pupae were singly isolated from the jars in 1.5 ml microcentrifuge tubes and provided with diluted honey ad libitum. This protocol allowed us to obtain unmated and naïve wasps of known age and sex. Mated females were obtained by placing a single 1-day-old female together with a male wasp for 12 h in a microcentrifuge tube. Subsequently, the wasps were again isolated and fed with diluted honey ad libitum. Each individual wasp was only used once in an experiment.

To rear predators of *L. pacifica* for the analysis of defensive compounds emitted by wasps, larvae of the Common Green Lacewing *Chrysoperla carnea* STEPHENS (Neuroptera: Chrysopidae) (obtained from Katz Biotech AG, Baruth, Germany) were maintained individually in 48-well plates to avoid cannibalism. They were kept in a climate and light controlled environment at 24 °C, 60% humidity and a 16:8 h L:D cycle, and fed ad libitum with *Sitotroga cerealella* OLIVIER eggs. Each *C. carnea* larva was used only once in an experiment.

Chemical analyses

For qualitative chemical analyses and behavioral experiments, extracts of males and females of *L. pacifica* were obtained by extracting each sex in batches of 30 to 100 individuals for 10 min in 10 µl dichloromethane (DCM) per individual. These pooled extracts were analyzed with an Agilent 7890 gas chromatograph (GC; Agilent Technologies, Germany), equipped with a non-polar capillary column (DB-5, 30 m, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, Germany) and coupled to an Agilent 5977A mass spectrometer (MS; Agilent Technologies, Germany). The injector temperature was set to 280 °C and samples were injected splitless. Helium was used as the carrier gas at a constant linear velocity of 50 cm s^{-1}. The GC oven was heated from 80 °C with 5 °C min^{-1} to 280 °C, where the temperature was held for 20 min. The MS was operated in electron impact (EI) mode at 70 eV and scanned a mass-range between 30 and 500 $m\ z^{-1}$.

Compounds in extracts were identified by comparing their mass spectra, diagnostic ions, and Kovats retention indices to those of synthetic reference compounds and known compounds from other species in the genus. To identify and separate the iridoid compounds enantioselectively, additional analyses were performed on a chiral capillary column (CycloSil-B, 30 m, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, Germany), in which injector temperature was set to 250 °C and the oven temperature was held at 80 °C for 4 min, before it was raised at 3° min^{-1} to 230 °C. Methyl-branched alkanes were identified by comparing their retention indices with data from the literature (Carlson et al. 1998) and interpretation of diagnostic ions (Nelson 1993). Double-bond positions of mono- and di-unsaturated compounds were determined by derivatizing samples with dimethyl disulfide (Carlson et al. 1989). Derivatized samples were then analyzed on a Shimadzu GC2030 coupled to a QP2020NX MS equipped with a SH-Rxi-5 ms column (30 m, 0.25 mm i.d., 0.25 µm film thickness). The other parameters were set as in the previous analysis, but the final

oven temperature was increased to 310 °C and the mass-range increased to 800 $m\ z^{-1}$.

For quantification of compounds produced by male and female *L. pacifica*, 15 wasps of each sex were extracted individually for 10 min in 20 µl of DCM with 20 ng μl^{-1} methyl undecanoate as internal standard. These samples were analyzed on the Agilent GC/MS system as described above and the amounts of compounds determined by comparing their integrated peak areas with those of the respective internal standard. For the calculation of absolute amounts of volatile compounds, external standard calibration curves were obtained by analyzing different concentrations of synthetic (+)-iridomyrmecin (2, 5, 10, 20, 50 ng μl^{-1}) together with the internal standard. For the absolute quantification of CHCs, external calibration curves with known amounts of 9-tricosene and tricosane, always 1, 5, 10, 25, 50 ng μl^{-1}, together with the internal standard, were conducted.

Fractionation of extracts

To identify the behaviorally active compounds, the females' extracts were separated into volatile polar compounds and non-polar CHC compounds by solid-phase extraction. Samples were dried under a gentle stream of nitrogen, re-dissolved in 50 µl of hexane, and applied on a cyanopropyl-modified silica gel column (50 mg, DSC-CN, Sigma-Aldrich, Taufkirchen, Germany), which had been pre-conditioned by rinsing with 2 ml of DCM and hexane. The non-polar CHC compounds of the samples were eluted from the column with 150 µl hexane, and subsequently the column was flushed with 500 µl of hexane. Then, the polar iridoid substances of the sample were eluted with 150 µl DCM. The fractions were analyzed by GC/MS as described above and their concentration re-adjusted to the concentration of the original extract.

Iridoids for defense

We analyzed the volatile organic compounds released from *L. pacifica* wasps when attacked by natural enemies to determine whether *L. pacifica* wasps emit iridoid compounds as deterrent allomones. To that end, two female wasps of *L. pacifica* were carefully placed in a 1 ml glass vial together with a previously starved third instar larva of the Common Green Lacewing (*Chrysoperla carnea*), after the wasps were left there for 30 s to acclimate. The amounts of compounds emitted by the female *L. pacifica* wasps were measured using dynamic headspace collection. For this, a headspace needle trap device (packed with 3 cm Tenax TA, NeedlEx, Shinwa Chemical Industries LTD, Japan) was inserted into the vial containing the wasps and the lacewing larvae. Air was drawn through the needle for 5 min at a rate of 6 ml min^{-1} by a sampling system (PAS Technology, Germany). An activated charcoal filter (50 mg, ORBO, Sigma-Aldrich) cleaned the air flowing into the vial. Volatiles emitted by the *L. pacifica* females were adsorbed in the needle, which was subsequently thermally desorbed in the hot injector of the Agilent GC/MS system and analyzed with the same settings as described above. As a control, two female wasps without a lacewing larva were put in the vial and their emitted compounds were collected and analyzed. Experiments were conducted 15 times per treatment (attack vs. control), and after each experiment, a new vial as well as new wasps and lacewing larvae were used. The activity of the lacewing larvae varied greatly between experiments, and so too did the amount of volatiles released by the wasps. Therefore, a second experiment with a standardized simulation of predatory attacks was performed in which the lacewing larvae were replaced with a small magnetic stir bar (8 × 3 mm). The stir bar was moved for 2 s every 30 s with a magnet from outside the vial to simulate an attack on the wasps. Chemicals were trapped and analyzed as in the previous experiment. Experiments were conducted 15 times per treatment (teasing vs. control), and after each experiment, the magnetic stir bar was washed twice with DCM and a new vial and new wasps were used. For the calculation of absolute amounts of volatile compounds, an external standard calibration curve was obtained by analyzing different concentrations of synthetic (+)-iridomyrmecin (1, 5, 10, 25, 50 ng μl^{-1}).

Female competition avoidance

The avoidance behavior of females towards conspecifics during host search was investigated using a Y-tube olfactometer. The glass Y-tube was positioned at a 30° slope with the arms pointed upwards and illuminated by two LED-tubes (white light, 350 lm, 5 W) from above. The inner diameter of the Y-tube was 1.5 cm, the base had a length of 6 cm, and the arms had a length of 9 cm and were spread at a 45° angle. Both ends of the arms were connected via Teflon tubes to separate Erlenmeyer flasks (50 ml), which contained host patches (~ 5 g of corn-based diet containing fresh *D. virilis* larvae). Humidified air was pumped through the flasks into the Y-tube at a flow rate of 30 ml min^{-1}. A single mated female wasp was carefully placed at the entrance of the base of the Y-tube and could decide between the two odor cues. The experimental test was stopped after 10 min (no choice) or once the female had crossed a decision line 2 cm beyond the branching point in each arm. After each test, the sample and control odor arm were alternated by turning the Y-tube. After every second test, the Y-tube was rinsed with ethanol and hot water and left to dry. To increase the number of responsive females, mated females were allowed to lay eggs in groups of 10–20 females on a host patch prior to the experiment. Each experiment was replicated until 30 females had crossed one of the decision lines. In the first experiment,

the females had to choose between the odor of a host patch with 20 live mated *L. pacifica* females and the odor of a host patch without wasps. In the second experiment, the females had to choose between the odor of a host patch with 2 µl of extract of females and the odor of a host patch with the solvent. Extract and solvent were applied to small discs of filter paper (5 mm diameter) and left to dry until the solvent evaporated. The paper discs were then placed directly into the arms of the Y-tube.

Female sex pheromone

Males of *L. pacifica* show wing fanning behavior, a high-frequency vibration of the wings, when they perceive and court a conspecific female. During all stages of the male courtship (perception, attraction, recognition, and approaching of the female, touching the females' antennae, mounting, antennal stroking, and, upon acceptance, copulation), wing fanning is maintained (Jenni 1951; van den Assem 1968; Isidoro et al. 1999). The duration of wing fanning indicates how much the male assumes that a female is nearby. We, therefore, used the duration of the wing fanning to measure the attractiveness of the females' compounds. Extracts of females (2 µl), fractions thereof (always representing 1/5th equivalent of a female), or a solvent control were applied to a small discs of filter paper (5 mm diameter), left for 30 s to let the solvent evaporate, and then placed in a glass arena (15 mm diameter, 4 mm height). Male wasps emerge 1–2 days before conspecific females (Böttinger and Stökl 2020), are directly sexually mature, and mating then takes place shortly after female emergence (pers. observation). Therefore, naïve 2–5-day-old males were used in this experiment. A single male wasp was introduced to the arena and its behavior recorded with a camera (Canon 70D, 100 mm macro objective) for 180 s. The total duration of the male's wing fanning behavior within these 180 s was analyzed using the video analysis software BORIS (Friard and Gamba 2016). Each extract, fraction and control were tested 20 times using a new male for each experiment. Impregnated filter papers and each male were only used once. After each replicate, the arena was rinsed with ethanol and hot water and left to dry at room temperature.

Statistics

Emitted amounts of volatile compounds of female wasps were compared between treatment groups (attacked vs. control; teased vs. control) using Mann–Whitney *U* tests. Decisions of female wasps in the Y-tube-experiments were analyzed with two-sided binomial tests. Differences in total wing fanning durations of males towards extracts, fractions, or the control were analyzed with a non-parametric Kruskal–Wallis ANOVA, followed by post hoc pairwise comparisons using Mann–Whitney *U* tests with Bonferroni-Holm correction (Benjamini and Hochberg 1995). All statistical analyses were performed using R version 3.3.0 (R Core Team 2017).

Results

Chemical analyses

In extracts of female and male wasps of *L. pacifica*, we identified compounds from mainly two substance classes, iridoids and CHCs. Additionally, we found (*Z*)-3,7-dimethyl-2,6-octadienal (neral) and (*E*)-3,7-dimethyl-2,6-octadienal (geranial). Our chemical analyses furthermore revealed clear sex-specific qualitative and quantitative differences in the chemical profiles of female and male *L. pacifica* wasps. Females produce 20 volatile compounds (iridoids and citral), with iridodial, actinidine and nepetalactone being among the most abundant. In males, which produce only 11 volatile compounds, citral, actinidine and nepetalactone 2 dominate the profile of volatile compounds. Quantitatively, the volatile compounds (iridoids, neral, geranial) made up 392.36 ± 206.47 ng (mean ± SD) or 45.05% of all compounds in females. In contrast, extracts of males contained on average only about 99.52 ± 40.17 ng (mean ± SD) volatile compounds (16.38% of total compounds), from which 48.57% were neral and geranial. Female *L. pacifica* thus produce not only a more complex iridoid blend than males, their extracts also contained substantially higher quantities of iridoids (Table 1, Fig. 2).

The CHC composition found in extracts of male and female *L. pacifica* was qualitatively similar, but females had more compounds and greater quantities of CHCs than males. The wasps' CHC profiles contained mainly mono- and di-unsaturated alkanes or methyl branched alkanes. Although the CHCs were dominated by 4-methyl triacontane, 9-hentriacontene, and 4-methyl dotriacontane in both sexes, males additionally produced high amounts of 9,19-pentatriacontadiene. In total, we identified 31 CHC compounds in the extracts of female and male wasps (Table 1).

Iridoids for defense

In these experiments, we tested whether females of *L. pacifica* emit iridoids as deterrents to defend themselves against natural predators. In the first experiment, we let single 3rd instar larvae of *C. carnea* attack two females of *L. pacifica* and analyzed the emitted compounds during these 5 min encounters. Females released not only iridoids, but also citral (the mixture of neral and geranial) as deterrents. Females released on average 1.78 ± 3.17 ng (mean ± SD) of citral and iridoids in a non-defensive

Table 1 Quantitative Analysis of compounds produced by male and female *Leptopilina pacifica* wasps

No	Compound	KRI	Diagn. Ions	Diagn. Ions DMDS	Mean amount (ng ± SD) per female	Mean amount (ng ± SD) per male
1	(*Z*)-3,7-dimethyl-2,6-octadienal (neral)	1247	69, 84, 94/5, 109		14.21 ± 7.48	17.81 ± 6.25
2	Unknown compound	1258	69, 84, 94, 109, 122/3		4.54 ± 3.18	–
3	Unknown compound	1265	69, 95, 109, 122/3		1.60 ± 0.94	–
4	(*E*)-3,7-dimethyl-2,6-octadienal (geranial)	1276	69, 84, 94, 109, 123, 137, 152		21.98 ± 11.47	30.52 ± 11.01
5	Iridodial	1293	67, 84, 86, 111, 135		0.97 ± 0.54	–
6	Iridodial 1	1303	67, 81, 109, 111, 135		71.07 ± 52.26	0.91 ± 0.68
7	Iridodial 2	1306	67, 81, 109, 111, 135		34.06 ± 18.29	1.69 ± 1.51
8	Unidentified iridoid	1326	69, 83/84, 97/98, 135		5.52 ± 2.54	1.75 ± 0.48
9	Unidentified iridoid	1333	69,81, 84, 107, 109, 111, 135		0.79 ± 1.26	–
10	Actinidine; in females coeluting with unidentified putative iridoid[a]	1341	117, 132, 147; 69, 95, 108/9, 137		69.20 ± 43.36	10.26 ± 8.72
11	Unknown putative iridoid[a]	1349	69, 83, 93, 108, 137		51.32 ± 36.92	–
12	Nepetalactone 1[b]	1363	81, 95, 109, 123, 166		5.25 ± 3.01	–
13	Nepetalactone 2[b]	1372	69, 81, 95, 109, 123, 166		66.56 ± 32.78	27.48 ± 11.71
14	Unknown compound	1381	67, 81, 84, 111, 135		1.63 ± 1.05	–
15	Unknown compound[c,d]	1391	81, 84, 109, 111, 135, 153		0.55 ± 0.52	–
16	Unidentified iridoid[c]	1407	84, 109, 111, 166		1.48 ± 0.98	0.83 ± 0.64
17	Unknown compound	1411	97, 109, 124, 139		4.45 ± 2.83	–
18	4*R*,4a*R*,7*R*,7a*S*-dihydronepetalactone[e]	1417	67, 81, 95, 110, 113, 153, 168		9.34 ± 4.92	2.21 ± 0.76
19	(+)-Iridomyrmecin	1450	67, 81, 95, 109		2.55 ± 1.32	1.49 ± 0.43
20	Unknown iridoid	1458	67, 81, 95, 110, 113, 126, 153, 168		25.29 ± 13.97	4.58 ± 1.48
21	*n*-Hexadecanoic acid	1961	57, 60, 73, 129, 213, 256		0.87 ± 2.02	–
22	4-Methyl tetracosane	2461	71, 309, 337 (M-15), 352 (M+)		0.54 ± 0.82	–
23	4-Methyl hexacosane	2661	337, 365 (M-15), 380 (M+)		1.62 ± 1.35	–
24	7,x,x-Trimethyl heptacosane	2785	113, 253, 337		0.95 ± 1.28	2.33 ± 2.03
25	x,x-Nonacosadiene	2837	96, 404 (M+)		1.54 ± 1.27	0.83 ± 1.11
26	4-Methyl octacosane	2861	71, 365, 393 (M-15), 408 (M+)		8.26 ± 5.29	9.11 ± 4.96
27	9-Nonacosene	2877	97, 253, 281, 406 (M+)	173, 327, 500 (M+)	7.96 ± 5.76	8.81 ± 5.49
28	7-Nonacosene	2884	97, 406 (M+)	145, 355	5.98 ± 3.57	–
29	Unknown CHC	2898	253, 341, 408 (M+)		–	0.56 ± 0.78
30	13-Methyl nonacosane; 15-methyl nonacosane	2927	196/7, 252/3; 224/5		2.20 ± 1.78	0.59 ± 0.82
31	4-Methyl nonacosane	2959	378/9, 407 (M-15), 422 (M+)		2.80 ± 1.37	3.08 ± 0.93
32	5,11-Dimethyl nonacosane; 5,17-dimethyl nonacosane	2977	85, 183, 281, 379, 421 (M-15); 85, 197, 267, 379, 421 (M-15)		1.76 ± 2.06	–
33	14-Methyl triacontane; 15-methyl triacontane	3028	211, 253, 421 (M-15), 434 (M+); 225, 239, 421 (M-15), 434 (M+)		0.53 ± 1.03	–
34	x,x-Hentriacontadiene	3048	96, 432 (M+)		7.26 ± 6.42	–
35	x,x-Hentriacontadiene	3051	96, 432 (M+)		3.92 ± 5.37	–
36	4-Methyl triacontane	3060	393, 421 (M-15), 436 (M+)		157.22 ± 57.99	186.39 ± 34.78
37	9-Hentriacontene	3078	97, 434 (M+)	173, 355, 528 (M+)	98.21 ± 59.67	52.63 ± 14.75
38	7-Hentriacontene	3085	97, 434 (M+)	145, 383, 528 (M+)	46.33 ± 33.35	19.48 ± 5.71

Table 1 (continued)

No	Compound	KRI	Diagn. Ions	Diagn. Ions DMDS	Mean amount (ng ± SD) per female	Mean amount (ng ± SD) per male
39	Putative cholesterol	3097	255, 275, 386		1.09 ± 1.88	–
40	13-Methyl hentriacontane; 15-methyl hentriacontane	3126	196/7, 280/1; 224/5, 252/3		7.41 ± 6.55	4.30 ± 3.68
41	Unknown compound[c,d]	3137	275, 301, 386, 353, 368		5.31 ± 2.94	8.82 ± 2.95
42	4-Methyl hentriacontane	3158	281, 386, 407, 435 (M-15), 450		0.41 ± 0.57	–
43	x-Methyl dotriacontane	3176	85, 253, 281, 351, 407, 449 (M-15)		1.08 ± 1.09	–
44	14-Methyl dotriacontane; 15-methyl dotriacontane	3232	211, 281, 449 (M-15), 464 (M+); 225, 267, 449 (M-15), 464 (M+)		5.44 ± 7.53	2.04 ± 2.61
45	7,17-Tritriacontadiene	3249	96, 460 (M+)	145, 271, 283, 409, 455, 507 (M-141), 554 (M-94), 648 (M+)	35.33 ± 23.83	19.10 ± 8.36
46	4-Methyl dotriacontane	3258	449 (M-15), 421		21.50 ± 11.54	32.68 ± 12.32
47	x-Tritriacontene	3278	97, 462 (M+)		16.35 ± 11.90	12.60 ± 12.82
48	x-Tritriacontene	3286	97, 462 (M+)		1.35 ± 2.02	2.21 ± 1.40
49	x-Tetratriacontene	3319	97, 476 (M+)		1.55 ± 2.84	4.16 ± 4.71
50	13-Methyl tritriacontane; 15-methyl tritriacontane; 17-methyl tritriacontane	3325	197, 309; 225, 281; 253		5.73 ± 7.13	2.48 ± 3.33
51	9,19-Pentatriacontadiene	3449	96, 488 (M+)	173, 271, 311, 409, 535 (M-141), 676 (M+)	20.09 ± 12.33	136.01 ± 39.84
52	x,x-Pentatriacontadiene	3450	96, 488 (M+)		8.01 ± 17.49	–
	Total amount				870.98 ± 339.54	607.73 ± 126.48

$N = 15$ males and females of *L. pacifica* singly extracted in 20 µl of DCM with 20 ng μl^{-1} methyl undecanoate as internal standard

KRI Kovats retention index on a non-polar DB-5 GC column. *Diagn. Ions* diagnostic ions used in the identification of the compound. *Diagn. Ions DMDS* diagnostic ions of unsaturated compounds after derivatization with DMDS. *x* = The position of methyl branch(es) and/or double bond(s) could not be determined. Numbers of compounds correspond to Fig. 2

[a]Compound also found in *L. boulardi* (Weiss et al. 2015a)

[b]Unknown absolute configuration

[c]Compound also found in *L. japonica* (Böttinger et al. 2019)

[d]Compound also found in *L. ryukyuensis* (Böttinger et al. 2019)

[e]Compound also found in *Alloxysta victrix* (Zimmermann et al. 2012)

context when left undisturbed, but when attacked by the *C. carnea* larvae, they released on average 7.33 ng ± 12.63 ng (mean ± SD), a significant increase (Mann–Whitney *U* test, $W = 64$, $P = 0.045$, Fig. 3).

The intensity of the attack by the *C. carnea* larvae was highly variable. Therefore, we measured the amount of released deterrent compounds of *L. pacifica* females when teased with a small magnetic stir bar, instead of being attacked by the larvae. Females released on average 0.40 ± 1.14 ng (mean ± SD) of total iridoids and citral components when left undisturbed, but when teased with the magnetic stir bar, the amount of iridoids and citral components released increased significantly to 8.89 ± 17.13 ng (mean ± SD; Mann–Whitney *U* test, $W = 9$, $P < 0.001$, Fig. 4).

Competition avoidance of females

Mated host-searching female *L. pacifica* did not avoid the odor of conspecific female wasps in the Y-tube olfactometer when given the choice between the odor of unexploited host patches and host patches occupied by 20 living conspecific females ($P = 0.86$, Fig. 5). However, *L. pacifica* females avoided the odor of the host patch, when the extract of *L. pacifica* females instead of living females was added to the host patch odor ($P < 0.01$, Fig. 5).

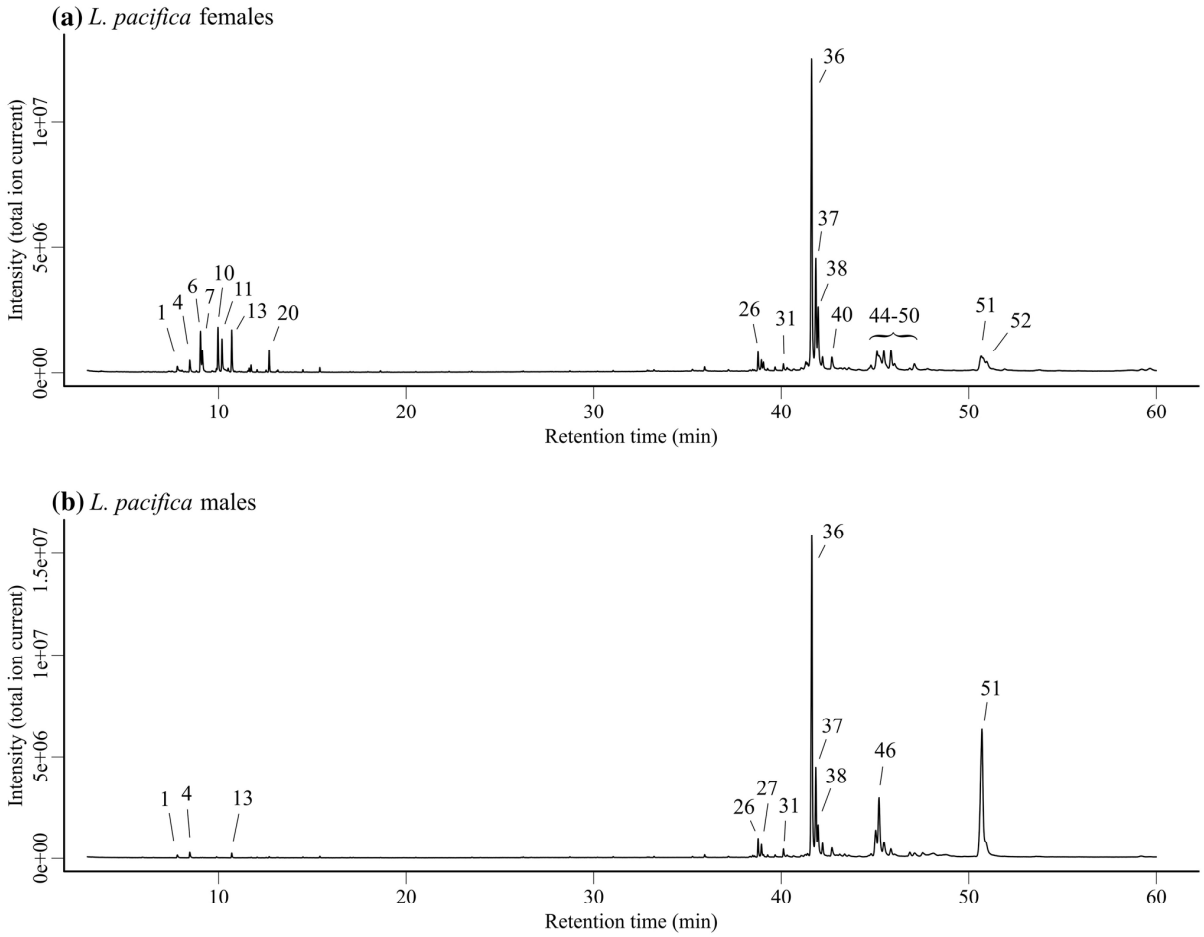

Fig. 2 Chemical compounds produced by *L. pacifica* wasps. Total ion current chromatograms of extracts of **a** *L. pacifica* females and **b** *L. pacifica* males, analyzed on a non-polar DB-5 GC column. Peak numbers correspond to Table 1

Sex pheromone

To investigate the compounds of females that are used by males to find and court mating partners, female extracts, fractions thereof and solvent control were applied on filter paper discs and presented to males. Male wasps of *L. pacifica* showed courtship behavior, i.e., wing fanning, when presented with the odor of conspecific females. The longest duration of wing fanning was displayed towards the whole body extracts of female *L. pacifica* wasps; however, there was no statistical difference in the duration of wing fanning exhibited when males were presented only with the females' CHCs present in the hexane fraction of female extracts (Kruskal–Wallis test, chi-squared = 53.577, $df = 3$, $P < 0.001$, Fig. 6). The solvent control, as well as the iridoid compounds contained in the DCM fraction of extracts of females, elicited significantly less wing fanning. The CHCs serve, therefore, as the female sex pheromone in *L. pacifica*.

Discussion

Weiss et al. (2013) recently found that the parasitoid wasp *L. heterotoma* relies on a single iridoid compound for several functions, allowing them to reconstruct the evolution of pheromone communication in this species. The compound (−)-iridomyrmecin serves in this species as a defensive secretion (Stökl et al. 2012), as a cue for females to avoid competition during host search, and as the main component of the female sex pheromone (Weiss et al. 2013, see Fig. 1). All *Leptopilina* species studied to date produce iridoids for defense (Stökl et al. 2012; Weiss et al. 2013, 2015a; Pfeiffer et al. 2018; Böttinger et al. 2019), but only three species use the iridoids in their female sex pheromone (Weiss et al. 2013, 2015a; Böttinger et al. 2019; Fig. 1). It is assumed, therefore, that defense is the primary function of these iridoid compounds, and that their use as sex pheromones to attract mates evolved

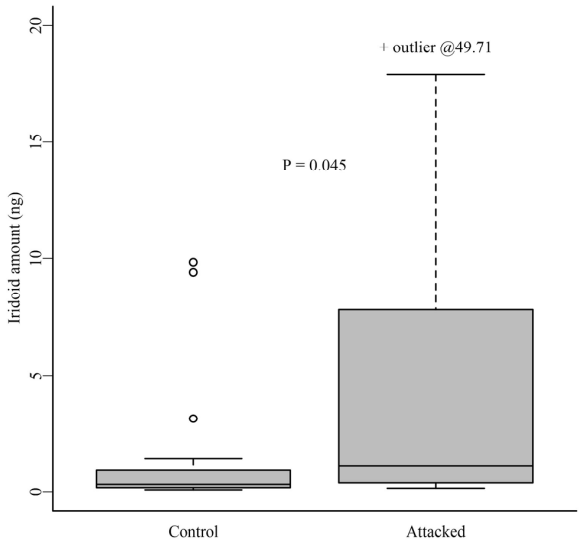

Fig. 3 Iridoids for defense released upon attack. Box-and-whisker plots showing median (horizontal line), inter-quartile range (box), outliers (unfilled circles), and maximum/minimum of the 1.5 × inter-quartile range (whiskers) of the released iridoid amounts (ng) of two females of *L. pacifica* in a small vial when left alone (Control) or when attacked 5 min from a lacewing larva (Attacked). *P* values are given for Mann–Whitney *U* tests. Each experiment $N = 15$

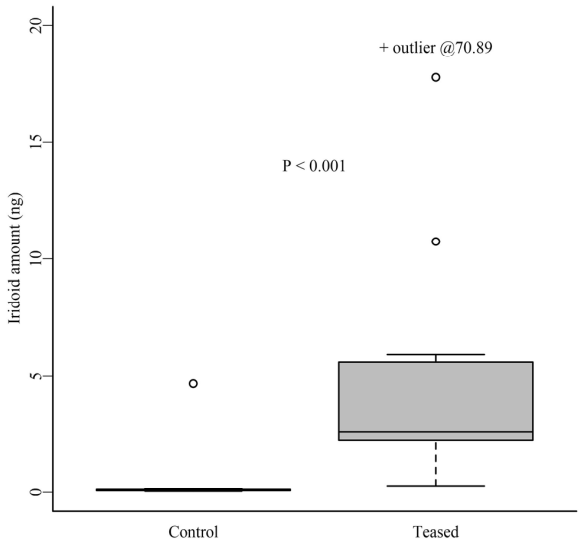

Fig. 4 Iridoids for defense released upon teasing. Box-and-whisker plots showing median (horizontal line), inter-quartile range (box), outliers (unfilled circles), and maximum/minimum of the 1.5 × inter-quartile range (whiskers) of the released iridoid amounts (ng) of two females of *L. pacifica* in a small vial when left alone (Control) or when slightly teased 10 times in 5 min with a small magnetic stir bar (Teased). *P* values are given for Mann–Whitney *U* tests. Each experiment $N = 15$

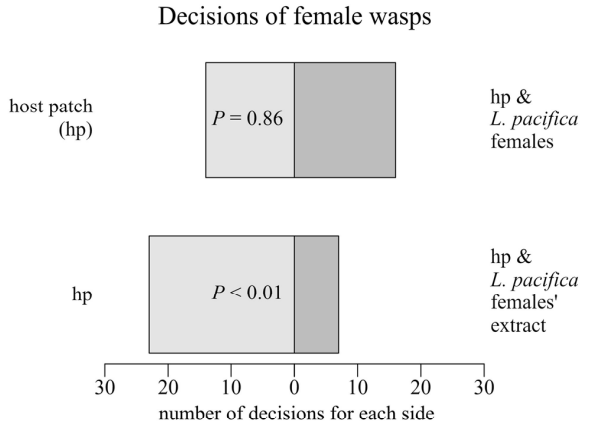

Fig. 5 Competition avoidance of females. Frequency of decision for control (light grey bars) or sample (dark grey bars) of females of *L. pacifica* in a Y-tube experiment when choosing between the odor of unexploited host patches (hp) or host patches with either 20 conspecific females or the extract of conspecific females. *P* values are given for two-sided binomial tests. Each experiment $N = 30$

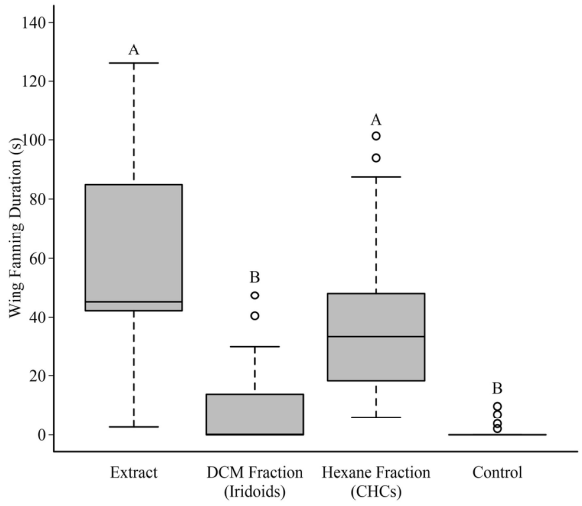

Fig. 6 Female sex pheromone. Box-and-whisker plots showing median (horizontal line), inter-quartile range (box), outliers (unfilled circles), and maximum/minimum of the 1.5 × inter-quartile range (whiskers) of the duration of courtship behavior (i.e., wing fanning) displayed by males of *L. pacifica* towards whole body extracts of conspecific females, as well as towards the iridoid containing DCM fractions and the CHC containing hexane fractions of the female extracts and the solvent control. Different letters indicate a significant difference (Kruskal–Wallis ANOVA followed by pairwise Mann–Whitney *U* tests with Bonferroni–Holm correction, $P < 0.05$). Each experiment $N = 20$

as a secondary function in *L. heterotoma* females (Weiss et al. 2013). Here, we investigated whether the evolution of pheromone communication led to a comparable result in

L. pacifica, the sister species of *L. heterotoma* (Novković et al. 2011; Wachi et al. 2015). Accordingly, we analyzed the chemical compounds produced by males and females, and the functions these compounds fulfill in communication in this species. Our results indeed show that *L. pacifica* wasps also produce several iridoid compounds and use them as defensive secretion. However, in contrast to female *L. heterotoma*, female *L. pacifica* do not avoid host patches occupied by conspecifics and do not use the iridoids in their sex pheromone, relying on CHCs instead (Fig. 1).

Congruent with chemical analyses of *L. heterotoma* (Stökl et al. 2012), the predominant volatile compounds of *L. pacifica* wasps were iridoid substances. However, the variety of different iridoid compounds produced by *L. pacifica* females is higher than in any other species of the genus *Leptopilina* (Table 1; Fig. 2; Stökl et al. 2012; Weiss et al. 2013, 2015a; Pfeiffer et al. 2018; Böttinger et al. 2019). Instead of having one main iridoid compound, as in *L. heterotoma,* where the compound (−)-iridomyrmecin constitutes more than 80% of the volatiles of females (Stökl et al. 2012; Weiss et al. 2015a), females of *L. pacifica* produce a complex mixture of different iridoid compounds with six iridoids being produced in similar amounts (Table 1). Interestingly, iridomyrmecin is not found among these six, which is in contrast to all other species of *Leptopilina* studied thus far. Furthermore, we found a differing stereochemistry of iridomyrmecin in *L. pacifica*; females and males produce (+)-iridomyrmecin. Most species, including *L. heterotoma* and *L. boulardi*, produce (−)-iridomyrmecin, while its enantiomer (+)-iridomyrmecin has been found in only two species thus far (Fig. 1). Additionally, we found citral, which is a mixture of the terpenoid *cis–trans*-isomers (*Z*)-3,7-dimethyl-2,6-octadienal (neral) and (*E*)-3,7-dimethyl-2,6-octadienal (geranial) in the extracts of *L. pacifica* wasps. Citral has not been found in any other species of *Leptopilina*.

The CHCs produced by male and female *L. pacifica* consisted mainly of mono- and di-unsaturated alkanes or methyl branched alkanes and, although they qualitatively resemble a number of the CHCs found in other species of the genus (Table 1; Fig. 2; Weiss et al. 2013, 2015a; Pfeiffer et al. 2018; Böttinger et al. 2019), their qualitative and quantitative composition is species specific. Extracts of male *L. pacifica* contained high amounts of 9,19-pentatriacontadiene, which seems to be a compound typically found in males of the genus *Leptopilina*. This compound was found in large amounts in male *L. japonica* and *L. ryukyuensis* (Böttinger et al. 2019), as well in *L. clavipes* and *L. heterotoma* (Weiss et al. 2015b; Pfeiffer et al. 2018). 9,19-pentatriacontadiene presumably comprises a part of the species-specific male antennal aphrodisiac pheromone that gets transferred between the male and female antennae during courtship to elicit readiness for mating in conspecific females (Isidoro et al. 1999; Weiss et al. 2015b).

In two different dynamic headspace analyses, we investigated which compounds get emitted by female *L. pacifica* when they are attacked. First, we determined which compounds get emitted by wasps when they are attacked by larval *C. carnea*, representing attacks from natural enemies. We found significantly more iridoid and citral compounds in headspace analyses of attacked wasps than of undisturbed control wasps; thus, the present study clearly demonstrates that these compounds are used for defense. However, we found wasps emitting different amounts of deterrent compounds in each run of the experiment. This was possibly due to the variation in the intensity of the attacks by the *C. carnea* larvae, as they sometimes heavily attacked the wasps, while other larvae did not attack the wasps at all. Therefore, in a second experiment, we simulated natural attacks in a standardized experimental design by teasing the wasps with a magnetic stir bar, leading again to emissions of females' citral and iridoids. As both experiments led to similar amounts of released defensive secretions, but with a lower variation in the stir bar experiment, the standardized experiment may provide a more sensitive test for assessing the deterrent compounds of small insects than the natural predator attack experiment. Iridoids are typical defensive compounds that are used for defense in several insect species. Iridomyrmecin and two iridodials were first isolated from the defensive secretion of the ant *Iridomyrmex humilis* (Pavan 1952; Cavill et al. 1976). Furthermore, iridoid compounds such as iridodials, iridomyrmecin, actinidine and nepetalactone were found to be used for defense in chrysomelid beetle larvae (e.g., Sugawara et al. 1979; Pasteels et al. 1982; Veith et al. 1994), aphids (Dawson et al. 1987), thrips (Tschuch et al. 2008), stick insects (Meinwald et al. 1962; Smith et al. 1979; Chow and Lin 1986; Prescott et al. 2009), in the parasitic wasp genus *Alloxysta* (Hymenoptera, Charipidae) (Völkl et al. 1994; Petersen 2000), ants (Wheeler et al. 1977; Tomalski et al. 1987), as well as in various staphylinid species (Bellas et al. 1974). Also citral, whose occurrence we show here for the first time in a species of *Leptopilina*, is used as an alarm pheromone in mites (e.g., Kuwahara et al. 1980, 1983; Raspotnig 2006) and as an alarm releaser and compound of the defensive secretion in ants, such as *Acanthomyrmex claviger* (Ghent 1961) and *Atta sexdens* (Butenandt et al. 1959; Blum et al. 1968). We conclude, therefore, that defense is the primary function of iridoid and citral compounds in *Leptopilina*. The amounts of emitted deterrent volatiles in both attack or simulated predatory disturbances were found in quantitatively lower amounts than in the total body extracts of female *L. pacifica* (see Figs. 3 and 4 and Table 1). However, we cannot assume wasps would entirely deplete their

defensive compound arsenal upon an attack, but rather emit an amount of deterrent secretions just sufficient to deter the attacking enemy and escape the dangerous situation (Stökl et al. 2015). The physiological mode of action of these emitted iridoid compounds is probably similar to that of iridoid glycosides, which denatures proteins and nucleic acids (Dobler et al. 2011). Wasps of the genus *Leptopilina* were previously shown to be able to adjust the amounts of emitted allomones depending on the size of their attacking enemy (Stökl et al. 2015), which could explain why we found different amounts of deterrent compounds in the different runs of the attack experiment (Fig. 3).

Although members of the same genus often have similar chemical compounds due to shared biosynthetic pathways (Tillman et al. 1999), and closely related species often use the same main pheromonal compounds (Smadja and Butlin 2009), pheromones of sister species can be considerably different (Symonds and Elgar 2008; Menzel et al. 2017; Butterworth et al. 2020). Indeed, studies found substantially different compositions of the volatile defensive compounds not only between the sister species *L. heterotoma* and *L. pacifica*, but within the whole genus *Leptopilina* (Stökl et al. 2012, 2015; Weiss et al. 2013, 2015a; Pfeiffer et al. 2018; Böttinger et al. 2019; this study). There is extensive variation in the quantity of different deterrent compounds, ranging from basically one main predominant compound (−)-iridomyrmecin in *L. heterotoma, L. boulardi* (Stökl et al. 2012, 2015; Weiss et al. 2015a), and *L. clavipes* (Pfeiffer et al. 2018), to the more complex array of iridoids in *L. victoriae* (Weiss et al. 2015a) and *L. japonica* (Böttinger et al. 2019), and peaking in the complex mixture of citral and iridoid compounds found here in *L. pacifica*. This is surprising, as we would predict only modest interspecific variation in the composition of defensive secretions, as individuals should benefit from the recognition of defensive secretions of con- and hetero-specifics. Therefore, species-specificity of these deterrent compounds should not be necessary and selection on interspecific variation of deterrent compounds and alarm cues should be low (Regnier and Law 1968; Blum 1969; Vander Meer and Le Alonso 1998). However, although there are several examples of interspecifically identical defensive or alarm substances in ants (Wilson and Pavan 1959), pentatomid stink bugs (Ishiwatari 1974), and aphids (Vandermoten et al. 2012), extensive variation in alarm pheromone composition among closely related leaf-cutting ant species has recently been documented (Norman et al. 2017). Interspecific variation of the volatile defensive secretion within the genus *Leptopilina* suggests, therefore, that iridoid compounds serve not only for defense but also other purposes, e.g., as sex pheromone.

In *L. heterotoma* (Weiss et al. 2013), *L. clavipes* (Pfeiffer et al. 2018), *L. boulardi* (unpublished) and *L. ryukyuensis* (Böttinger et al. 2019), female wasps avoided competition with conspecific females (Fig. 1). This competition avoidance is mediated by the females' emissions of iridoid volatiles even in undisturbed situations during egg-laying. In *L. pacifica*, no such avoidance behavior was observed in the experiment using live females. However, we found a significant avoidance of the odor of the host patch with the females' extract (as opposed to live females). Thus, female *L. pacifica* have the potential to exhibit avoidance behavior, but it was not triggered in our experimental setting. This could be explained by the relatively lower emission of the deterrent iridoid and citral volatile compounds of undisturbed wasps of *L. pacifica*, as can be seen in the control wasp headspace analyses (Figs. 3 and 4). It remains possible that a higher number of females on the host patch would trigger the avoidance behavior. However, the number of females on the host patch was already quite high (20 females). It seems plausible, therefore, that under natural conditions, females on the host patch do not emit enough volatiles to elicit the avoidance behavior in searching females. The positive result we found using the females' extract might then be regarded as an artefact. Interestingly, females of the sister species *L. heterotoma* show a clear avoidance of live conspecific females (Weiss et al. 2013). The lack of avoidance behavior in *L. pacifica* could indicate that this species is more competitive and does not need to avoid superparasitism. Alternatively, the amount of iridoids in the extract presented to the female subjects might have resembled the odor of attacked or dead conspecifics. Under this scenario, the females avoided the host patch not because of anticipated competition with other females, but because of the danger of a predatory attack.

Iridoid compounds serve not only as a deterrent secretion and a cue for female competition avoidance in *L. heterotoma*, but also as the main component of the female sex pheromone (Stökl et al. 2012; Weiss et al. 2013). As speciation of the sister species *L. heterotoma* and *L. pacifica* may have been accompanied by a differentiation of sex pheromones, we aimed in this study to analyze the sex pheromone compounds of *L. pacifica* females. We found that the sex pheromone of female *L. pacifica* wasps consists of CHC compounds, whereas the iridoid compounds and citral are not needed to elicit courtship behavior in males. This is in contrast to the sister species *L. heterotoma*, in which females' CHCs elicited almost no interest of males, whereas the iridoids (with (−)-iridomyrmecin as main component) triggered courtship behavior of males (Weiss et al. 2013, 2015a). This stark divergence in female sex pheromones could be the result of a saltational shift during speciation as observed in several other insect species (bark beetles: e.g., Symonds and Elgar 2004; wasps: e.g., Buellesbach et al. 2013; ants: e.g., Menzel et al. 2017; blowflies: e.g., Butterworth et al. 2020; stick insects: e.g.,

Schwander et al. 2013). Even within the same class of compounds (iridoids or CHCs), a saltational shift in pheromone composition is easily achievable. As such, the switch from iridoids to CHCs may be explained by selection for either long-range attracting volatile iridoids or short-range CHCs depending on differences in the mating system of the species. Species mating directly on the host patch on which they emerge would not have the necessity of a long-range sex pheromone, and a CHC-based sex pheromone would be sufficient to ensure sex and species recognition. In contrast, species with high dispersal rates after hatching would not be able to find their mates if they relied on low volatile CHC sex pheromones for orientation. These species must have evolved a volatile sex pheromone ranging over longer distances, a function for which the iridoid compounds are well suited. Support for this hypothesis was recently found in the species *L. heterotoma*, *L. japonica*, *L. pacifica*, and *L. ryukyuensis* (Böttinger and Stökl 2020). The dispersal behavior of male wasps correlated with the volatility of the female sex pheromones, with males of species with volatile iridoid sex pheromones, *L. heterotoma* and *L. japonica*, starting to disperse directly after emergence. In contrast, male wasps of species with CHC-based sex pheromones, *L. pacifica* and *L. ryukyuensis*, delayed their dispersal until the emergence of conspecific females (Böttinger and Stökl 2020). A comparative genetic and ecological analysis approach would help to disentangle the role of the selective forces shaping the evolution of pheromone usage in the genus *Leptopilina*.

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Author contributions LB and JS conceived and designed the research plan. LB performed the quantitative and qualitative chemical analyses as well as the chemical defense experiment. FII conducted the behavioral analyses for the sex pheromones and the female competition avoidance. LB and FH analyzed the data. LB wrote the manuscript. JS edited and all authors approved the manuscript.

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Compliance with ethical standards

Conflicts of interest/Competing interests The authors declare that they have no conflict of interest.

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Supplementary Table 1, 1/4 Quantitative Analysis of compounds produced by all male and female *Leptopilina pacifica* wasps. Each wasp was extracted for 10 min in 20 µl of a 20 ng µl^{-1} methyl undecanoate standard. We used three calibration curves to quantify the compounds: one using (+)-iridomyrmecin for all iridoid compounds, one using 9-tricosene for unsaturated CHCs and one using tricosane for saturated CHCs. Unidentified compounds were also quantified using the most likely curve. For integrated peak areas see Supplementary Table 2.

No.	Compound	KRI	Diagn. Ions	Diagn. Ions DMDS	Mean amount (ng) per female														
					01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
1	(*Z*)-3,7-dimethyl-2,6-octadienal (neral)	1247	69, 84, 94/5, 109		16,76	25,68	11,43	21,70	8,48	12,25	4,32	19,01	13,34	–	16,97	6,15	9,73	25,26	22,06
2	unknown compound	1258	69, 84, 94, 109, 122/3		5,47	9,06	6,36	13,55	3,60	3,29	1,55	2,83	4,50	–	3,70	2,61	2,16	5,31	4,11
3	unknown compound	1265	69, 95, 109, 122/3		1,09	1,81	1,52	3,67	2,13	2,06	0,72	0,70	1,42	–	0,99	1,00	1,49	3,34	2,07
4	(*E*)-3,7-dimethyl-2,6-octadienal (geranial)	1276	69, 84, 94, 109, 123, 137, 152		24,89	40,59	26,75	39,11	11,61	19,47	6,00	19,35	19,79	2,71	31,98	9,23	14,24	35,73	28,19
5	iridodial	1293	67, 84, 86, 111, 135		1,12	1,98	1,91	1,65	0,50	1,05	0,42	1,11	1,02	0,86	1,14	0,56	0,50	–	0,64
6	iridodial 1	1303	67, 81, 109, 111, 135		85,10	224,36	80,47	115,77	40,40	39,27	8,06	68,42	52,94	10,62	110,31	26,33	38,96	97,32	67,66
7	iridodial 2	1306	67, 81, 109, 111, 135		39,08	0,00	50,95	62,27	26,35	37,03	9,98	42,56	38,31	6,72	46,92	20,94	24,51	61,25	44,03
8	unidentified iridoid	1326	69, 83/84, 97/98, 135		6,20	12,08	7,19	10,16	4,94	5,06	5,87	3,64	3,58	2,51	5,88	3,63	3,16	4,37	4,58
9	unidentified iridoid actinidine; in females coeluting with unidentified putative iridoid[1]	1333	69,81, 84, 107, 109, 111, 135		2,55	2,37	–	4,04	0,58	–	–	–	–	–	2,06	0,29	–	–	–
10		1341	117, 132, 147; 69, 95, 108/9, 137		83,67	167,12	102,33	142,07	49,80	51,23	13,88	55,16	51,23	2,72	96,35	31,24	38,47	82,12	70,66
11	unknown putative iridoid[1]	1349	69, 83, 93, 108, 137		64,44	148,62	70,79	98,08	35,94	35,12	9,52	36,88	28,91	2,77	76,45	19,47	25,16	74,44	43,22
12	nepetalactone 1[2]	1363	81, 95, 109, 123, 166		3,34	7,31	5,03	8,41	3,07	7,10	1,87	5,17	6,21	–	8,90	2,41	2,35	11,51	6,10
13	nepetalactone 2[2]	1372	69, 81, 95, 109, 123, 166		68,60	119,97	82,19	114,94	38,52	58,92	23,80	70,95	61,48	8,42	97,43	27,50	40,66	98,66	86,39
14	unknown compound	1381	67, 81, 84, 111, 135		1,68	3,50	3,41	2,88	1,38	–	2,08	1,10	1,22	–	2,16	1,05	0,59	2,21	1,24
15	unknown compound[3,4]	1391	81, 84, 109, 111, 135, 153		0,79	1,10	0,55	1,40	–	–	–	–	–	–	1,25	0,63	0,43	1,19	0,97
16	unidentified iridoid[3]	1407	84, 109, 111, 166		1,31	1,36	1,68	1,19	–	1,59	0,78	1,53	1,56	–	3,49	1,06	0,73	2,71	3,21
17	unknown compound	1411	97, 109, 124, 139		3,23	6,52	6,33	6,18	4,09	3,03	–	9,09	2,29	9,13	7,88	1,02	2,84	4,04	1,05
18	4*R*,4a*R*,7*R*,7a*S*-dihydronepetalactone[5]	1417	67, 81, 95, 110, 113, 153, 168		4,92	9,03	7,87	7,72	3,80	15,35	6,53	11,11	11,29	0,83	14,96	5,05	7,23	18,98	15,38
19	(+)-iridomyrmecin	1450	67, 81, 95, 109		2,42	4,39	3,60	3,14	1,46	2,85	0,52	2,48	2,48	0,30	4,18	1,28	1,39	4,78	3,02
20	unknown iridoid	1458	67, 81, 95, 110, 113, 126, 153, 168		12,57	24,19	22,60	20,15	8,92	41,50	13,52	31,54	30,72	1,69	41,98	15,49	19,01	50,55	44,86
21	n-hexadecanoic acid	1961	57, 60, 73, 129, 213, 256		3,95	7,46	–	–	–	–	–	–	–	–	0,73	–	–	0,93	–
22	4-methyl tetracosane	2461	71, 309, 337 (M-15), 352 (M+)		1,15	2,16	0,12	0,74	0,54	2,69	–	–	–	–	0,21	–	–	0,53	–
23	4-methyl hexacosane	2661	337, 365 (M-15), 380 (M+)		2,58	4,99	–	2,98	1,86	3,37	–	1,42	1,64	–	0,70	0,65	1,68	1,12	1,29
24	7,x,x-trimethyl heptacosane	2785	113, 253, 337		–	–	–	0,96	–	1,18	–	–	–	–	1,10	3,48	1,12	3,69	2,79
25	x,x-nonacosadiene	2837	96, 404 (M+)		2,89	4,05	–	2,24	2,72	1,17	–	–	1,67	–	0,80	1,30	1,55	1,16	3,51
26	4-methyl octacosane	2861	71, 365, 393 (M-15), 408 (M+)		9,32	21,08	1,62	17,56	14,69	8,41	1,82	6,27	6,44	5,64	7,29	4,71	7,48	5,34	6,28
27	9-nonacosene	2877	97, 253, 281, 406 (M+)	173, 327, 500 (M+)	2,58	7,45	0,87	2,57	7,48	9,11	3,69	4,12	13,22	7,26	9,48	4,99	10,61	10,63	25,32
28	7-nonacosene	2884	97, 406 (M+)	145, 355	2,03	6,34	0,51	2,71	6,96	7,61	2,93	4,17	8,58	6,12	7,93	3,21	7,55	7,26	15,82
29	unknown CHC	2898	253, 341, 408 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
30	13-methyl nonacosane; 15-methyl nonacosane	2927	196/7, 252/3; 224/5		3,33	6,83	0,24	2,64	3,63	1,36	–	4,90	1,53	1,12	1,15	0,79	1,58	1,41	2,44
31	4-methyl nonacosane	2959	378/9, 407 (M-15), 422 (M+)		2,11	5,01	0,42	4,09	3,76	2,45	–	4,82	3,10	2,04	3,79	2,06	2,48	2,76	3,16
32	5,11-dimethyl nonacosane; 5,17-dimethyl nonacosane	2977	85, 183, 281, 379, 421 (M-15); 85, 197, 267, 379, 421 (M-15)		2,69	4,27	0,45	2,48	4,73	–	–	2,27	–	–	–	–	–	3,15	6,40
33	14-methyl triacontane; 15-methyl triacontane	3028	211, 253, 421 (M-15), 434 (M+); 225, 239, 421 (M-15), 434 (M+)		1,01	3,99	–	0,89	1,35	–	–	–	–	–	–	–	0,64	–	–

Supplementary Table 1, 2/4 Quantitative Analysis of compounds produced by all male and female *Leptopilina pacifica* wasps. Each wasp was extracted for 10 min in 20 µl of a 20 ng µl^{-1} methyl undecanoate standard. We used three calibration curves to quantify the compounds: one using (+)-iridomyrmecin for all iridoid compounds, one using 9-tricosene for unsaturated CHCs and one using tricosane for saturated CHCs. Unidentified compounds were also quantified using the most likely curve. For integrated peak areas see Supplementary Table 2.

					Mean amount (ng) per female														
No.	**Compound**	**KRI**	**Diagn. Ions**	**Diagn. Ions DMDS**	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
34	x,x-hentriacontadiene	3048	96, 432 (M+)		4,09	11,58	1,13	–	6,32	9,44	2,06	6,97	8,19	12,58	3,47	1,73	7,82	6,55	27,02
35	x,x-hentriacontadiene	3051	96, 432 (M+)		3,71	12,75	0,56	–	–	–	–	–	7,06	–	3,45	1,90	5,37	4,81	19,23
36	4-methyl triacontane	3060	393, 421 (M-15), 436 (M+)		140,52	220,20	36,54	176,95	223,41	129,56	20,35	200,23	194,36	163,55	202,25	133,19	173,25	152,02	191,96
37	9-hentriacontene	3078	97, 434 (M+)	173, 355, 528 (M+)	63,25	126,05	16,52	34,93	113,81	99,37	23,38	82,11	149,44	98,03	109,55	59,41	116,19	109,87	271,17
38	7-hentriacontene	3085	97, 434 (M+)	145, 383, 528 (M+)	27,89	0,00	8,32	16,78	75,21	44,25	10,82	46,05	88,11	47,54	57,54	33,08	55,20	50,50	133,72
39	putative cholesterol	3097	255, 275, 386		–	2,76	–	–	–	–	–	–	–	5,50	–	4,36	3,69	–	–
40	13-methyl hentriacontane; 15-methyl hentriacontane	3126	196/7, 280/1; 224/5, 252/3		13,99	23,22	0,68	17,02	15,20	2,19	–	7,17	5,48	3,92	4,17	2,25	6,57	4,38	4,94
41	unknown compound[3,4]	3137	275, 301, 386, 353, 368		6,59	11,40	0,35	6,59	7,93	3,98	–	4,62	4,36	3,43	4,72	3,60	6,56	6,01	9,51
42	4-methyl hentriacontane	3158	281, 386, 407, 435 (M-15), 450		0,67	2,06	–	0,63	1,03	–	–	–	–	–	0,62	0,31	–	0,88	–
43	x-methyl dotriacontane	3176	85, 253, 281, 351, 407, 449 (M-15)		1,35	3,97	–	1,65	2,63	–	–	–	1,14	–	0,93	0,44	1,00	1,24	1,79
44	14-methyl dotriacontane; 15-methyl dotriacontane	3232	211, 281, 449 (M-15), 464 (M+); 225, 267, 449 (M-15), 464 (M+)		14,43	24,30	0,95	16,36	14,40	–	–	1,86	1,97	1,55	1,18	1,15	1,64	1,78	0,00
45	7,17-tritriacontadiene	3249	96, 460 (M+)	145, 271, 283, 409, 455, 507 (M-141), 554 (M-94), 648 (M+)	29,38	62,55	4,46	17,82	48,09	25,44	3,57	23,46	50,09	41,10	22,97	15,23	44,69	41,40	99,65
46	4-methyl dotriacontane	3258	449 (M-15), 421		22,59	46,10	1,93	22,86	37,09	14,67	2,45	18,70	23,98	16,80	18,09	12,38	24,33	24,67	35,85
47	x-tritriacontene	3278	97, 462 (M+)		7,49	34,58	–	3,71	25,11	13,86	2,56	–	29,50	26,51	19,70	9,33	15,20	22,03	35,73
48	x-tritriacontene	3286	97, 462 (M+)		–	–	–	–	–	–	–	–	–	–	4,73	2,66	3,24	3,89	5,80
49	x-tetratriacontene	3319	97, 476 (M+)		–	6,30	–	4,12	3,44	–	–	9,42	–	–	–	–	–	–	–
50	13-methyl tritriacontane; 15-methyl tritriacontane; 17-methyl tritriacontane	3325	197, 309; 225, 281; 253		13,65	23,88	1,14	16,41	13,63	–	–	3,49	2,51	2,01	2,34	1,13	2,03	2,32	1,36
51	9,19-pentatriacontadiene	3449	96, 488 (M+)	173, 271, 311, 409, 535 (M-141), 676 (M+)	12,10	30,22	–	14,85	41,22	24,17	3,07	27,46	23,74	9,77	29,24	17,36	32,52	33,67	2,02
52	x,x-pentatriacontadiene	3450	96, 488 (M+)		–	–	–	–	–	–	–	–	35,07	23,82	–	–	–	–	61,25
			Sum		824,57	1526,60	569,75	1068,62	921,81	740,44	186,13	842,14	993,49	527,57	1093,10	497,64	767,57	1087,77	1417,48

N=15 males and females of *L. pacifica* singly extracted in 20 µl of DCM with 20 ng µl^{-1} methyl undecanoate as internal standard. KRI = Kovats retention index on a non-polar DB-5 GC column. Diagn. Ions = diagnostic ions used in the identification of the compound. Diagn. Ions DMDS = diagnostic ions of unsaturated compounds after derivatisation with DMDS. [1]Compound also found in *L. boulardi* (Weiss et al. 2015a); [2]Unknown absolute configuration; [3]Compound also found in *L. japonica* (Böttinger et al. 2019); [4]Compound also found in *L. ryukyuensis* (Böttinger et al. 2019); [5]Compound also found in *Alloxysta victrix* (Zimmermann et al. 2012). x = The position of methyl branch(es) and/or double bond(s) could not be determined. Numbers of compounds correspond to Fig. 2.

Supplementary Table 1, 3/4 Quantitative Analysis of compounds produced by all male and female *Leptopilina pacifica* wasps. Each wasp was extracted for 10 min in 20 µl of a 20 ng µl^{-1} methyl undecanoate standard. We used three calibration curves to quantify the compounds: one using (+)-iridomyrmecin for all iridoid compounds, one using 9-tricosene for unsaturated CHCs and one using tricosane for saturated CHCs. Unidentified compounds were also quantified using the most likely curve. For integrated peak areas see Supplementary Table 2.

					Mean amount (ng) per male														
No.	**Compound**	**KRI**	**Diagn. Ions**	**Diagn. Ions DMDS**	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
1	(*Z*)-3,7-dimethyl-2,6-octadienal (neral)	1247	69, 84, 94/5, 109		29,03	19,41	23,12	18,27	22,95	19,61	25,58	8,55	4,25	15,95	11,62	14,03	18,85	15,53	20,44
2	unknown compound	1258	69, 84, 94, 109, 122/3		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	unknown compound	1265	69, 95, 109, 122/3		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	(*E*)-3,7-dimethyl-2,6-octadienal (geranial)	1276	69, 84, 94, 109, 123, 137, 152		49,50	31,32	39,06	31,03	39,90	31,99	50,74	15,30	7,79	26,20	25,09	22,41	27,33	27,44	32,71
5	iridodial	1293	67, 84, 86, 111, 135		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	iridodial 1	1303	67, 81, 109, 111, 135		1,07	1,74	1,53	0,85	1,14	1,81	1,73	–	0,78	1,81	0,77	–	–	–	0,47
7	iridodial 2	1306	67, 81, 109, 111, 135		2,89	4,46	2,91	0,80	4,03	3,47	2,57	–	0,86	1,00	1,53	–	–	–	0,79
8	unidentified iridoid	1326	69, 83/84, 97/98, 135		1,80	1,58	2,22	1,44	2,24	2,00	2,16	1,38	1,03	1,90	2,49	2,25	1,50	0,75	1,50
9	unidentified iridoid	1333	69,81, 84, 107, 109, 111, 135		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	actinidine; in females coeluting with unidentified putative iridoid[1]	1341	117, 132, 147; 69, 95, 108/9, 137		28,48	16,31	12,84	12,33	22,59	12,62	23,52	2,21	1,84	5,08	–	4,81	4,21	2,20	4,81
11	unknown putative iridoid[1]	1349	69, 83, 93, 108, 137		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	nepetalactone 1[2]	1363	81, 95, 109, 123, 166		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	nepetalactone 2[2]	1372	69, 81, 95, 109, 123, 166		47,56	33,33	36,27	29,95	45,81	26,87	44,16	13,73	5,65	21,14	18,97	19,17	24,88	19,50	25,18
14	unknown compound	1381	67, 81, 84, 111, 135		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15	unknown compound[3,4]	1391	81, 84, 109, 111, 135, 153		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	unidentified iridoid[3]	1407	84, 109, 111, 166		1,68	1,20	0,99	0,97	1,67	1,22	1,44	–	–	0,96	1,50	–	–	–	0,74
17	unknown compound	1411	97, 109, 124, 139		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	4*R*,4a*R*,7*R*,7a*S*-dihydronepetalactone[5]	1417	67, 81, 95, 110, 113, 153, 168		3,13	2,07	2,56	2,34	3,11	2,53	3,68	1,06	0,75	1,92	1,91	1,78	1,79	1,75	2,82
19	(+)-iridomyrmecin	1450	67, 81, 95, 109		2,06	1,51	1,81	1,67	1,84	1,21	1,91	0,83	0,43	1,05	1,64	1,49	1,42	1,80	1,64
20	unknown iridoid	1458	67, 81, 95, 110, 113, 126, 153, 168		5,40	2,97	4,43	5,64	4,39	2,51	5,45	3,25	1,61	4,03	4,18	6,08	6,70	5,09	6,88
21	n-hexadecanoic acid	1961	57, 60, 73, 129, 213, 256		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22	4-methyl tetracosane	2461	71, 309, 337 (M-15), 352 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23	4-methyl hexacosane	2661	337, 365 (M-15), 380 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	7,x,x-trimethyl heptacosane	2785	113, 253, 337		–	–	1,32	1,44	1,91	3,77	3,12	–	–	2,62	2,10	4,59	7,69	3,48	2,93
25	x,x-nonacosadiene	2837	96, 404 (M+)		1,00	–	1,12	–	–	–	2,45	–	2,80	–	–	–	2,59	2,47	–
26	4-methyl octacosane	2861	71, 365, 393 (M-15), 408 (M+)		5,58	16,33	6,75	9,79	12,46	21,20	14,83	2,94	6,02	6,81	2,69	9,59	5,93	7,50	8,28
27	9-nonacosene	2877	97, 253, 281, 406 (M+)	173, 327, 500 (M+)	5,17	3,47	2,10	2,19	3,81	8,62	7,83	7,97	10,83	9,29	5,26	19,18	17,41	18,52	10,49
28	7-nonacosene	2884	97, 406 (M+)	145, 355	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
29	unknown CHC	2898	253, 341, 408 (M+)		0,48	–	–	–	–	–	–	–	–	2,41	–	1,62	1,36	1,34	1,25
30	13-methyl nonacosane; 15-methyl nonacosane	2927	196/7, 252/3; 224/5		–	1,03	0,58	0,76	0,00	3,26	1,06	–	–	–	–	0,64	–	0,63	0,90
31	4-methyl nonacosane	2959	378/9, 407 (M-15), 422 (M+)		2,24	3,29	2,24	3,71	3,27	5,01	3,05	1,78	2,30	3,66	1,40	3,93	2,95	4,15	3,21
32	5,11-dimethyl nonacosane; 5,17-dimethyl nonacosane	2977	85, 183, 281, 379, 421 (M-15); 85, 197, 267, 379, 421 (M-15)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33	14-methyl triacontane; 15-methyl triacontane	3028	211, 253, 421 (M-15), 434 (M+); 225, 239, 421 (M-15), 434 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Supplementary Table 1, 4/4 Quantitative Analysis of compounds produced by all male and female *Leptopilina pacifica* wasps. Each wasp was extracted for 10 min in 20 µl of a 20 ng µl^{-1} methyl undecanoate standard. We used three calibration curves to quantify the compounds: one using (+)-iridomyrmecin for all iridoid compounds, one using 9-tricosene for unsaturated CHCs and one using tricosane for saturated CHCs. Unidentified compounds were also quantified using the most likely curve. For integrated peak areas see Supplementary Table 2.

					Mean amount (ng) per male														
No.	**Compound**	**KRI**	**Diagn. Ions**	**Diagn. Ions DMDS**	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
34	x,x-hentriacontadiene	3048	96, 432 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
35	x,x-hentriacontadiene	3051	96, 432 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
36	4-methyl triacontane	3060	393, 421 (M-15), 436 (M+)		141,25	185,42	184,39	210,16	182,92	256,09	202,40	156,44	174,36	214,52	104,87	227,98	178,67	196,48	179,90
37	9-hentriacontene	3078	97, 434 (M+)	173, 355, 528 (M+)	45,75	40,38	28,25	35,50	39,19	63,57	53,79	56,07	61,80	48,92	41,08	68,08	69,32	86,01	51,76
38	7-hentriacontene	3085	97, 434 (M+)	145, 383, 528 (M+)	17,42	11,03	11,62	13,94	17,42	22,67	19,69	27,75	21,51	18,31	14,84	29,49	22,03	28,92	15,55
39	putative cholesterol	3097	255, 275, 386		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
40	13-methyl hentriacontane; 15-methyl hentriacontane	3126	196/7, 280/1; 224/5, 252/3		2,21	7,58	6,00	7,12	7,09	13,80	8,04	1,97	2,72	0,62	1,10	2,09	–	1,80	2,36
41	unknown compound[3,4]	3137	275, 301, 386, 353, 368		5,46	7,16	7,06	7,93	8,08	13,08	10,71	4,10	8,52	11,24	3,40	12,06	11,90	12,34	9,19
42	4-methyl hentriacontane	3158	281, 386, 407, 435 (M-15), 450		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
43	x-methyl dotriacontane	3176	85, 253, 281, 351, 407, 449 (M-15)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
44	14-methyl dotriacontane; 15-methyl dotriacontane	3232	211, 281, 449 (M-15), 464 (M+); 225, 267, 449 (M-15), 464 (M+)		1,29	3,24	2,10	3,79	2,58	9,72	2,98	–	–	–	4,84	–	–	–	–
45	7,17-tritriacontadiene	3249	96, 460 (M+)	145, 271, 283, 409, 455, 507 (M-141), 554 (M-94), 648 (M+)	15,56	20,04	10,60	14,29	17,16	34,47	24,97	13,29	20,53	12,99	–	28,14	21,30	30,32	22,81
46	4-methyl dotriacontane	3258	449 (M-15), 421		30,00	24,26	19,30	26,54	22,52	42,07	35,28	37,20	49,24	17,65	13,92	52,66	39,22	53,54	26,81
47	x-tritriacontene	3278	97, 462 (M+)		–	19,41	15,63	24,85	20,67	38,58	29,84	–	0,00	20,85	–	–	–	–	19,16
48	x-tritriacontene	3286	97, 462 (M+)		2,16	1,47	1,98	1,47	–	–	1,67	1,58	4,12	2,17	1,08	4,25	2,61	4,43	4,10
49	x-tetratriacontene	3319	97, 476 (M+)		2,56	9,36	6,29	7,22	8,05	16,46	7,94	–	–	–	–	1,00	–	1,30	2,16
50	13-methyl tritriacontane; 15-methyl tritriacontane; 17-methyl tritriacontane	3325	197, 309; 225, 281; 253		–	6,42	3,47	5,46	4,36	10,05	7,46	–	–	–	–	–	–	–	–
51	9,19-pentatriacontadiene	3449	96, 488 (M+)	173, 271, 311, 409, 535 (M-141), 676 (M+)	109,74	131,39	129,74	139,31	120,93	222,03	155,14	116,19	156,52	83,36	50,58	190,06	140,79	169,23	125,20
52	x,x-pentatriacontadiene	3450	96, 488 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			Sum		560,48	607,22	568,30	620,77	622,09	890,30	755,18	473,60	546,26	536,45	316,87	727,37	610,46	696,53	584,02

N=15 males and females of *L. pacifica* singly extracted in 20 µl of DCM with 20 ng µl^{-1} methyl undecanoate as internal standard. KRI = Kovats retention index on a non-polar DB-5 GC column. Diagn. Ions = diagnostic ions used in the identification of the compound. Diagn. Ions DMDS = diagnostic ions of unsaturated compounds after derivatisation with DMDS. [1]Compound also found in *L. boulardi* (Weiss et al. 2015a); [2]Unknown absolute configuration; [3]Compound also found in *L. japonica* (Böttinger et al. 2019); [4]Compound also found in *L. ryukyuensis* (Böttinger et al. 2019); [5]Compound also found in *Alloxysta victrix* (Zimmermann et al. 2012). x = The position of methyl branch(es) and/or double bond(s) could not be determined. Numbers of compounds correspond to Fig. 2.

Supplementary Table 2, 1/4 Integrated peak areas of compounds produced by male and female *Leptopilina pacifica* wasps. Corresponds to Supplementary Table 1

No.	Compound	KRI	Diagn. Ions	Diagn. Ions DMDS	Integrated peak area per female														
					01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
1	(*Z*)-3,7-dimethyl-2,6-octadienal (neral)	1247	69, 84, 94/5, 109		26385094	43016372	8203480	36915912	14998793	22301603	1567405	20856773	20926358	–	29816721	10449241	16149817	58670825	33413197
2	unknown compound	1258	69, 84, 94, 109, 122/3		8609143	15173253	4566116	23048441	6370540	5979865	563824	3103491	7058697	–	6505378	4432635	3588564	12325223	6222656
3	unknown compound	1265	69, 95, 109, 122/3		1713017	3024359	1089445	6241387	3770204	3754464	262053	762768	2228021	–	1739443	1701447	2464636	7762904	3139762
4	(*E*)-3,7-dimethyl-2,6-octadienal (geranial)	1276	69, 84, 94, 109, 123, 137, 152		39186858	67991815	19203330	66527680	20548575	35434489	2179138	21234638	31053745	4075004	56189261	15702775	23626263	82986816	42706528
5	iridodial	1293	67, 84, 86, 111, 135		1768231	3315480	1372968	2802060	886864	1907979	152919	1221092	1607282	1299045	1998989	960521	836165	–	973842
6	iridodial 1	1303	67, 81, 109, 111, 135		133972480	375785619	57775013	196950070	71495299	71460939	2924588	75080308	83050673	16001479	193781149	44767375	64655697	226056858	102497710
7	iridodial 2	1306	67, 81, 109, 111, 135		61528198	–	36581740	105943716	46632914	67383903	3622694	46697021	60097414	10118911	82422876	35610996	40681897	142287704	66696885
8	unidentified iridoid	1326	69, 83/84, 97/98, 135		9764675	20240263	5164477	17288042	8746333	9199810	2131290	3992159	5616938	3780845	10324591	6164460	5243442	10154044	6933601
9	unidentified iridoid	1333	69,81, 84, 107, 109, 111, 135		4006479	3970324	–	6875262	1018614	–	–	–	–	–	3622665	491858	–	–	–
10	actinidine; in females coeluting with unidentified putative iridoid[1]	1341	117, 132, 147; 69, 95, 108/9, 137		131721462	279912126	73475619	241700339	88114933	93239740	5039958	60531507	80367687	4091403	169259044	53117556	63848685	190755621	107035351
11	unknown putative iridoid[1]	1349	69, 83, 93, 108, 137		101436930	248929491	50830101	166857061	63590096	63911105	3455393	40465673	45361379	4170951	134310509	33105184	41749131	172929270	65470409
12	nepetalactone 1[2]	1363	81, 95, 109, 123, 166		5256203	12242032	3608378	14312936	5434331	12927659	679122	5673910	9741798	–	15627524	4103189	3896867	26725806	9236548
13	nepetalactone 2[2]	1372	69, 81, 95, 109, 123, 166		107997742	200945224	59013764	195544251	68168695	107228494	8640660	77854628	96458867	12687406	171160758	46751914	67474330	229177628	130872736
14	unknown compound	1381	67, 81, 84, 111, 135		2640242	5865929	2446565	4907808	2443078	–	756349	1208740	1914803	–	3795001	1783450	979062	5122958	1878382
15	unknown compound[3,4]	1391	81, 84, 109, 111, 135, 153		1243132	1847009	394893	2377318	–	–	–	–	–	–	2196383	1070765	711964	2771424	1472160
16	unidentified iridoid[3]	1407	84, 109, 111, 166		2059905	2275157	1207687	2031280	–	2894562	281852	1682939	2442133	–	6134452	1805266	1218548	6296210	4868233
17	unknown compound	1411	97, 109, 124, 139		5078425	10918882	4547678	10514954	7234497	5520212	–	9971868	3598310	13750983	13835707	1741717	4709506	9381358	1592119
18	4*R*,4a*R*,7*R*,7a*S*-dihydronepetalactone[5]	1417	67, 81, 95, 110, 113, 153, 168		7739118	15126699	5653494	13133574	6727991	27935407	2369731	12194847	17708889	1243263	26275431	8580925	11994902	44088897	23291792
19	methyl undecanoate IS	1427	74, 87		674027358	717140734	307416124	728406324	757616230	779188660	155434033	469812988	671726953	644895780	752166539	728027180	710591882	994585090	648611995
20	(+)-iridomyrmecin	1450	67, 81, 95, 109		3805191	7359114	2585051	5343706	2587611	5189440	187782	2724294	3893463	447477	7341173	2176319	2313564	11112064	4578293
21	unknown iridoid	1458	67, 81, 95, 110, 113, 126, 153, 168		19794104	40512938	16224442	34275247	15790401	75522818	4906595	34604831	48195608	2542549	73756397	26338262	31544798	117417530	67958475
22	n-hexadecanoic acid	1961	57, 60, 73, 129, 213, 256		10251669	20597832	–	–	–	–	–	–	–	–	2102853	–	–	3569578	–
23	4-methyl tetracosane	2461	71, 309, 337 (M-15), 352 (M+)		2976580	5976117	139584	2071074	1581892	8059208	–	–	–	–	605729	–	–	2042781	–
24	4-methyl hexacosane	2661	337, 365 (M-15), 380 (M+)		6700021	13780023	–	8362265	5438312	10112230	–	2573029	4248475	–	2035921	1820618	4594072	4275454	3220276
25	7,x,x-trimethyl heptacosane	2785	113, 253, 337		–	–	–	2681966	–	3533386	–	–	–	–	3184889	9751921	3055750	14138307	6973469
26	x,x-nonacosadiene	2837	96, 404 (M+)		5019034	7474576	–	4199712	5298817	2346156	–	–	2888584	–	1552341	2436227	2826473	2981639	5860333
27	4-methyl octacosane	2861	71, 365, 393 (M-15), 408 (M+)		24184268	58214419	1911785	49237877	42842092	25223649	1089009	11342159	16665915	14002583	21098148	13191613	20465081	20456934	15695731
28	9-nonacosene	2877	97, 253, 281, 406 (M+)	173, 327, 500 (M+)	4482784	13756272	684878	4816220	14581428	18261567	1477125	4978129	22857662	12056602	18361504	9355222	19404410	27212692	42274215
29	7-nonacosene	2884	97, 406 (M+)	145, 355	3520942	11698454	403734	5073647	13571000	15260394	1170365	5039507	14834829	10162687	15348885	6020379	13815025	18590218	26404089
30	unknown CHC	2898	253, 341, 408 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
31	13-methyl nonacosane; 15-methyl nonacosane	2927	196/7, 252/3; 224/5		8646177	18864567	285732	7398120	10574933	4078047	–	8861341	3969808	2792975	3344763	2204707	4320992	5390376	6104635
32	4-methyl nonacosane	2959	378/9, 407 (M-15), 422 (M+)		5483485	13819986	495151	11480848	10962633	7350066	–	8717633	8009748	5060592	10978488	5786409	6782272	10576100	7893739
33	5,11-dimethyl nonacosane; 5,17-dimethyl nonacosane	2977	85, 183, 281, 379, 421 (M-15); 85, 197, 267, 379, 421 (M-15)		6983755	11782962	530160	6951905	13785744	–	–	4101235	–	–	–	–	–	12065198	15995377
34	14-methyl triacontane; 15-methyl triacontane	3028	211, 253, 421 (M-15), 434 (M+); 225, 239, 421 (M-15), 434 (M+)		2614073	11017557	–	2499187	3937380	–	–	–	–	–	–	–	1748657	–	–
35	x,x-hentriacontadiene	3048	96, 432 (M+)		7094284	21376970	891288	–	12326232	18934116	825778	8433670	14159182	20889388	6711578	3244709	14305202	16756246	45113481
36	x,x-hentriacontadiene	3051	96, 432 (M+)		6441787	23530228	440200	–	–	–	–	–	12201747	–	6674731	3553280	9814774	12305886	32100430
37	4-methyl triacontane	3060	393, 421 (M-15), 436 (M+)		364667485	608007804	43254923	496264731	651689508	388706128	12176054	362206422	502682350	406108458	585718662	373335314	474003592	582163184	479399799
38	9-hentriacontene	3078	97, 434 (M+)	173, 355, 528 (M+)	109724131	232671229	13068264	65489406	221927181	199294848	9355368	99285882	258380350	162714781	212085856	111320713	212508419	281257330	452710608
39	7-hentriacontene	3085	97, 434 (M+)	145, 383, 528 (M+)	48389955	–	6583137	31459897	146653727	88737678	4329011	55687363	152331087	78909122	111392584	61993175	100955349	129287143	223244019
40	putative cholesterol	3097	255, 275, 386		–	7608297	–	–	–	–	–	–	–	13651286	–	12234283	10085691	–	–

Supplementary Table 2, 2/4 Integrated peak areas of compounds produced by male and female *Leptopilina pacifica* wasps. Corresponds to Supplementary Table 1

No.	Compound	KRI	Diagn. Ions	Diagn. Ions DMDS	Integrated peak area per female														
					01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
41	13-methyl hentriacontane; 15-methyl hentriacontane	3126	196/7, 280/1; 224/5, 252/3		36307923	64115229	804065	47740426	44331251	6566847	–	12966778	14172068	9729293	12076149	6307198	17969530	16760521	12324515
42	unknown compound[3,4]	3137	275, 301, 386, 353, 368		17108498	31471653	416312	18475775	23132623	11941798	–	8356640	11282822	8506544	13666662	10102249	17946452	23005107	23753151
43	4-methyl hentriacontane	3158	281, 386, 407, 435 (M-15), 450		1735192	5694684	–	1762173	2997556	–	–	–	–	–	1792392	861471	–	3381340	–
44	x-methyl dotriacontane	3176	85, 253, 281, 351, 407, 449 (M-15)		3493755	10965283	–	4634204	7668298	–	–	–	2945929	–	2695234	1236777	2733565	4740887	4469347
45	14-methyl dotriacontane; 15-methyl dotriacontane	3232	211, 281, 449 (M-15), 464 (M+); 225, 267, 449 (M-15), 464 (M+)		37456208	67107446	1126535	45895496	42012743	–	–	3358190	5100284	3851251	3418737	3222877	4489515	6813296	–
46	7,17-tritriacontadiene	3249	96, 460 (M+)	145, 271, 283, 409, 455, 507 (M-141), 554 (M-94), 648 (M+)	50977781	115453407	3531167	33405454	93778323	51030211	1426414	28369949	86610214	68226327	44462471	28546102	81740499	105971285	166364618
47	4-methyl dotriacontane	3258	449 (M-15), 421		58615020	127281909	2279703	64119825	108187927	44001232	1468548	33822873	62025154	41726002	52402214	34699846	66565153	94490144	89541698
48	x-tritriacontene	3278	97, 462 (M+)		12988617	63820307	–	6957220	48967755	27796229	1025477	–	50995091	44000541	38144058	17476745	27798704	56406670	59642194
49	x-tritriacontene	3286	97, 462 (M+)		–	–	–	–	–	–	–	–	–	–	9150736	4984301	5919035	9945700	9688506
50	x-tetratriacontene	3319	97, 476 (M+)		–	11637852	–	7721287	6717673	–	–	11394238	–	–	–	–	–	–	–
51	13-methyl tritriacontane; 15-methyl tritriacontane; 17-methyl tritriacontane	3325	197, 309; 225, 281; 253		35433326	65941846	1349332	46034645	39765651	–	–	6315293	6503032	4991087	6788813	3175855	5559334	8890987	3397584
52	9,19-pentatriacontadiene	3449	96, 488 (M+)	173, 271, 311, 409, 535 (M-141), 676 (M+)	20987269	55784817	–	27839968	80374862	48464619	1230085	33208237	41049504	16213078	56599243	32536155	59484256	86205058	3371419
53	x,x-pentatriacontadiene	3450	96, 488 (M+)		–	–	–	–	–	–	–	–	60640255	39546108	–	–	–	–	102259227

N=15 males and females of *L. pacifica* singly extracted in 20 µl of DCM with 20 ng µl^{-1} methyl undecanoate as internal standard. KRI = Kovats retention index on a non-polar DB-5 GC column. Diagn. Ions = diagnostic ions used in the identification of the compound. Diagn. Ions DMDS = diagnostic ions of unsaturated compounds after derivatisation with DMDS. IS = internal standard. [1]Compound also found in *L. boulardi* (Weiss et al. 2015a); [2]Unknown absolute configuration; [3]Compound also found in *L. japonica* (Böttinger et al. 2019); [4]Compound also found in *L. ryukyuensis* (Böttinger et al. 2019); [5]Compound also found in *Alloxysta victrix* (Zimmermann et al. 2012). x = The position of methyl branch(es) and/or double bond(s) could not be determined. Numbers of compounds correspond to Fig. 2.

Supplementary Table 2, 3/4 Integrated peak areas of compounds produced by male and female *Leptopilina pacifica* wasps. Corresponds to Supplementary Table 1

No.	Compound	KRI	Diagn. Ions	Diagn. Ions DMDS	Integrated peak area per male														
					01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
1	(*Z*)-3,7-dimethyl-2,6-octadienal (neral)	1247	69, 84, 94/5, 109		35276875	31278125	36182554	32012512	42778538	36305817	42107662	13892243	8231834	35830284	12473906	34296699	29838614	19187032	46871433
2	unknown compound	1258	69, 84, 94, 109, 122/3		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	unknown compound	1265	69, 95, 109, 122/3		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	(*E*)-3,7-dimethyl-2,6-octadienal (geranial)	1276	69, 84, 94, 109, 123, 137, 152		60154237	50462027	61138209	54363951	74386429	59214703	83509776	24858783	15098753	58857176	26926497	54781131	43250418	33895876	75024311
5	iridodial	1293	67, 84, 86, 111, 135		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	iridodial 1	1303	67, 81, 109, 111, 135		1301873	2810061	2402424	1486692	2130613	3356550	2852266	–	1502191	4061879	825647	–	–	–	1070113
7	iridodial 2	1306	67, 81, 109, 111, 135		3510411	7185506	4560286	1410295	7506382	6418068	4223668	–	1663550	2251641	1645912	–	–	–	1811243
8	unidentified iridoid	1326	69, 83/84, 97/98, 135		2185584	2545899	3471321	2525840	4175219	3702063	3552572	2245130	2004610	4261853	2677175	5487594	2366657	928246	3434113
9	unidentified iridoid	1333	69,81, 84, 107, 109, 111, 135		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	actinidine; in females coeluting with unidentified putative iridoid[1]	1341	117, 132, 147; 69, 95, 108/9, 137		34610307	26277713	20100070	21605527	42113309	23349926	38709009	3586557	3556498	11402247	–	11756345	6656345	2722000	11024321
11	unknown putative iridoid[1]	1349	69, 83, 93, 108, 137		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	nepetalactone 1[2]	1363	81, 95, 109, 123, 166		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	nepetalactone 2[2]	1372	69, 81, 95, 109, 123, 166		57797650	53697940	56761175	52465424	85413512	49743609	72677206	22310751	10954336	47490530	20362476	46858498	39373596	24090898	57751257
14	unknown compound	1381	67, 81, 84, 111, 135		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15	unknown compound[3,4]	1391	81, 84, 109, 111, 135, 153		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	unidentified iridoid[3]	1407	84, 109, 111, 166		2037805	1940916	1548648	1707794	3117064	2252065	2370959	–	–	2162964	1609103	–	–	–	1693982
17	unknown compound	1411	97, 109, 124, 139		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	4*R*,4a*R*,7*R*,7a*S*-dihydronepetalactone[5]	1417	67, 81, 95, 110, 113, 153, 168		3807641	3333734	4011088	4091412	5806983	4676300	6058836	1728193	1461917	4303073	2052134	4356536	2839821	2159502	6460811
19	methyl undecanoate IS	1427	74, 87		520327991	689854271	670118654	750134300	798255223	792499247	704672722	695552804	829754597	961931721	459549333	1,047E+09	677612106	528905876	9,82E+08
20	(+)-iridomyrmecin	1450	67, 81, 95, 109		2504215	2432540	2834847	2933600	3428455	2236988	3137612	1351164	839334	2369842	1756731	3638348	2250575	2226116	3750308
21	unknown iridoid	1458	67, 81, 95, 110, 113, 126, 153, 168		6564901	4786458	6935552	9883366	8189943	4636866	8971309	5286039	3115494	9060411	4487744	14871446	10607701	6290867	15787079
22	n-hexadecanoic acid	1961	57, 60, 73, 129, 213, 256		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23	4-methyl tetracosane	2461	71, 309, 337 (M-15), 352 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	4-methyl hexacosane	2661	337, 365 (M-15), 380 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	7,x,x-trimethyl heptacosane	2785	113, 253, 337		–	–	3413715	4165962	5855796	11505520	8453826	–	–	9704180	3719862	18491476	20053288	7086359	11066497
26	x,x-nonacosadiene	2837	96, 404 (M+)		1340311	–	1930948	–	–	–	4451407	–	5976439	–	–	–	4521379	3366410	–
27	4-methyl octacosane	2861	71, 365, 393 (M-15), 408 (M+)		11186334	43383724	17404063	28282009	38290065	64696798	40224088	7866335	19232513	25238649	4758409	38654984	15482022	15280939	31303663
28	9-nonacosene	2877	97, 253, 281, 406 (M+)	173, 327, 500 (M+)	6919841	6157385	3613699	4228153	7829418	17590200	14203075	14261966	23139233	23001536	6227017	51661316	30371280	25207404	26518836
29	7-nonacosene	2884	97, 406 (M+)	145, 355	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
30	unknown CHC	2898	253, 341, 408 (M+)		957039	–	–	–	–	–	–	–	–	8916033	–	6509801	3541732	2728699	4719134
31	13-methyl nonacosane; 15-methyl nonacosane	2927	196/7, 252/3; 224/5		–	2742672	1495407	2196112	–	9937666	2869085	–	–	–	–	2561085	–	1276809	3396063
32	4-methyl nonacosane	2959	378/9, 407 (M-15), 422 (M+)		4478374	8750508	5788125	10724055	10052014	15297900	8280909	4770056	7344852	13554939	2471140	15831282	7701498	8443503	12129443
33	5,11-dimethyl nonacosane; 5,17-dimethyl nonacosane	2977	85, 183, 281, 379, 421 (M-15); 85, 197, 267, 379, 421 (M-15)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
34	14-methyl triacontane; 15-methyl triacontane	3028	211, 253, 421 (M-15), 434 (M+); 225, 239, 421 (M-15), 434 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
35	x,x-hentriacontadiene	3048	96, 432 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
36	x,x-hentriacontadiene	3051	96, 432 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
37	4-methyl triacontane	3060	393, 421 (M-15), 436 (M+)		282988850	492490713	475754127	606994001	562223335	781425458	549146384	418967938	557034306	794532707	185565011	918664470	466156977	400122293	6,8E+08
38	9-hentriacontene	3078	97, 434 (M+)	173, 355, 528 (M+)	61274757	71691595	48729952	68541707	80516767	129669504	97558929	100376990	131975264	121112917	48587924	183383903	120891791	117093231	1,31E+08
39	7-hentriacontene	3085	97, 434 (M+)	145, 383, 528 (M+)	23335504	19591029	20046323	26912868	35788063	46235819	35703711	49686865	45946769	45344011	17548111	79425156	38429150	39368310	39311620
40	putative cholesterol	3097	255, 275, 386		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Supplementary Table 2, 4/4 Integrated peak areas of compounds produced by male and female *Leptopilina pacifica* wasps. Corresponds to Supplementary Table 1

No.	Compound	KRI	Diagn. Ions	Diagn. Ions DMDS	Integrated peak area per male														
					01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
41	13-methyl hentriacontane; 15-methyl hentriacontane	3126	196/7, 280/1; 224/5, 252/3		4429972	20144752	15478810	20559888	21779308	42113331	21816921	5272757	8704891	2292617	1946618	8435325	–	3661505	8924217
42	unknown compound[3,4]	3137	275, 301, 386, 353, 368		10935407	19028629	18217352	22899379	24837785	39920981	29053574	10976675	27216725	41616489	6017317	48612145	31057582	25133644	34730764
43	4-methyl hentriacontane	3158	281, 386, 407, 435 (M-15), 450		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
44	x-methyl dotriacontane	3176	85, 253, 281, 351, 407, 449 (M-15)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
45	14-methyl dotriacontane; 15-methyl dotriacontane	3232	211, 281, 449 (M-15), 464 (M+); 225, 267, 449 (M-15), 464 (M+)		2592962	8613439	5430017	10935653	7922590	29669407	8085206	–	–	–	8567645	–	–	–	–
46	7,17-tritriacontadiene	3249	96, 460 (M+)	145, 271, 283, 409, 455, 507 (M-141), 554 (M-94), 648 (M+)	20843947	35590079	18278760	27598960	35254326	70305774	45282666	23787147	43847411	32149511	–	75811853	37154369	41273162	57659227
47	4-methyl dotriacontane	3258	449 (M-15), 421		60095004	64451251	49806376	76644405	69210216	128380360	95715312	99636393	157306574	65374015	24637118	212199235	102316453	109021262	1,01E+08
48	x-tritriacontene	3278	97, 462 (M+)		–	34469819	26951522	47984897	42478191	78701126	54120928	–	–	51624789	–	–	–	–	48441167
49	x-tritriacontene	3286	97, 462 (M+)		2886221	2618648	3423206	2830332	–	–	3027804	2824301	8809093	5375786	1277227	11445844	4548073	6035958	10350988
50	x-tetratriacontene	3319	97, 476 (M+)		3432495	16611272	10854771	13946407	16547520	33570539	14408198	–	–	–	–	2701815	–	1767886	5457152
51	13-methyl tritriacontane; 15-methyl tritriacontane; 17-methyl tritriacontane	3325	197, 309; 225, 281; 253		–	17058243	8940894	15764749	13407303	30666123	20250742	–	–	–	–	–	–	–	–
52	9,19-pentatriacontadiene	3449	96, 488 (M+)	173, 271, 311, 409, 535 (M-141), 676 (M+)	146974281	233298213	223778529	268972640	248455431	452888767	281383719	208005087	334269699	206381826	59823062	511959749	245554081	230381691	3,16E+08
53	x,x-pentatriacontadiene	3450	96, 488 (M+)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

N=15 males and females of *L. pacifica* singly extracted in 20 µl of DCM with 20 ng µl^{-1} methyl undecanoate as internal standard. KRI = Kovats retention index on a non-polar DB-5 GC column. Diagn. Ions = diagnostic ions used in the identification of the compound. Diagn. Ions DMDS = diagnostic ions of unsaturated compounds after derivatisation with DMDS. IS = internal standard. [1]Compound also found in *L. boulardi* (Weiss et al. 2015a); [2]Unknown absolute configuration; [3]Compound also found in *L. japonica* (Böttinger et al. 2019); [4]Compound also found in *L. ryukyuensis* (Böttinger et al. 2019); [5]Compound also found in *Alloxysta victrix* (Zimmermann et al. 2012). x = The position of methyl branch(es) and/or double bond(s) could not be determined. Numbers of compounds correspond to Fig. 2.

5.3 Publication 3

Dispersal from Natal Patch Correlates with the Volatility of Female Sex Pheromones in Parasitoid Wasps

Lea C. Böttinger, Johannes Stökl

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Dispersal From Natal Patch Correlates With the Volatility of Female Sex Pheromones in Parasitoid Wasps

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Chemical communication via pheromones is considered the oldest and most widespread form of communication in nature. However, the way that the enormous diversity of species-specific pheromones evolved is still of debate. One possible process driving pheromone evolution is the mate-finding and dispersal behavior, as long-distance mate-finding requires highly volatile compounds. In contrast, less volatile compounds might be sufficient attractants in species that search for mates within proximity. In the parasitoid wasp genus *Leptopilina*, the composition of species-specific sex pheromones ranges from highly volatile iridoid compounds through combinations of iridoids with low volatile cuticular hydrocarbons (CHCs) to only CHCs. To study the selective forces shaping the composition of sex pheromones in *Leptopilina*, we examined the dispersal behavior, i.e., the proportion of male and female wasps dispersing after emergence, in four species with known sex pheromone compositions. If males and females disperse immediately, long-range mate attraction might become necessary, favoring volatile iridoids over CHCs. If mating occurs directly on the host patch, short-range mate attraction by low volatile CHCs might suffice. Our analyses have revealed that the dispersal behavior of *Leptopilina* males and females after emergence does indeed differ between species with differently volatile sex pheromones. Specifically, males of species with iridoid sex pheromones start to disperse immediately before their females' emergence, whereas males of species with CHC sex pheromones delay dispersal until their conspecific females emerge. While the differences in female dispersal behavior turned out to be species-specific, differences in male dispersal correlated with the volatility of female-produced sex pheromones of each species. This study significantly contributes to our understanding of the evolution of sex pheromones by differences in dispersal behavior.

Keywords: sex pheromone, mating system, evolution, chemical communication, insects, *Leptopilina*, *Drosophila*

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INTRODUCTION

Semiochemicals that mediate a variety of intra- and interspecific communication such as the attraction and finding of mating partners, hosts, or prey, or the deterrence of enemies, are ubiquitous in nature and consequently show a remarkable diversity (Wyatt, 2014). The thousands of chemicals transmitting information between organisms belong

to various compound classes (see, e.g., El-Sayed, 2018 at http://www.pherobase.com, which lists >3500 semiochemical compounds), are used alone or in combination, and range from small, light, volatile molecules to large, non-volatile compounds with higher molecular weights. Thus, these chemical messengers have a variety of different physicochemical properties (e.g., solubility in water for sex pheromones in fish, volatility in airborne signals, Mollo et al., 2017), although the prerequisites of semiochemicals should be similar for each form and function of chemical communication. Ultimately, they have to be emitted and delivered at the right concentration in order to be perceived and to elicit a response.

Despite having similar functions, the effective range of pheromones and their detection is often markedly different. If signals merely have to be detected by organisms in close vicinity to each other, non- or semi-volatile compounds such as long-chained cuticular hydrocarbons (CHCs) can suffice as contact pheromones (Blomquist and Bagnères, 2010), whereas long-range pheromones need to be volatile, to vaporize rapidly, and to spread widely in order to inform conspecifics over greater distances (alarm pheromones: e.g., Fujiwara-Tsujii et al., 2006; sex pheromones: e.g., Allison and Cardé, 2016; aggregation pheromones: e.g., Gries et al., 2015). For example, sex pheromones can be expected to be volatile, if mates need to be attracted and/or found over long distances, whereas non-volatile compounds are sufficient to elicit courtship at close range once the mating partner has been found. Because of the necessity for species-specificity in mate-finding, the compounds used for sexual communication are extremely diverse and show large variations in composition, even among closely related species (Symonds and Elgar, 2008).

The evolution of pheromone diversity is still little understood. Up to date, studies concerning differences in pheromone compositions between closely related species have focused on phylogenetic comparisons and genetic analyses with explanations such as large, saltational shifts in pheromone evolution during speciation or differential gene regulation leading to changes between species in enzymatic pathways generating the pheromone (e.g., Lassance et al., 2010, see also Symonds and Elgar, 2008). Another explanation for the chemical composition of pheromones is provided by the precursor hypothesis, which states that compounds used for communication evolve from compounds previously fulfilling non-communicative purposes in the same species (Wyatt, 2010; Bradbury and Vehrencamp, 2011; Steiger et al., 2011; Stökl and Steiger, 2017). Examples are hormones that are excreted in urine and that evolved as sex pheromones in females of the goldfish and Atlantic salmon (Stacey and Sorensen, 2009), or defensive secretions that evolved as alarm pheromones (Löfqvist, 1976), but also as sex pheromones (Boppré, 1986; Ruther et al., 2001; Geiselhardt et al., 2008) or aggregation pheromones (Wheeler and Cardé, 2013). However, research is limited with regard to the degree to which the evolution of the impressive variety of sex pheromone compositions is driven by ecological or life-history traits and behavior. One important factor that has so far been neglected in explaining this diversity is the dispersal behavior of organisms during mate-finding, a characteristic that might be closely correlated with the volatility of pheromonal compounds. As the volatility of sex pheromones will restrict communication via these chemicals to a certain distance within which the compounds can be perceived, it consequently also restricts dispersal distances. Therefore, dispersal behavior might be a life history trait that has shaped pheromone composition and its volatility and physicochemical properties.

Ideally, this hypothesis can be tested by comparing the dispersal behavior of closely-related species with known sex pheromones of different volatilities. Such an example is found in the genus *Leptopilina*, which are parasitic wasps attacking *Drosophila* larvae (Carton et al., 1986) and which comprises several closely related species with sex pheromones of different volatilities (Weiss et al., 2013, 2015; Pfeiffer et al., 2018; Böttinger et al., 2019; Böttinger et al. submitted; unpublished). Although all *Leptopilina* species studied until now produce iridoid compounds and CHCs, an enormous variation in their sex pheromone compositions has been found. *Leptopilina heterotoma* (Weiss et al., 2013) and *Leptopilina japonica* (Böttinger et al., 2019) use solely their species-specific volatile iridoid compounds as their female sex pheromones, whereas combinations of iridoids and non-volatile CHCs (*Leptopilina boulardi*, Weiss et al., 2015) or only CHCs serve as sex pheromones in several species such as *Leptopilina clavipes* (Pfeiffer et al., 2018), *Leptopilina guineaensis* (unpublished), *Leptopilina pacifica* (Böttinger et al. submitted), *Leptopilina ryukyuensis* (Böttinger et al., 2019), and *Leptopilina victoriae* (Weiss et al., 2015). The CHCs used as sex pheromones in the latter species consist of methyl-branched alkanes and mono- or di-unsaturated alkenes with chain lengths ranging from 27 to 37 carbons (with the exception of some trace compounds; Weiss et al., 2015; Pfeiffer et al., 2018; Böttinger et al., 2019; Böttinger et al. submitted; unpublished) and are thus of low volatility. Consequently, *Leptopilina* sex pheromones consisting of iridoids can be perceived over long distances, whereas those consisting of CHCs can only be perceived in close vicinity of the releasing individual. Wasps of this genus are quasi-gregarious (van den Assem et al., 1980), as each of them develops solitarily in separate *Drosophila* larvae, which are however mostly clumped together. Therefore, male wasps searching for mates can either stay on their natal host patch to wait for conspecific females to emerge, as males generally emerge before females (Carton et al., 1986), or can disperse to mate with females from other host patches (Fauvergue et al., 1999). Accordingly, female wasps can disperse directly after emergence or mate on their host patch before they disperse to search for hosts (Hardy and Godfray, 1990). To find their mating partners after leaving their natal host patch, wasps could either follow the long-range pheromones of their prospective partner or use host-patch-derived volatiles as attractants, because host patches are probably optimal places for mate encounters. They can subsequently use short-range pheromones for mate recognition. We propose the following two hypotheses: If males and females of *Leptopilina* disperse unmated immediately after emergence, long-range mate attraction might become necessary, favoring sex pheromones containing volatile iridoids. If mating occurs directly on the host patch and dispersal is delayed,

short-range mate attraction by low volatile CHCs could be sufficient.

In this study, we wished to tackle these hypotheses by comparing the dispersal behaviors of male and female *Leptopilina* wasps of four closely related species with differently volatile sex pheromones (highly volatile iridoids: *L. heterotoma* and *L. japonica*, low volatile CHCs: *L. pacifica* and *L. ryukyuensis*). For *L. heterotoma* (volatile iridoid sex pheromone) and *L. boulardi* (iridoids with CHCs as female sex pheromone) it is known that males and females disperse actively by flight from their natal patches shortly after emergence at a similar rate over several meters (Fauvergue et al., 1999). In field releases marked female *L. heterotoma* wasps dispersed upwind and were caught foraging at baits up to 18 m away from their point of release (Papaj and Vet, 1990). However, nothing is known about dispersal behaviors of species with non-volatile sex pheromones, and about the way that dispersal can shape the evolution of sex pheromone diversity. We therefore tested (1) whether males and females of species that use highly volatile iridoids in the female sex-pheromone disperse earlier or at a higher rate than species that use low volatile CHCs as sex pheromones, (2) whether dispersal of males and females is delayed or occurs to a lesser extent in species that use very low volatile CHCs as sex pheromones and in which no long-range attraction is possible, (3) the odor sources to which the wasps are attracted, as they might explain the reason for the dispersal of wasps.

MATERIALS AND METHODS

Species

The wasp strain of *L. heterotoma* is a long established lab strain that was originally collected in Leiden, Netherlands. The strain of *L. japonica* (subspecies *L. j. japonica*, Novković et al., 2011) was collected in Tokyo, Japan in 2006, whereas *L. ryukyuensis* and *L. pacifica* were collected on Iriomote-jima, Japan in 2006 and 2011, respectively. The Japanese species were kindly provided by Prof. K. T. Kimura. Wasps of the species *L. heterotoma*, *L. japonica*, and *L. ryukyuensis* were reared on larvae of *Drosophila simulans*, whereas *L. pacifica* wasps were reared on *Drosophila virilis* larvae. Wasps and flies were kept in a climate- and light-controlled room (Zettner; Bayreuth, Germany) at 25°C and ∼60% humidity and under a 16:8 h L:D cycle. About 30 flies of both sexes were placed into a jar containing fresh corn-based diet as the host patch medium (for ingredients, see Böttinger et al., 2019) and removed after 48 h. Subsequently, about 10 mated females of either *L. heterotoma*, *L. japonica*, *L. pacifica*, or *L. ryukyuensis* were placed into the jar to parasitize their host fly larvae. To obtain unmated and naïve wasps of a defined sex and age for the experiments, parasitized pupae were singly isolated prior to eclosion into 1.5 ml microcentrifuge tubes. Filter papers (5 mm diameter) with a 50:50 honey water solution were added, so that individual wasps could feed *ad libitum*. Mated females were obtained by placing a 2- to 6-day-old male wasp in a microcentrifuge tube containing a freshly hatched female wasp and leaving them together for approximately 12 h.

Dispersal Experiment

In this experiment, we investigated the dispersal of wasps from their place of emergence. Dispersal is hereby defined as the leaving of the natal patch. Flies of *D. simulans* and *D. virilis* were allowed to lay eggs for 48 h on the host patch medium in small jars (70 × 40 mm height and diameter). Subsequently, 10 female wasps of either *L. heterotoma*, *L. japonica*, *L. pacifica*, or *L. ryukyuensis* were placed in the jar for 48 h to parasitize the *Drosophila* larvae. The jars were then transferred to transparent plastic boxes (size ca. 155 × 100 mm, 125 mm height). A micropipette tip (200 µl) was inserted into each jar lid through a hole (3 mm diameter) with the small tip end directed outwards. Previously, the small tip end had been cut and its opening diameter thus enlarged, so that the wasps once they emerged could climb out and exit the jars via these tips but could not return into the original jar. Each jar was checked three times daily (at 9 am, 3 pm, 9 pm), and the number and the sex of the wasps in the jar as well as outside the jar in the transparent box was recorded. Dispersed wasps were then removed from the transparent plastic box. The experiment was conducted 30 times per species and lasted 126 h after the emergence of the first wasp in each jar.

Attraction Olfactometer Experiment

To assess the odor sources that can mediate mate-finding, other than a long-range sex pheromone, attraction assays were performed with a Y tube olfactometer in which wasps were allowed to decide between two presented odors. The Y-tube was made from glass (15 mm inner diameter) and consisted of a base (60 mm length) and two arms (90 mm length, divided by a 45° angle). It was positioned at a 30° slope with the arms pointing upward and illuminated from above by two LED tubes (350 lm, 5 W). Each arm was connected via Teflon tubes to separate Erlenmeyer flasks (50 ml) containing artificial host patches (5 g *Drosophila* rearing substrate, on which 10 *D. simulans* females were allowed to oviposit for 48 h). Humidified air was pumped through the flasks into the Y-tube arms at an air flow rate of 30 ml min^{-1}. To test the attraction of males to the odor of females, 2- to 6-day-old male wasps were given the choice between the odor of a host patch and the odor of a host patch with 20 conspecific virgin female wasps (1–3 days old). In a follow-up experiment, instead of live females, 2 µl female extract [equivalent to 1/5th of a female; pooled extracts with 10 µl Dichloromethane (DCM) per female] was applied to a filter paper disk (5 mm diameter) and positioned into the end of one arm of the Y-tube. As a control, 2 µl DCM was applied to a filter paper disk and positioned in the other arm. Impregnated filter paper disks were left to evaporate for 30 s before being used for the experiment. Likewise, the attraction of females to males was tested by presenting two host patches in separate Erlenmeyer flasks, with one additionally containing 20 conspecific males (2–6 days old), to 1- to 3-day-old virgin females. To check whether males and females meet on host patches, their attraction to host patches with eggs and larvae of their host fly species was tested by presenting them with either the host patch or moist cotton pads. To differentiate whether female wasps disperse to find host patches before or after mating, the

attraction of virgin and mated females to the host patch odor was compared here. For each run of the experiments, one male or female wasp was carefully placed into the entrance of the base of the Y-tube olfactometer by using an aspirator. Subsequently, the wasp was allowed to choose between the control odor (host patch, host patch with DCM solvent, or moist cotton) and the treatment odor (host patch with 20 conspecific individuals of the opposite sex, host patch with extract of conspecific females, or host patch). The experiment was stopped after the wasps had either crossed a decision line, which was marked within each Y-tube arm at a distance of 2 cm beyond the branching point, or after 5 min (attraction to odor of mating partners) or 10 min (attraction to odor of host patches), if no choice occurred, and the respective decisions were recorded. After each run, the Y-tube was turned, and the control and treatment odor were swapped. The Y-tube was cleaned with ethanol and hot water following every second run. Each individual wasp was only used once ($N = 30$ for each species and treatment).

Statistics

To test for differences between the dispersal curves of female and male wasps of each species, we used the log-rank test as implemented in the R function *survdiff*.

To analyze differences in male dispersal depending on the presence of females, the proportion of males dispersing in each observation interval within 48 h before and 48 h after the emergence of the first female was calculated. We compared the mean proportions of dispersed males in these 48-h intervals per species using Wilcoxon signed rank tests (Bonferroni corrected for multiple comparisons). Furthermore, Wilcoxon signed rank tests (Bonferroni corrected) were used to analyze male dispersal differences dependent on the volatility of female sex pheromones, in comparing pooled data for species using CHCs (*L. pacifica*, *L. ryukyuensis*) and iridoids (*L. heterotoma*, *L. japonica*) as sex pheromones.

For the analysis of the dispersal of female wasps, the proportion of females that dispersed at every time point as well as the mean proportion of dispersed females within 24-h intervals (up to 96 h) after the emergence of the first female was calculated for each jar. The differences in female dispersal between all species were examined in each 24-h interval with Kruskal–Wallis rank sum tests. As *post hoc* tests we used pairwise Wilcoxon signed rank tests (Bonferroni corrected). Additionally, we analyzed the differences in the dispersal quotients of female wasps of species with differently volatile sex pheromones (iridoids: *L. heterotoma* and *L. japonica*, CHCs: *L. pacifica* and *L. ryukyuensis*) by using Wilcoxon signed rank tests (Bonferroni corrected).

The decisions of wasps in the Y-tube olfactometer were analyzed using a two-sided binomial test. For each experiment, $N = 30$. All statistics were carried out in R Version 3.3.0.

RESULTS

The first male wasps of *L. pacifica* emerged on average 34.0 ± 12.6 h (mean ± SD) before their conspecific females in the same jars, whereas males of *L. japonica* emerged 39.4 ± 18.1 h (mean ± SD), those of *L. ryukyuensis* 42.0 ± 13.9 h (mean ± SD), and *L. heterotoma* 46.2 ± 17.1 h (mean ± SD) on average before their females.

In total, 57.7% of the males and 57.9% of the females of *L. heterotoma* dispersed, whereas 76.6% of the males and 39.0% of the females of *L. japonica*, 46.9% of the males and 38.0% of the females of *L. ryukyuensis*, and 63.2% of the males and 19.7% of the females of *L. pacifica* dispersed (**Figure 1**). Dispersal differed significantly between male and female wasps in all species (**Figure 1**; Log-rank curve comparison tests between female and male wasps in *L. heterotoma*: $\chi^2(1) = 4.3$, $P = 0.040$; *L. japonica*: $\chi^2(1) = 344$, $P < 0.001$; *L. ryukyuensis*: $\chi^2(1) = 31.6$, $P < 0.001$; *L. pacifica*: $\chi^2(1) = 411$, $P < 0.001$). Males of all species started to disperse before the emergence of their conspecific females (**Figure 1**) and dispersed more quickly than their females. However, whereas males of the species *L. heterotoma* and *L. japonica* dispersed directly after emergence from their host patches and jars (**Figures 1A,B**), males of *L. ryukyuensis* and *L. pacifica* tended to stay longer after emergence on their host patches, before they started to disperse (**Figures 1C,D**). Females of all species started to disperse directly after their emergence, whereby the proportion of dispersing female wasps of *L. heterotoma* was about 1.5 to 3 times higher than those of the other species (**Figure 1**).

The dispersal of males of *L. heterotoma*, *L. japonica*, *L. pacifica* and *L. ryukyuensis* showed differences between all species and depended in two of the species on the presence of females (**Figure 2**). The dispersal of males of species with highly volatile sex pheromones, namely of *L. heterotoma* and *L. japonica*, did not change with the presence of females (Wilcoxon signed rank tests: $V = 129$, $P = 0.196$; $V = 160$, $P = 1$, respectively; **Figure 2**), as the proportions of dispersing males from their natal patch stayed the same before and after the emergence of females (**Figure 2**). In contrast, in *L. pacifica* and *L. ryukyuensis*, which use low volatile CHCs as sex pheromones, significantly fewer males dispersed before the emergence of the first female than afterward (Wilcoxon signed rank tests: $V = 25$, $P < 0.001$; $V = 47$, $P = 0.007$, respectively; **Figure 2**). Moreover, when we combined the data for species with volatile iridoids and low volatile CHCs as sex pheromones, we obtained the same result (**Figure 3**). In species in which the females had volatile iridoid sex pheromones, the males started to disperse directly after emergence from the natal patch, before the females emerged, and dispersed at a similar rate after the females emerged (Wilcoxon signed rank tests: $V = 571$, $P = 0.257$). However, if they had sex pheromones of low volatility, males dispersed less before female emergence and significantly more after the presence of conspecific females (Wilcoxon signed rank tests: $V = 143$, $P < 0.001$; **Figure 3**).

When checked for species-specific effects, the proportions of dispersed female wasps differed among the four investigated species within all 24-h time intervals after female emergence (Kruskal–Wallis rank sum tests, 0–24 h: $\chi^2 = 14.073$, df = 3, $P = 0.003$; 24–48 h: $\chi^2 = 25.181$, df = 3, $P < 0.001$; 48–72 h: $\chi^2 = 28.953$, df = 3, $P < 0.001$; 72–96 h: $\chi^2 = 30.331$, df = 3, $P < 0.001$; **Figure 4**). The proportions of dispersed females of *L. heterotoma* were higher than those of the other

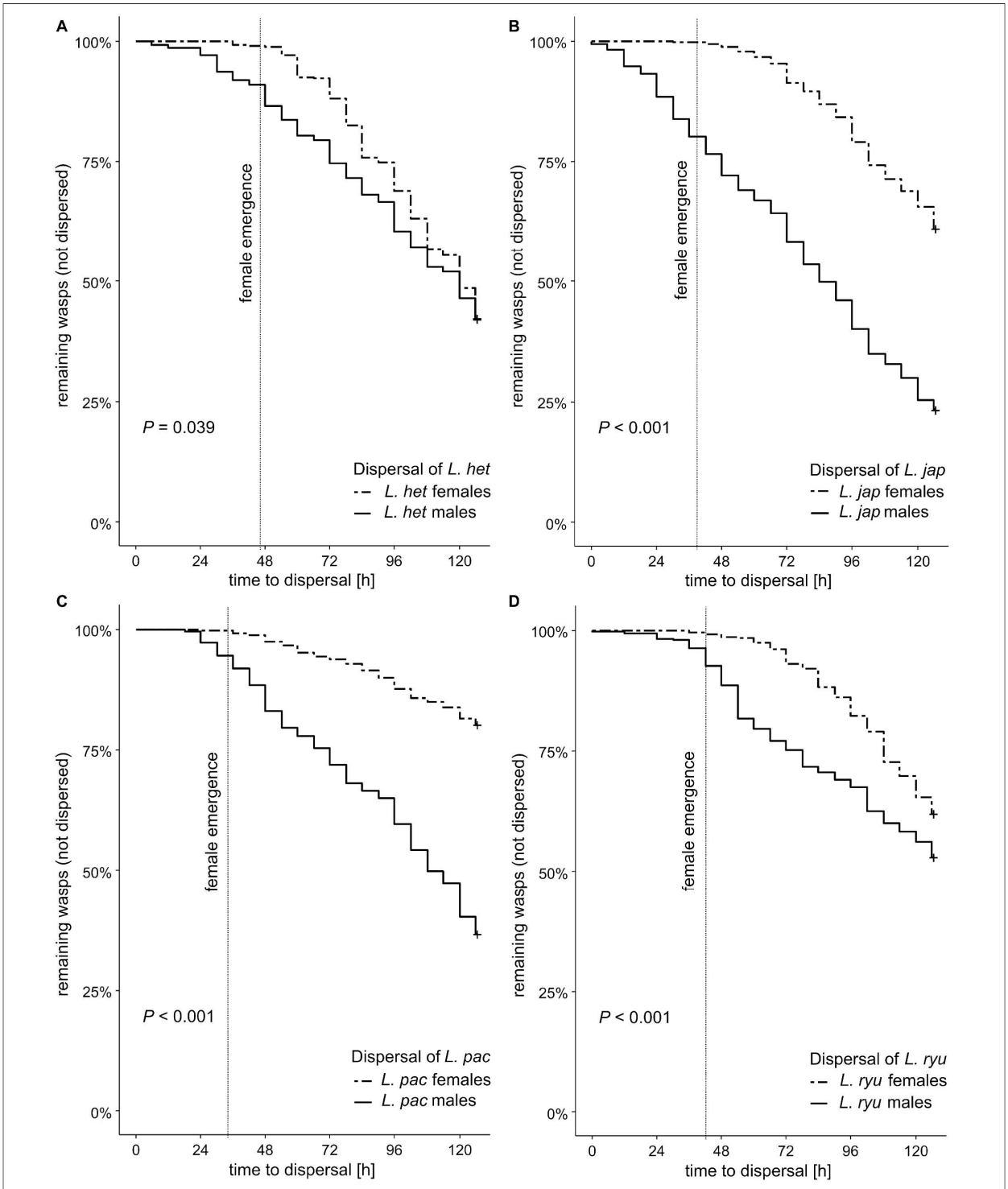

FIGURE 1 | Kaplan Meier plots showing the probability of dispersing for female and male wasps of **(A)** *L. heterotoma* (*L. het*), **(B)** *L. japonica* (*L. jap*), **(C)** *L. pacifica* (*L. pac*), and **(D)** *L. ryukyuensis* (*L. ryu*) within the first 126 h after emergence of the first wasp per jar. Numbers of females and males were summed up over all jars. The mean time point of female emergence per species is indicated with a dotted vertical line. Statistical differences between dispersal curves of the different sexes were analyzed with log rank tests. For each experiment, $N = 30$.

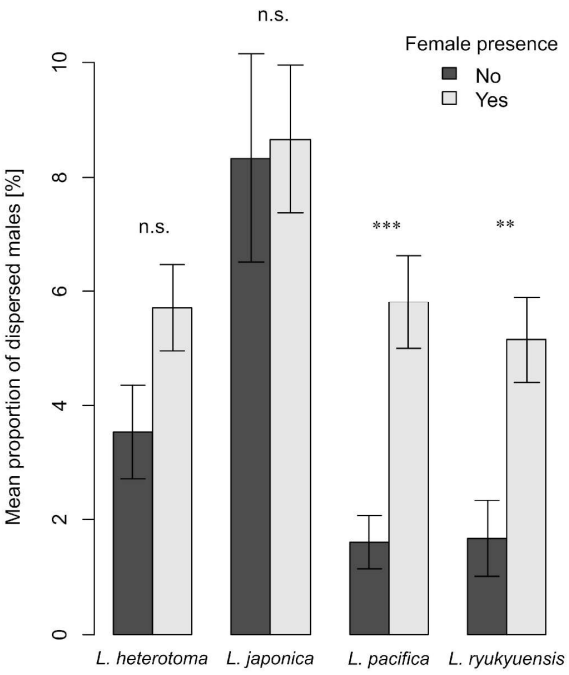

FIGURE 2 | Barplots showing means and standard errors of the mean proportions of dispersed males within the 48 h before (dark gray bars) and within the 48 h after (light gray bars) female emergence in each jar for *L. heterotoma*, *L. japonica*, *L. pacifica*, and *L. ryukyuensis*. Dispersal before and after female emergence was compared with Wilcoxon signed rank tests (Bonferroni corrected). Asterisks indicate a significant difference ** $P < 0.01$, *** $P < 0.001$, n.s. not significant. For each experiment, $N = 30$.

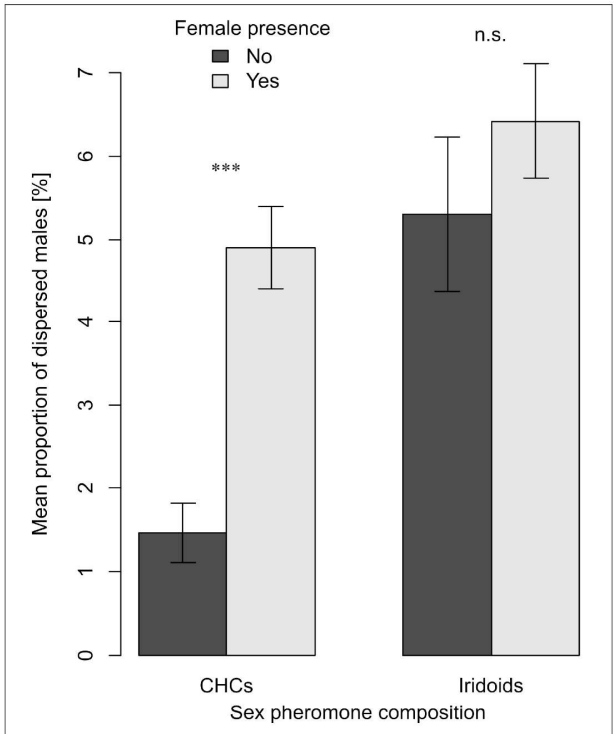

FIGURE 3 | Barplots showing means and standard errors of the mean proportions of dispersed males within the 48 h before (dark gray bars) and within the 48 h after (light gray bars) female emergence in each jar for species using CHCs (*L. pacifica* and *L. ryukyuensis*) or iridoids (*L. heterotoma*, *L. japonica*) in the female sex pheromone. Dispersal before and after female emergence was compared with Wilcoxon signed rank tests (Bonferroni corrected). Asterisks indicate a significant difference *** $P < 0.001$, n.s. not significant. For each experiment, $N = 60$.

three species, however, after Bonferroni corrections of *P*-values these differences were only significant within the first 48 h after emergence (pairwise Wilcoxon signed rank tests: each $P < 0.05$; **Figure 4**). The mean proportion of dispersed *L. japonica* females did never differ from that of *L. pacifica* (within 0–24 h and 24–48 h: each $P = 1$; 48–72 h: $P = 0.108$; 72–96 h: $P = 0.428$) or *L. ryukyuensis* (each $P = 1$; **Figure 4**). Proportions of dispersed female individuals did not differ between *L. pacifica* and *L. ryukyuensis* within the first 48 h after emergence (within 0–24 h and 24–48 h: each $P = 1$), but afterward those of *L. pacifica* were lower than those of *L. ryukyuensis* (within 48–72 h: $P = 0.045$; 72–96 h: $P < 0.001$). Albeit these species-specific differences in female dispersal, we found that when we combined species according to the volatility of their sex pheromones, the mean proportion of dispersed female wasps of species with highly volatile iridoid sex pheromones, namely of *L. heterotoma* and *L. japonica*, was significantly higher than that of females of *L. pacifica* and *L. ryukyuensis*, which have sex pheromones consisting of less volatile CHCs (**Figure 5**). This difference was visible in all 24-h intervals after the first female emergence in each jar (Wilcoxon signed rank tests, 0–24 h: $V = 278$, $P = 0.006$; 24–48 h: $V = 417$, $P = 0.005$; 48–72 h: $V = 360$, $P < 0.001$; 72–96 h: $V = 486$, $P = 0.011$; **Figure 5**).

To assess the odor sources that enable mate-finding after the dispersal of males and females, attraction assays were performed in which wasps could decide between two presented odors. Males of all species were attracted to the odor of their living conspecific females (*L. heterotoma*: $P < 0.001$; *L. japonica*: $P < 0.001$; *L. ryukyuensis*: $P < 0.001$; *L. pacifica*: $P = 0.016$; **Figure 6A**) and to the extract of their females (*L. heterotoma*: $P < 0.001$; *L. japonica*: $P < 0.001$; *L. ryukyuensis*: $P = 0.016$; *L. pacifica*: $P < 0.001$; **Figure 6B**), irrespective of the composition of the female sex pheromone, but not to the odor of host patches (*L. heterotoma*: $P = 0.362$; *L. japonica*: $P = 0.361$; *L. ryukyuensis*: $P = 0.585$; *L. pacifica*: $P = 0.856$; **Figure 6C**). Females, however, were not attracted to the odor of males (*L. heterotoma*: $P = 0.585$; *L. japonica*: $P = 0.855$; *L. ryukyuensis*: $P = 1$; *L. pacifica*: $P = 0.099$; **Figure 7A**), but to the odor of host patches (virgin *L. heterotoma*: $P = 0.016$; mated *L. heterotoma*: $P = 0.016$; virgin *L. japonica*: $P = 0.005$; mated *L. japonica*: $P < 0.001$; virgin *L. ryukyuensis*: $P < 0.001$; mated *L. ryukyuensis*: $P < 0.001$; virgin *L. pacifica*: $P < 0.001$; mated *L. pacifica*: $P < 0.001$; **Figures 7B,C**). No difference was noted in this decision according to the mating status of the female wasps (**Figures 7B,C**).

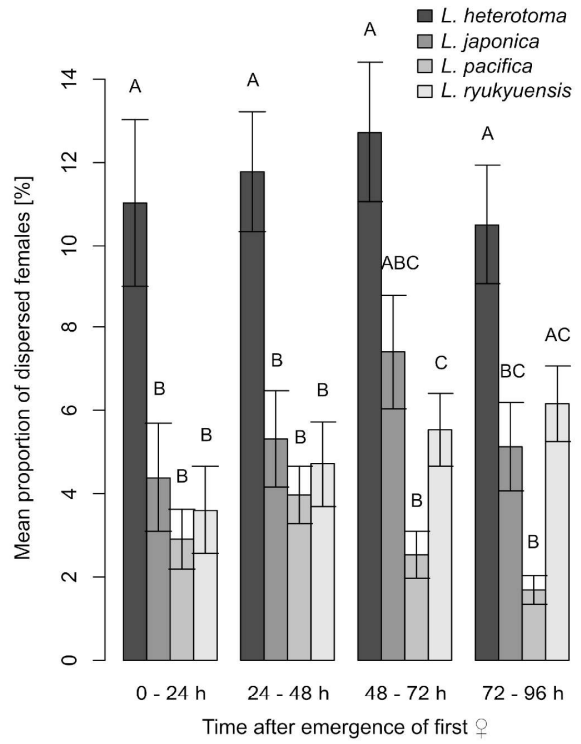

FIGURE 4 | Barplots showing means and standard errors of the mean proportions of dispersed females within 24-h intervals after emergence of the first female in each jar for each of the species *L. heterotoma*, *L. japonica*, *L. pacifica*, and *L. ryukyuensis*. Differences between species within time intervals were analyzed using Kruskal–Wallis rank sum tests with *post hoc* pairwise Wilcoxon signed rank tests (Bonferroni corrected). Different letters indicate a significant difference, $P < 0.05$. For each experiment, $N = 30$.

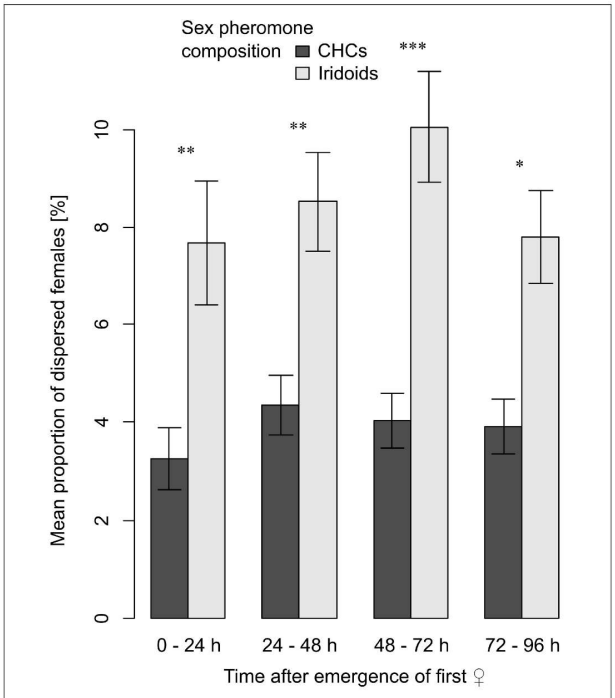

FIGURE 5 | Barplots showing means and standard errors of the mean proportions of dispersed females within 24-h intervals after emergence of the first female in each jar for species using CHCs (*L. pacifica* and *L. ryukyuensis*, dark gray bars) or iridoids (*L. heterotoma*, *L. japonica*, light gray bars) in the female sex pheromone. Dispersal in each time interval was compared with Wilcoxon signed rank tests (Bonferroni corrected). Asterisks indicate a significant difference * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For each experiment, $N = 60$.

DISCUSSION

Dispersal Can Explain Sex Pheromone Composition

Within the genus *Leptopilina*, all species studied to date have different combinations of low volatile CHCs and/or volatile iridoids as female-produced sex pheromones (Weiss et al., 2013, 2015; Pfeiffer et al., 2018; Böttinger et al., 2019; Böttinger et al. submitted). The evolution of this female sex pheromone diversity might have been shaped by the dispersal behavior of male and female wasps, as we have found a correlation between the post-emergence dispersal of males (**Figures 2**, **3**) and females (**Figure 5**) and the volatility of the sex pheromone of the females. We show here that males of the species *L. pacifica* and *L. ryukyuensis*, in which the long-chained CHCs of the females elicit courtship (Böttinger et al., 2019; Böttinger et al. submitted), delay their dispersal until females emerge at their host patch. In contrast, in the species *L. heterotoma* and *L. japonica*, whose female sex pheromones consist of volatile iridoid compounds (Weiss et al., 2013; Böttinger et al., 2019), a high proportion of males disperse before the first females emerge. No change occurs in the rate of male dispersal after the emergence of the females. Furthermore, the dispersal of female wasps with highly volatile iridoid sex pheromones is continuously more pronounced than that of females with low volatile CHC-based sex pheromones when species are pooled according to the sex pheromone volatility (**Figure 5**). However, this difference is due to the high proportions of dispersing *L. heterotoma* females compared to those of the other species (**Figure 4**) and not due to the sex pheromone differences of females. Therefore, the differences in the sex pheromone compositions of the genus *Leptopilina* can be explained by the different dispersal patterns of male wasps. Consequently, post-emergence dispersal behavior might have shaped the evolution of the female sex pheromones in this genus. To our knowledge, this is the first time that the volatility of female sex pheromones has been associated with the dispersal behavior of males.

Emergence of Females Is Important for Male Dispersal

We have found that the time point of female emergence (about 2 days after male emergence in all species) demonstrates a

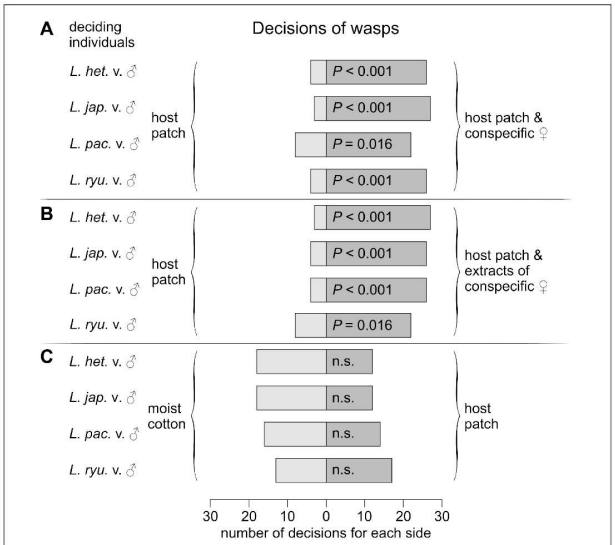

FIGURE 6 | Frequency of decision for control (light gray bars) or sample (dark gray bars) of males of *L. heterotoma*, *L. japonica*, *L. pacifica*, or *L. ryukyuensis* in a Y-tube experiment with a choice between the odor of unexploited host patches or host patches with either **(A)** 20 conspecific females or **(B)** extracts of their females; **(C)** Decisions of males of *L. heterotoma*, *L. japonica*, *L. pacifica*, or *L. ryukyuensis* for host patches or clean humidified air. *P*-values are given for two-sided binomial tests. For each experiment, *N* = 30. *L. het.* = *Leptopilina heterotoma*, *L. jap.* = *Leptopilina japonica*, *L. pac.* = *Leptopilina pacifica*, *L. ryu.* = *Leptopilina ryukyuensis*, v. = virgin, mc = moist cotton, n.s. = not significant.

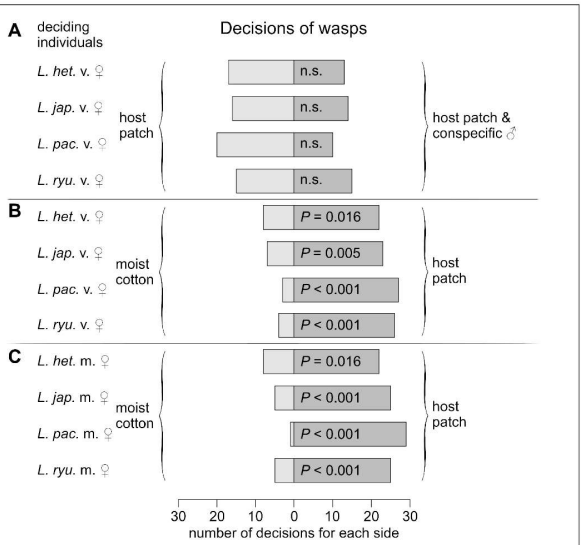

FIGURE 7 | Frequency of decision for control (light gray bars) or sample (dark gray bars) of virgin females of *L. heterotoma*, *L. japonica*, *L. pacifica*, or *L. ryukyuensis* in a Y-tube experiment with a choice between the odor of unexploited host patches or **(A)** host patches with 20 conspecific males or **(B)** clear humidified air provided by moist cotton pads, **(C)** Decisions of mated females of each of the species when choosing between the odor of unexploited host patches or clean humidified air. *P*-values are given for two-sided binomial tests. For each experiment, *N* = 30. *L. het.* = *Leptopilina heterotoma*, *L. jap.* = *Leptopilina japonica*, *L. pac.* = *Leptopilina pacifica*, *L. ryu.* = *Leptopilina ryukyuensis*, v. = virgin, m. = mated, n.s. = not significant.

changing point in the dispersal of males in the species with low volatile CHCs as sex pheromones. In these species (*L. pacifica* and *L. ryukyuensis*), the small proportion of males dispersing before the emergence of conspecific females indicates that males of these species stay at the host patches to wait for the females to emerge in order to ensure successful mate-finding and mating. This can be explained by the fact that mate location over long distances is not possible through the use of long-chained CHCs as sex pheromones. The risk of dispersing and not being able to find mating partners when CHCs are used as sex pheromones is probably high, and it is thus beneficial for males of these species to wait for the emergence of potential mates. In many other species of parasitoid wasps, males wait for conspecific females to emerge to mate with them. In *Cotesia waterstoni*, for example, male wasps wait at their place of emergence, recognize cocoons of conspecific females because of the leakage of CHCs of the females through the wall of the cocoon (Howard, 1992), and chew these open to mate with the females (Evans, 1964; Gordh and Móczár, 1990). Furthermore, in the parasitoid wasp *Lariophagus distinguendus*, males emerge before females and recognize the pheromones of the females while the latter are still pupated (Steiner et al., 2005). Also, approximately 50–60% of individuals in the gregarious parasitoid wasp, *Cotesia glomerata*, mate on the natal patch (Kitano, 1976; Tagawa and Hidaka, 1982; Gu and Dorn, 2003). The dispersal of the remaining newly emerged wasps of *C. glomerata* before mating is assumed to be induced by pheromones to reduce fierce competition, as males as well as mated females use the compound heptanal to repel siblings (Xu et al., 2019). Mating on the host patch of emergence leads to local mate competition (LMC; Hamilton, 1967) among male wasps for access to females. This LMC can lead to physical fights among male wasps (Godfray, 1994; Boulton et al., 2015), resulting in some cases, as in fig wasps or most wasps of the genus *Melittobia*, in the killing of competitors (Herre et al., 1997; Matthews et al., 2009). However, nothing is not known about male-male combat for mating opportunities or competition-mediated dispersal in male *Leptopilina* wasps. LMC can also result in female-biased sex ratios, as in, for example, the jewel wasp *Nasonia vitripennis* (Ruther et al., 2009). Here, offspring sex ratios can be adjusted, depending on the foundress number, in order to minimize competition between brothers. If only one foundress female lays eggs, the minimal necessary number of males to inseminate all female offspring of the brood will be produced (Ruther et al., 2009 and references therein). However, although LMC has been described in some *Leptopilina* species, consequences such as strong female-biased sex ratios have not been observed to date (Fauvergue et al., 1999).

Iridoid Sex Pheromones Are Used for Long-Range Attraction

In contrast to the species with CHCs as sex pheromones, males of *Leptopilina* species with iridoids as sex pheromones,

namely *L. heterotoma* and *L. japonica*, dispersed directly after emergence and showed no difference in their rate of dispersal after the emergence of their females (**Figures 2**, **3**). This result was expected, as the dispersal of male wasps (before female emergence) is attributed to mate-searching; long-range mate-finding for males of these species is possible because of the volatile iridoid sex pheromone emitted by the females. Males of *L. boulardi* and *L. heterotoma* have previously been shown to be attracted to volatiles of virgin females in the field (Fauvergue et al., 1999). Male *Leptopilina* wasps benefit from (off-patch) mate-searching as they mate several times, while females mate only once (van den Assem, 1968). Therefore, males with iridoid female sex pheromones might benefit from direct dispersal, as they could have increased mating opportunities with unrelated conspecifics compared to males that stay at the natal patch. The mating strategy of *Leptopilina* species with iridoids as sex pheromones could be the avoidance of inbreeding through direct dispersal, as discussed below.

Long-Range Attraction in Species With CHC-Based Sex Pheromones

Males of species with CHC-based sex pheromones also disperse from the host patch, although later than males of species with iridoid-based sex pheromones. As mate searching is the most likely reason for males to disperse, a long range mate-finding mechanism must also exist in species that use CHCs as sex pheromones. Indeed, we have shown in the olfactometer experiments, that the males of all the studied *Leptopilina* species are attracted to the compounds of their conspecific females (**Figure 6**). This is not surprising for species with iridoids as long-range sex pheromones. However, even species that use the long-chained CHCs of their females as sex pheromones are attracted over longer distances (in the Y-tube olfactometer) to their females. We can exclude that the attraction is mediated by low-volatile CHCs, as long-chained CHCs are solely perceivable in the close vicinity of the emitting individual (e.g., Schiestl and Ayasse, 2000; Blomquist and Bagnères, 2010; Ruther et al., 2011; Kühbandner et al., 2012; Ruther, 2013). For example, in wasps of the genus *Lariophagus*, the activity of the female pheromone consisting of CHCs is restricted to a distance of 0–5 mm (Ruther et al., 2000; Steiner et al., 2005, 2007a). This suggests that, even in the *Leptopilina* species with CHCs as sex pheromones, other compounds lead the males to conspecific females. These compounds are either the iridoid compounds of the females or the odor of the host patch. In several insect species, host-plant volatiles or host-associated volatile cues are used for the location of mating partners from a distance. Several parasitic wasps with CHC-based female sex pheromones, such as *C. glomerata*, use volatiles emitted by plants damaged by their host insects as long-range attraction cues to find mates (Xu et al., 2017; Xu and Turlings, 2018). Moreover, males of *L. distinguendus* orient themselves to the same volatiles emitted by host larval feces to which females are also attracted (Steidle and Ruther, 2000; Steiner et al., 2007b). This is not, however, the case in males of the genus *Leptopilina*, for which we have found no attraction of males to odors of host patches (**Figure 6C**). Our results therefore suggest that, even in species with CHC-based sex pheromones, the iridoid compounds emitted by females are used for long-range attraction of males.

Dispersal of Females

Female dispersal of the four *Leptopilina* species is species-specific and not dependent on the volatility of the sex pheromone composition of females (**Figure 4**). Females of *L. heterotoma* (volatile iridoid sex pheromones) showed consistently the highest proportion of dispersed individuals compared to the other species (**Figure 4**). As in males, when the data for species with pheromones of similar volatility were pooled, we have found higher percentages of dispersing females in *L. heterotoma* and *L. japonica*, the species using iridoids as sex pheromones, than in the species that use CHCs as sex pheromones (**Figure 5**). Female dispersal is important and essential for the location and establishment of new hosts or food sources and for the enlargement of distribution areas of that species (Lidicker and Stenseth, 1992; Vinson, 1998). The dispersal of females is necessary as usually no hosts suitable for oviposition are present at the site of emergence (Hardy and Godfray, 1990). We can conclude that female wasps of the four investigated species, and probably also other species of the genus *Leptopilina*, leave their host patch to search for oviposition sites, as females of all species were not attracted to conspecific males in the olfactometer experiments (hence, they are not interested in mate-searching; **Figure 7A**), but are attracted to host patch odors, irrespective of their mating status (**Figures 7B,C**). This result is consistent with the findings of earlier studies showing that female *Leptopilina* wasps are attracted to volatile cues of their host habitats (Vet and van der Hoeven, 1983; Dicke et al., 1984; Vet and van Opzeeland, 1984). We assume that the female wasps that dispersed out of the jars were mated, as the encounter rate of males and females on the host patches was high, and in a study of Hardy and Godfray (1990), almost all female *L. heterotoma* wasps found at host patches were mated. However, even if females of *Leptopilina* disperse before mating, the attraction of males via the iridoid compounds is possible.

Evolution

The way in which the various sex pheromones, i.e., volatile iridoids and low-volatile CHCs, evolved in the genus *Leptopilina* is not clear (Weiss et al., 2013, 2015; Pfeiffer et al., 2018; Böttinger et al., 2019; Böttinger et al. submitted). The sex pheromone composition does not show a phylogenetic pattern, i.e., closely related sister-species do not have a markedly more similar or more different sex pheromone composition than more distantly related species (unpublished and Böttinger et al., 2019). The high diversity in sex pheromone composition between species would not have been necessary for species divergence, as species specificity could have easily been achieved within one class of compounds. The data presented here show that the dispersal of male wasps of the genus *Leptopilina* correlates with the volatility of the species-specific sex pheromones of the females. Immediate dispersal seems to be more beneficial for species with long-range iridoids as female sex pheromones, as in species with short-range CHC as female sex pheromones. Staying on the host patch after

emergence might result in sibling matings and inbreeding, if the number of foundress females on this host patch has been low, and the offspring consequently is highly related. Inbreeding is often considered as major ultimate cause and important driver of dispersal (e.g., Bengtsson, 1978; Greenwood, 1980; Pusey, 1987). For example, in the parasitoid wasp *C. glomerata,* inbreeding avoidance is the main trigger of natal dispersal (Ruf et al., 2011). Depending on the strength of inbreeding depression, individual wasps benefit from mating off-patch and, hence, disperse further away from the natal patch, or, alternatively, require mate-recognition compounds enabling the distinction of inbred kin or ideal mating partners. Such compounds might be the sex- and species-specific CHCs, as they can inform a prospective partner not only about the individual's condition and genetic make-up, but also about the relatedness of the two individuals (reviewed in Steiger and Stökl, 2014). Although offspring of inbred *Leptopilina* wasps do not show inbreeding depression or a strong female-biased sex ratio (Biémont and Bouletreau, 1980; Hey and Gargiulo, 1985; Fauvergue et al., 1999), we propose that *Leptopilina* species with iridoids as sex pheromones avoid inbreeding by their stronger urge to disperse and to out-breed, whereas the species with low volatile sex pheromones make use of the information presented in CHCs to circumvent mismating with too closely related conspecifics. These species therefore do not need to disperse directly after emergence, reducing the costs of mate search for males. Analyses of inbreeding consequences in species with CHCs as sex pheromones should yield further relevant insights.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

Both authors conceived the study, analyzed the data, and revised the manuscript. LB designed, performed the experiments, and wrote the first draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.557527/full#supplementary-material

Supplementary Table 1 contains the dataset of the dispersal experiment, corresponding to **Figures 1–5**. **Supplementary Tables 2–7** contain the results of the attraction assays in the olfactometer, corresponding to **Figures 6**, **7**.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Table 1, 1/16 (continued on the next pages) Numbers of male wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_ryu = *L. ryukyuensis*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
1	L_ryu	20	N/A	40	40	50	N/A	50	50	50	N/A	50	50	49	N/A	50	40	40	N/A	40	40	40	N/A
2	L_ryu	3	N/A	12	12	17	N/A	20	20	20	N/A	20	30	30	N/A	30	30	10	N/A	12	6	6	N/A
3	L_ryu	1	1	1	N/A	2	2	3	N/A	3	3	5	N/A	8	7	9	N/A	9	8	8	N/A	8	9
4	L_ryu	2	2	30	N/A	35	35	40	N/A	40	40	40	N/A	39	40	39	N/A	40	31	34	N/A	32	23
5	L_ryu	1	1	1	N/A	2	2	18	N/A	20	20	20	N/A	20	20	20	N/A	20	24	22	N/A	27	25
6	L_ryu	2	2	5	N/A	10	10	25	N/A	40	40	40	N/A	40	40	40	N/A	40	44	40	N/A	40	40
7	L_ryu	20	N/A	35	35	40	N/A	40	40	38	N/A	40	45	60	N/A	60	60	60	N/A	55	50	50	N/A
8	L_ryu	6	N/A	14	14	19	N/A	20	20	20	N/A	20	22	5	N/A	5	7	6	N/A	6	6	6	N/A
9	L_ryu	1	N/A	2	2	10	N/A	18	18	23	N/A	20	20	20	N/A	20	27	5	N/A	5	5	4	N/A
10	L_ryu	2	2	1	N/A	7	7	30	N/A	40	40	40	N/A	60	54	54	N/A	9	11	6	N/A	7	6
11	L_ryu	3	3	30	N/A	38	38	40	N/A	40	40	40	N/A	40	40	39	N/A	39	34	35	N/A	30	30
12	L_ryu	1	N/A	5	5	16	N/A	35	35	45	N/A	50	50	50	N/A	50	4	4	N/A	6	3	2	N/A
13	L_ryu	1	1	7	N/A	14	14	23	N/A	20	37	37	N/A	37	12	12	N/A	12	12	5	N/A	4	4
14	L_ryu	1	1	2	N/A	3	3	30	N/A	30	30	60	N/A	60	60	60	N/A	60	60	60	N/A	50	50
15	L_ryu	1	1	4	N/A	9	9	10	N/A	13	21	21	N/A	21	13	13	N/A	18	13	10	N/A	17	8
16	L_ryu	10	N/A	10	10	24	N/A	20	23	21	N/A	14	6	5	N/A	5	3	2	N/A	0	0	0	N/A
17	L_ryu	5	5	18	N/A	32	32	45	N/A	40	40	40	N/A	40	40	14	N/A	17	4	7	N/A	15	7
18	L_ryu	4	N/A	4	4	15	N/A	21	32	32	N/A	31	19	19	N/A	10	5	4	N/A	1	3	3	N/A
19	L_ryu	16	N/A	16	16	19	N/A	18	17	15	N/A	15	2	3	N/A	4	3	2	N/A	2	2	2	N/A
20	L_ryu	20	N/A	34	34	36	N/A	40	30	30	N/A	26	20	13	N/A	3	1	2	N/A	1	0	0	N/A
21	L_ryu	1	1	11	N/A	18	22	36	N/A	43	41	52	N/A	52	51	49	N/A	49	49	47	N/A	47	45
22	L_ryu	2	7	N/A	11	29	43	N/A	46	57	53	N/A	55	57	56	N/A	56	46	45	N/A	44	44	44
23	L_ryu	1	4	N/A	13	16	22	N/A	36	39	37	N/A	38	43	42	N/A	42	42	42	N/A	42	41	38
24	L_ryu	8	15	32	N/A	40	54	60	N/A	60	72	70	N/A	68	67	64	N/A	61	91	90	N/A	90	85
25	L_ryu	5	N/A	8	8	15	N/A	20	21	24	N/A	24	28	27	N/A	26	24	24	N/A	24	23	23	N/A
26	L_ryu	1	N/A	7	16	18	N/A	18	20	21	N/A	20	17	17	N/A	18	20	20	N/A	20	18	18	N/A
27	L_ryu	1	13	13	N/A	15	15	15	N/A	15	15	15	N/A	15	15	15	N/A	15	12	12	N/A	11	10
28	L_ryu	2	2	5	N/A	8	9	28	N/A	34	24	31	N/A	31	31	29	N/A	29	29	29	N/A	28	28
29	L_ryu	1	5	18	N/A	31	35	48	N/A	64	75	75	N/A	73	73	72	N/A	72	89	89	N/A	86	86
30	L_ryu	3	12	N/A	20	33	46	N/A	48	51	49	N/A	48	45	46	N/A	46	45	43	N/A	40	39	35

2/16 Numbers of male wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_ryu = *L. ryukyuensis*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
1	L_ryu	3	N/A	0	N/A	9	N/A	0	7	1	N/A	0	9	1	N/A	1	15	6	N/A	4	12	14	N/A
2	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	0	N/A	0	0	0	N/A
3	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	2	0	N/A	0	1	0	N/A	0	0
4	L_ryu	0	N/A	3	N/A	1	N/A	5	N/A	0	15	5	N/A	1	9	1	N/A	1	30	7	N/A	3	9
5	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	1	N/A	0	2	2	N/A	0	1	2	N/A	0	2
6	L_ryu	0	N/A	0	N/A	1	N/A	3	N/A	0	N/A	7	N/A	0	7	0	N/A	0	22	6	N/A	1	7
7	L_ryu	2	N/A	0	N/A	0	N/A	0	4	2	N/A	0	10	2	N/A	0	3	0	N/A	5	3	1	N/A
8	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A
9	L_ryu	0	N/A	0	N/A	1	N/A	0	N/A	1	N/A	0	0	1	N/A	0	0	0	N/A	0	0	0	N/A
10	L_ryu	0	N/A	1	N/A	0	N/A	2	N/A	0	N/A	1	N/A	2	6	0	N/A	0	1	1	N/A	0	1
11	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	1	1	0	N/A	0	1	1	N/A	0	5	3	N/A	2	8
12	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A
13	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
14	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	3	N/A	0	5	1	N/A	0	3	3	N/A	0	3
15	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
16	L_ryu	0	N/A	0	N/A	0	N/A	1	6	2	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A
17	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	0	N/A	0	0
18	L_ryu	0	N/A	0	N/A	1	N/A	0	2	0	N/A	1	3	0	N/A	0	0	0	N/A	0	0	0	N/A
19	L_ryu	0	N/A	0	N/A	0	N/A	1	1	2	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A
20	L_ryu	0	N/A	0	N/A	4	N/A	0	5	2	N/A	4	2	0	N/A	0	0	0	N/A	0	1	0	N/A
21	L_ryu	0	0	0	N/A	0	0	0	N/A	1	4	0	N/A	0	1	2	N/A	0	0	2	N/A	0	2
22	L_ryu	0	0	N/A	0	0	0	N/A	3	14	4	N/A	1	8	1	N/A	0	10	1	N/A	1	0	0
23	L_ryu	0	0	N/A	0	0	2	N/A	5	11	2	N/A	0	3	1	N/A	0	0	0	N/A	0	1	3
24	L_ryu	0	0	0	N/A	0	0	7	N/A	3	34	3	N/A	2	5	3	N/A	3	0	1	N/A	1	5
25	L_ryu	0	N/A	0	0	0	N/A	1	3	2	N/A	0	6	1	N/A	1	2	0	N/A	0	1	0	N/A
26	L_ryu	0	N/A	0	0	0	N/A	3	14	1	N/A	1	5	0	N/A	0	1	0	N/A	0	2	0	N/A
27	L_ryu	0	0	0	N/A	0	0	0	N/A	0	4	0	N/A	0	0	1	N/A	0	3	0	N/A	1	1
28	L_ryu	0	0	0	N/A	0	0	0	N/A	2	23	1	N/A	0	5	2	N/A	0	0	0	N/A	1	0
29	L_ryu	0	0	0	N/A	0	0	0	N/A	5	8	0	N/A	2	0	1	N/A	0	1	0	N/A	3	0
30	L_ryu	0	0	N/A	0	0	1	N/A	1	6	2	N/A	1	3	2	N/A	0	1	2	N/A	3	1	4

3/16 Numbers of female wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_ryu = *L. ryukyuensis*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t102	t96	t108	t114	t120	t126
1	L_ryu	0	N/A	0	0	0	N/A	1	11	11	N/A	16	16	15	N/A	26	24	25	N/A	20	20	20	N/A
2	L_ryu	0	N/A	0	0	0	N/A	0	1	1	N/A	7	2	7	N/A	8	11	15	N/A	16	21	21	N/A
3	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	3	N/A	3	12	12	N/A	10	12	10	N/A	10	14
4	L_ryu	0	0	0	N/A	0	0	5	N/A	6	8	9	N/A	19	18	10	N/A	20	20	20	N/A	19	15
5	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	2	N/A	3	10	10	N/A	10	10	10	N/A	12	12
6	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	3	N/A	8	4	4	N/A	10	10	12	N/A	12	13
7	L_ryu	0	N/A	0	0	1	N/A	1	3	5	N/A	19	10	16	N/A	21	20	20	N/A	20	20	20	N/A
8	L_ryu	0	N/A	0	0	1	N/A	3	13	13	N/A	17	14	16	N/A	18	15	19	N/A	17	17	17	N/A
9	L_ryu	0	N/A	0	0	0	N/A	0	0	0	N/A	5	7	9	N/A	18	13	18	N/A	20	19	19	N/A
10	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	6	N/A	6	22	25	N/A	16	24	14	N/A	17	11
11	L_ryu	0	0	0	N/A	0	0	1	N/A	3	3	3	N/A	10	10	9	N/A	15	12	20	N/A	20	20
12	L_ryu	0	N/A	0	0	0	N/A	0	0	0	N/A	0	9	9	N/A	22	11	25	N/A	29	42	40	N/A
13	L_ryu	0	0	0	N/A	0	0	0	N/A	2	5	5	N/A	10	7	15	N/A	15	15	12	N/A	15	15
14	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	4	N/A	6	14	14	N/A	30	30	30	N/A	40	40
15	L_ryu	0	0	0	N/A	0	0	0	N/A	0	3	5	N/A	5	5	6	N/A	7	5	5	N/A	6	6
16	L_ryu	0	N/A	0	0	1	N/A	3	7	8	N/A	11	10	11	N/A	7	4	5	N/A	4	4	4	N/A
17	L_ryu	0	0	0	N/A	0	0	0	N/A	0	7	13	N/A	11	12	13	N/A	18	18	20	N/A	14	16
18	L_ryu	0	N/A	0	0	0	N/A	0	0	0	N/A	12	0	12	N/A	12	12	18	N/A	16	16	16	N/A
19	L_ryu	0	N/A	0	0	7	N/A	11	17	17	N/A	16	16	17	N/A	12	12	8	N/A	8	8	8	N/A
20	L_ryu	0	N/A	0	0	2	N/A	5	12	16	N/A	16	16	24	N/A	27	18	16	N/A	16	6	6	N/A
21	L_ryu	0	0	0	N/A	0	0	0	N/A	2	7	10	N/A	11	18	20	N/A	26	18	26	N/A	22	27
22	L_ryu	0	0	N/A	0	0	1	N/A	4	19	20	N/A	17	23	30	N/A	42	29	42	N/A	41	41	40
23	L_ryu	0	0	N/A	0	0	0	N/A	2	4	10	N/A	12	14	19	N/A	43	33	40	N/A	45	43	39
24	L_ryu	0	0	0	N/A	0	0	4	N/A	7	12	16	N/A	20	34	41	N/A	79	38	79	N/A	75	74
25	L_ryu	0	N/A	0	0	3	N/A	2	11	11	N/A	9	18	17	N/A	36	21	36	N/A	35	35	35	N/A
26	L_ryu	0	N/A	0	0	0	N/A	1	7	7	N/A	7	13	13	N/A	27	13	26	N/A	19	24	24	N/A
27	L_ryu	0	0	0	N/A	0	0	2	N/A	3	15	15	N/A	13	14	15	N/A	21	14	21	N/A	17	22
28	L_ryu	0	0	0	N/A	0	0	2	N/A	2	9	12	N/A	12	32	31	N/A	36	30	36	N/A	35	33
29	L_ryu	0	0	0	N/A	0	0	4	N/A	11	16	26	N/A	23	40	40	N/A	44	40	44	N/A	51	49
30	L_ryu	0	0	N/A	0	0	3	N/A	4	21	21	N/A	21	23	27	N/A	39	29	39	N/A	39	39	37

4/16 Numbers of female wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_ryu = *L. ryukyuensis*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
1	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	1	0	2	N/A	3	2	1	N/A	6	0	1	N/A
2	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A
3	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	2	0	N/A	0	1
4	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	1	N/A	3	0	9	N/A	4	2	7	N/A	8	4
5	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
6	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1
7	L_ryu	0	N/A	0	N/A	0	N/A	0	0	1	N/A	2	0	3	N/A	3	2	5	N/A	5	1	2	N/A
8	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	2	0	1	N/A
9	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	1	0	0	N/A	0	1	0	N/A
10	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	0	5	N/A	4	11	4	N/A	1	2
11	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	1	N/A	1	3	3	N/A	1	1
12	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	1	0	0	N/A	0	0	3	N/A	1	0	2	N/A
13	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
14	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	1	N/A	0	6
15	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	0	N/A	0	0
16	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	0	N/A	0	0	0	N/A
17	L_ryu	0	N/A	0	N/A	0	N/A	0	N/A	0	0	0	N/A	1	1	0	N/A	0	1	3	N/A	1	2
18	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	2	0	N/A	0	0	1	N/A
19	L_ryu	0	N/A	0	N/A	0	N/A	0	1	2	N/A	1	0	0	N/A	0	3	0	N/A	1	1	0	N/A
20	L_ryu	0	N/A	0	N/A	0	N/A	0	0	0	N/A	0	1	4	N/A	0	1	0	N/A	1	1	0	N/A
21	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	3	0	0	N/A	2	0	0	N/A	5	0
22	L_ryu	0	0	N/A	0	0	0	N/A	2	0	0	N/A	4	0	1	N/A	1	0	0	N/A	6	0	1
23	L_ryu	0	0	N/A	0	0	0	N/A	0	0	0	N/A	6	0	3	N/A	3	1	3	N/A	13	2	4
24	L_ryu	0	0	0	N/A	0	0	0	N/A	0	2	0	N/A	2	0	3	N/A	8	0	0	N/A	4	1
25	L_ryu	0	N/A	0	0	1	N/A	1	0	0	N/A	2	0	1	N/A	3	1	0	N/A	11	0	0	N/A
26	L_ryu	0	N/A	0	0	0	N/A	1	0	0	N/A	0	0	0	N/A	2	1	1	N/A	7	1	0	N/A
27	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	2	1	0	N/A	1	2	0	N/A	4	0
28	L_ryu	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	1	N/A	1	1	0	N/A	1	2
29	L_ryu	0	0	0	N/A	0	0	1	N/A	2	0	0	N/A	3	1	0	N/A	0	2	0	N/A	0	2
30	L_ryu	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	2

5/16 Numbers of male wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_jap = *L. japonica.* Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
31	L_jap	1	1	25	N/A	30	30	26	N/A	28	23	23	N/A	12	12	8	N/A	6	5	5	N/A	1	1
32	L_jap	9	21	12	N/A	32	37	31	N/A	45	45	45	N/A	20	22	15	N/A	15	15	15	N/A	12	12
33	L_jap	3	9	12	N/A	24	39	39	N/A	50	50	36	N/A	40	34	33	N/A	52	52	52	N/A	52	52
34	L_jap	1	N/A	5	21	23	N/A	53	54	54	N/A	28	30	30	N/A	30	30	30	N/A	48	48	48	N/A
35	L_jap	1	6	7	N/A	22	44	44	N/A	40	40	40	N/A	40	40	40	N/A	62	62	62	N/A	60	60
36	L_jap	4	19	19	N/A	39	48	48	N/A	40	40	40	N/A	40	40	40	N/A	57	57	57	N/A	38	38
37	L_jap	1	1	1	N/A	1	5	7	N/A	7	10	10	N/A	20	15	15	N/A	16	16	16	N/A	10	10
38	L_jap	1	4	6	N/A	14	20	20	N/A	26	25	20	N/A	20	22	22	N/A	34	34	34	N/A	28	28
39	L_jap	20	N/A	30	30	30	N/A	30	30	25	N/A	25	30	30	N/A	25	18	16	N/A	8	8	8	N/A
40	L_jap	5	5	N/A	11	20	20	N/A	23	40	40	N/A	15	14	14	N/A	0	0	0	N/A	1	1	1
41	L_jap	1	7	11	N/A	29	44	44	N/A	44	45	45	N/A	40	40	40	N/A	55	55	55	N/A	36	36
42	L_jap	11	24	24	N/A	38	40	40	N/A	40	40	40	N/A	40	40	39	N/A	49	49	49	N/A	41	41
43	L_jap	3	3	30	N/A	20	40	36	N/A	32	34	34	N/A	20	16	10	N/A	12	7	7	N/A	2	2
44	L_jap	1	1	35	N/A	40	40	40	N/A	40	40	39	N/A	30	30	25	N/A	24	26	22	N/A	24	24
45	L_jap	3	N/A	30	40	40	N/A	45	45	41	N/A	40	40	40	N/A	40	45	20	N/A	45	45	45	N/A
46	L_jap	3	3	30	N/A	35	35	34	N/A	34	33	30	N/A	30	30	20	N/A	24	24	19	N/A	24	24
47	L_jap	3	3	N/A	16	27	33	N/A	33	33	35	N/A	35	40	40	N/A	40	40	40	N/A	30	30	30
48	L_jap	5	N/A	30	25	15	N/A	30	40	35	N/A	35	30	35	N/A	35	30	11	N/A	31	31	31	N/A
49	L_jap	10	10	40	N/A	60	50	46	N/A	44	40	36	N/A	36	20	20	N/A	20	12	7	N/A	6	6
50	L_jap	27	N/A	45	40	35	N/A	67	55	52	N/A	48	21	23	N/A	10	10	9	N/A	5	5	5	N/A
51	L_jap	2	2	N/A	1	2	1	N/A	1	4	4	N/A	3	4	4	N/A	4	3	1	N/A	1	3	4
52	L_jap	4	2	N/A	3	25	26	N/A	25	29	26	N/A	26	31	28	N/A	26	24	18	N/A	24	19	16
53	L_jap	3	1	N/A	1	13	16	N/A	15	32	30	N/A	28	27	27	N/A	25	22	21	N/A	20	18	13
54	L_jap	0	0	N/A	0	0	0	N/A	0	2	1	N/A	1	3	2	N/A	2	6	4	N/A	5	6	6
55	L_jap	13	11	N/A	10	44	38	N/A	36	53	52	N/A	50	71	52	N/A	42	38	12	N/A	10	10	10
56	L_jap	1	1	N/A	1	3	3	N/A	3	2	4	N/A	30	1	1	N/A	2	5	2	N/A	2	1	0
57	L_jap	2	2	N/A	2	2	2	N/A	2	5	6	N/A	6	6	5	N/A	5	6	4	N/A	3	3	3
58	L_jap	0	1	N/A	1	4	4	N/A	4	5	6	N/A	6	5	5	N/A	4	4	3	N/A	3	2	2
59	L_jap	6	3	N/A	2	18	18	N/A	19	26	24	N/A	21	23	14	N/A	14	10	8	N/A	7	5	4
60	L_jap	14	14	N/A	12	36	32	N/A	26	34	33	N/A	28	28	26	N/A	24	24	21	N/A	17	17	16

6/16 Numbers of male wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_jap = *L. japonica.* Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
31	L_jap	0	N/A	7	N/A	3	3	4	N/A	0	5	1	N/A	0	1	3	N/A	1	1	1	N/A	0	0
32	L_jap	0	8	9	N/A	1	21	6	N/A	2	5	5	N/A	3	7	7	N/A	2	2	3	N/A	0	3
33	L_jap	0	0	0	N/A	0	1	0	N/A	0	1	0	N/A	1	0	1	N/A	0	1	1	N/A	0	0
34	L_jap	0	N/A	0	1	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A
35	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	3	0	N/A	0	1	0	N/A	0	0
36	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	4	0	N/A	0	1
37	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0
38	L_jap	0	0	0	N/A	0	0	0	N/A	0	1	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1
39	L_jap	3	N/A	1	3	4	N/A	10	6	5	N/A	1	6	9	N/A	1	8	2	N/A	0	4	2	N/A
40	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0
41	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	0	N/A	0	0	0	N/A	0	1
42	L_jap	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	0	0	1	N/A	1	0	0	N/A	0	0
43	L_jap	0	N/A	3	N/A	3	5	4	N/A	4	3	1	N/A	0	0	1	N/A	1	1	0	N/A	0	0
44	L_jap	0	N/A	1	N/A	1	4	7	N/A	2	4	1	N/A	2	2	4	N/A	1	10	4	N/A	3	6
45	L_jap	0	N/A	1	4	2	N/A	2	9	4	N/A	3	5	8	N/A	2	15	25	N/A	5	16	24	N/A
46	L_jap	0	N/A	4	N/A	3	5	1	N/A	0	1	3	N/A	1	4	11	N/A	0	0	5	N/A	1	0
47	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0
48	L_jap	0	N/A	0	5	9	N/A	4	4	5	N/A	2	5	7	N/A	3	4	19	N/A	2	5	15	N/A
49	L_jap	0	N/A	13	N/A	7	6	6	N/A	2	10	4	N/A	2	16	11	N/A	1	8	5	N/A	0	0
50	L_jap	5	N/A	4	5	5	N/A	4	12	3	N/A	4	10	4	N/A	1	3	1	N/A	0	1	0	N/A
51	L_jap	0	1	N/A	1	2	1	N/A	0	2	0	N/A	0	1	0	N/A	0	1	0	N/A	0	0	0
52	L_jap	0	2	N/A	0	4	0	N/A	1	11	3	N/A	1	9	3	N/A	2	2	9	N/A	4	5	3
53	L_jap	0	2	N/A	0	0	0	N/A	1	3	2	N/A	2	2	0	N/A	2	3	7	N/A	1	2	5
54	L_jap	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	1	2	N/A	0	0	2	N/A	1	0	2
55	L_jap	0	2	N/A	1	8	6	N/A	7	7	1	N/A	2	8	19	N/A	10	5	3	N/A	0	2	0
56	L_jap	0	0	N/A	0	1	0	N/A	0	1	1	N/A	1	2	0	N/A	0	0	3	N/A	0	0	0
57	L_jap	0	0	N/A	0	3	0	N/A	0	1	0	N/A	0	3	0	N/A	1	1	2	N/A	1	0	0
58	L_jap	2	0	N/A	0	2	0	N/A	0	0	0	N/A	0	2	0	N/A	1	0	0	N/A	0	1	0
59	L_jap	0	3	N/A	1	1	0	N/A	0	9	2	N/A	3	8	9	N/A	2	4	2	N/A	4	2	1
60	L_jap	0	1	N/A	2	3	4	N/A	6	2	1	N/A	5	4	2	N/A	2	2	3	N/A	1	3	1

7/16 Numbers of female wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30 , L_jap = *L. japonica*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t102	t96	t108	t114	t120	t126
31	L_jap	0	N/A	0	N/A	0	2	7	N/A	7	11	19	N/A	16	11	15	N/A	11	12	7	N/A	8	8
32	L_jap	0	0	0	N/A	0	0	0	N/A	0	8	10	N/A	14	14	9	N/A	22	22	22	N/A	29	29
33	L_jap	0	0	0	N/A	0	0	0	N/A	0	3	7	N/A	9	7	8	N/A	16	16	16	N/A	26	26
34	L_jap	0	N/A	0	0	0	N/A	0	0	0	N/A	3	3	5	N/A	8	5	8	N/A	14	14	14	N/A
35	L_jap	0	0	0	N/A	0	0	0	N/A	1	1	2	N/A	5	1	5	N/A	15	15	15	N/A	21	21
36	L_jap	0	0	0	N/A	0	0	0	N/A	0	1	4	N/A	6	6	6	N/A	11	11	11	N/A	23	23
37	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	6	1	6	N/A	13	13	13	N/A	15	15
38	L_jap	0	0	0	N/A	0	0	0	N/A	0	3	3	N/A	15	7	14	N/A	13	13	13	N/A	20	20
39	L_jap	0	N/A	0	0	4	N/A	4	6	4	N/A	10	15	18	N/A	22	29	20	N/A	19	19	19	N/A
40	L_jap	0	0	N/A	0	0	0	N/A	0	0	1	N/A	1	1	1	N/A	0	0	0	N/A	1	1	1
41	L_jap	0	0	0	N/A	0	1	1	N/A	1	5	8	N/A	10	8	10	N/A	11	11	11	N/A	20	20
42	L_jap	0	0	0	N/A	0	2	2	N/A	3	8	8	N/A	9	8	9	N/A	12	12	12	N/A	25	25
43	L_jap	0	N/A	0	N/A	0	0	0	N/A	2	6	17	N/A	18	25	25	N/A	20	25	15	N/A	14	14
44	L_jap	0	N/A	0	N/A	0	0	3	N/A	3	5	2	N/A	5	5	5	N/A	5	5	5	N/A	4	4
45	L_jap	0	N/A	0	0	0	N/A	0	1	1	N/A	1	7	10	N/A	10	10	9	N/A	20	20	20	N/A
46	L_jap	0	N/A	0	N/A	0	2	2	N/A	0	3	3	N/A	5	5	6	N/A	10	10	10	N/A	13	13
47	L_jap	0	0	N/A	0	0	0	N/A	1	3	3	N/A	8	5	8	N/A	13	13	13	N/A	21	21	21
48	L_jap	0	N/A	0	1	2	N/A	2	2	2	N/A	3	12	10	N/A	21	17	17	N/A	25	25	25	N/A
49	L_jap	0	N/A	0	N/A	0	1	1	N/A	1	5	5	N/A	7	7	10	N/A	15	11	15	N/A	8	8
50	L_jap	0	N/A	0	1	1	N/A	0	10	15	N/A	14	17	23	N/A	16	10	14	N/A	14	14	14	N/A
51	L_jap	0	0	N/A	0	0	0	N/A	0	1	0	N/A	0	1	2	N/A	4	2	3	N/A	3	3	3
52	L_jap	0	0	N/A	0	2	3	N/A	3	5	5	N/A	5	13	14	N/A	21	11	23	N/A	23	20	15
53	L_jap	0	0	N/A	0	1	1	N/A	0	0	0	N/A	0	13	12	N/A	26	14	41	N/A	38	36	38
54	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	1	N/A	6	1	6	N/A	5	11	10
55	L_jap	0	0	N/A	0	1	1	N/A	1	5	4	N/A	7	22	21	N/A	34	26	31	N/A	16	21	15
56	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	2	N/A	2	2	2
57	L_jap	0	0	N/A	0	1	1	N/A	1	3	4	N/A	3	4	4	N/A	5	3	5	N/A	5	6	7
58	L_jap	0	0	N/A	0	0	0	N/A	0	1	1	N/A	3	1	3	N/A	3	4	2	N/A	4	3	3
59	L_jap	0	0	N/A	0	0	0	N/A	0	1	1	N/A	4	1	4	N/A	8	13	13	N/A	18	17	15
60	L_jap	0	2	N/A	2	4	8	N/A	9	11	10	N/A	10	23	23	N/A	35	21	31	N/A	32	29	25

8/16 Numbers of female wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_jap = *L. japonica*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
31	L_jap	0	N/A	0	N/A	0	0	0	N/A	1	2	2	N/A	3	5	6	N/A	3	3	4	N/A	1	0
32	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	2	5	N/A	4	4	2	N/A	1	4
33	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	1	N/A	0	0	1	N/A	0	0
34	L_jap	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A
35	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
36	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1
37	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
38	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	1	N/A	2	0
39	L_jap	0	N/A	0	0	0	N/A	0	1	1	N/A	0	5	7	N/A	0	7	2	N/A	4	1	4	N/A
40	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	0
41	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
42	L_jap	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
43	L_jap	0	N/A	0	N/A	0	0	0	N/A	0	0	1	N/A	0	2	3	N/A	2	7	5	N/A	0	0
44	L_jap	0	N/A	0	N/A	0	0	0	N/A	0	0	3	N/A	0	0	0	N/A	0	0	0	N/A	0	0
45	L_jap	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	4	N/A	0	1	1	N/A	0	1	0	N/A
46	L_jap	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	0	2
47	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	0	N/A	0	0	0	N/A	0	0	0
48	L_jap	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	2	N/A	0	1	4	N/A	2	0	1	N/A
49	L_jap	0	N/A	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	2	N/A	2	2	0	N/A	2	1
50	L_jap	0	N/A	0	0	0	N/A	0	0	0	N/A	1	2	8	N/A	1	5	10	N/A	2	2	3	N/A
51	L_jap	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0
52	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	0	N/A	3	1	2	N/A	0	3	5
53	L_jap	0	0	N/A	0	0	0	N/A	1	0	0	N/A	0	1	1	N/A	0	4	3	N/A	3	2	3
54	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	1	5
55	L_jap	0	0	N/A	0	0	0	N/A	0	1	3	N/A	0	1	1	N/A	0	1	8	N/A	4	2	6
56	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	1	0
57	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	1	N/A	1	1	1	N/A	0	0	2
58	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	1	N/A	0	1	0
59	L_jap	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	2	0	N/A	0	0	0	N/A	2	1	2
60	L_jap	0	0	N/A	0	1	1	N/A	1	1	1	N/A	0	2	0	N/A	2	1	4	N/A	5	0	4

9/16 Numbers of male wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_het = *L. heterotoma*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
61	L_het	6	6	8	N/A	40	50	50	N/A	50	50	50	N/A	50	50	50	N/A	57	57	57	N/A	37	37
62	L_het	1	2	1	N/A	36	44	45	N/A	45	50	50	N/A	50	50	50	N/A	45	45	45	N/A	45	45
63	L_het	15	20	29	N/A	50	60	56	N/A	60	60	60	N/A	60	60	60	N/A	60	60	60	N/A	60	60
64	L_het	24	28	28	N/A	40	50	50	N/A	50	50	50	N/A	60	60	60	N/A	70	70	70	N/A	56	56
65	L_het	5	6	N/A	16	16	16	N/A	28	28	28	N/A	32	32	32	N/A	32	32	32	N/A	29	29	29
66	L_het	4	7	7	N/A	25	25	30	N/A	40	45	45	N/A	45	45	45	N/A	65	65	65	N/A	58	58
67	L_het	12	20	24	N/A	40	45	45	N/A	45	50	50	N/A	50	50	50	N/A	40	40	40	N/A	34	34
68	L_het	1	1	N/A	4	5	5	N/A	15	16	18	N/A	22	24	24	N/A	39	39	39	N/A	41	41	41
69	L_het	9	19	20	N/A	58	60	70	N/A	70	70	70	N/A	70	67	67	N/A	40	40	40	N/A	50	50
70	L_het	1	1	N/A	1	2	1	N/A	7	8	8	N/A	17	17	17	N/A	22	22	22	N/A	24	24	24
71	L_het	4	4	4	N/A	12	12	12	N/A	19	19	19	N/A	24	24	24	N/A	23	23	23	N/A	26	26
72	L_het	15	23	30	N/A	40	60	60	N/A	60	60	60	N/A	60	60	60	N/A	40	40	40	N/A	45	45
73	L_het	16	21	19	N/A	25	35	35	N/A	35	30	30	N/A	30	30	30	N/A	30	30	30	N/A	30	30
74	L_het	12	20	28	N/A	39	50	50	N/A	50	50	50	N/A	50	50	50	N/A	33	33	33	N/A	25	25
75	L_het	8	22	22	N/A	42	46	46	N/A	50	50	50	N/A	50	50	50	N/A	40	40	40	N/A	45	45
76	L_het	2	1	N/A	21	22	25	N/A	40	50	50	N/A	50	40	40	N/A	35	35	35	N/A	19	19	19
77	L_het	15	18	18	N/A	45	60	60	N/A	60	60	60	N/A	60	60	60	N/A	55	55	55	N/A	40	40
78	L_het	43	40	40	N/A	50	50	50	N/A	50	50	50	N/A	45	45	45	N/A	40	40	40	N/A	55	55
79	L_het	10	9	10	N/A	10	29	29	N/A	28	28	28	N/A	35	35	35	N/A	17	17	17	N/A	0	0
80	L_het	1	1	0	N/A	9	13	12	N/A	12	24	24	N/A	38	38	38	N/A	27	27	27	N/A	28	28
81	L_het	4	8	8	N/A	32	35	40	N/A	40	40	39	N/A	37	37	36	N/A	28	23	21	N/A	24	N/A
82	L_het	1	6	6	N/A	21	26	26	N/A	37	36	26	N/A	25	25	25	N/A	25	24	24	N/A	24	23
83	L_het	1	1	1	N/A	2	3	3	N/A	5	19	19	N/A	36	47	46	N/A	54	53	50	N/A	50	48
84	L_het	5	5	N/A	13	15	16	N/A	27	26	25	N/A	25	25	24	N/A	24	23	23	N/A	20	19	19
85	L_het	3	5	N/A	36	52	55	N/A	52	45	41	N/A	37	35	34	N/A	31	31	27	N/A	24	22	21
86	L_het	1	7	7	N/A	42	45	44	N/A	61	60	60	N/A	57	55	55	N/A	54	54	54	N/A	41	41
87	L_het	3	3	N/A	25	43	43	N/A	40	40	40	N/A	36	36	36	N/A	30	29	29	N/A	27	27	24
88	L_het	2	2	N/A	12	17	17	N/A	27	27	27	N/A	27	28	28	N/A	28	27	27	N/A	27	26	26
89	L_het	1	1	N/A	9	17	17	N/A	28	36	42	N/A	43	36	35	N/A	48	48	78	N/A	32	32	31
90	L_het	6	6	6	N/A	46	43	43	N/A	28	28	25	N/A	25	14	10	N/A	26	21	15	N/A	17	N/A

10/16 Numbers of male wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_het = *L. heterotoma*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
61	L_het	0	2	2	N/A	2	8	1	N/A	6	1	2	N/A	7	2	9	N/A	6	3	3	N/A	10	4
62	L_het	0	0	1	N/A	0	0	0	N/A	2	4	2	N/A	7	2	2	N/A	3	1	1	N/A	2	1
63	L_het	0	0	0	N/A	0	6	4	N/A	5	0	6	N/A	4	1	3	N/A	3	3	0	N/A	3	7
64	L_het	0	0	0	N/A	0	2	0	N/A	5	4	4	N/A	9	1	10	N/A	18	6	13	N/A	5	10
65	L_het	0	0	N/A	0	0	1	N/A	0	1	0	N/A	2	1	0	N/A	8	5	0	N/A	7	0	0
66	L_het	0	0	0	N/A	0	0	0	N/A	0	1	4	N/A	1	2	3	N/A	6	1	2	N/A	4	1
67	L_het	0	1	1	N/A	5	18	4	N/A	7	4	9	N/A	4	1	5	N/A	8	3	3	N/A	7	4
68	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	0	N/A	1	2	1	N/A	3	2	1
69	L_het	0	0	0	N/A	0	3	5	N/A	5	3	5	N/A	18	3	4	N/A	4	1	10	N/A	4	8
70	L_het	0	0	N/A	1	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	0	0	1
71	L_het	1	0	0	N/A	1	0	0	N/A	1	0	1	N/A	2	1	0	N/A	3	1	0	N/A	1	0
72	L_het	0	0	0	N/A	0	4	1	N/A	7	4	5	N/A	2	2	7	N/A	6	7	12	N/A	10	5
73	L_het	0	0	2	N/A	4	0	0	N/A	1	2	0	N/A	2	1	2	N/A	8	3	1	N/A	5	1
74	L_het	0	0	0	N/A	0	1	1	N/A	7	0	4	N/A	2	0	0	N/A	6	4	3	N/A	9	5
75	L_het	0	0	0	N/A	0	0	0	N/A	0	1	1	N/A	4	3	1	N/A	3	1	1	N/A	8	3
76	L_het	0	0	N/A	0	1	1	N/A	8	2	0	N/A	2	2	10	N/A	4	4	3	N/A	3	3	4
77	L_het	0	0	0	N/A	0	2	4	N/A	5	3	6	N/A	5	2	6	N/A	5	0	7	N/A	9	6
78	L_het	1	9	3	N/A	9	4	0	N/A	1	2	2	N/A	0	1	0	N/A	4	2	1	N/A	3	1
79	L_het	0	1	1	N/A	0	5	3	N/A	3	1	2	N/A	3	2	0	N/A	2	0	0	N/A	0	0
80	L_het	0	0	0	N/A	0	0	1	N/A	0	3	1	N/A	2	3	0	N/A	1	2	1	N/A	3	1
81	L_het	0	0	0	N/A	0	0	1	N/A	0	0	1	N/A	2	0	1	N/A	1	2	2	N/A	0	N/A
82	L_het	0	0	0	N/A	2	0	0	N/A	3	1	0	N/A	1	0	0	N/A	0	1	0	N/A	0	1
83	L_het	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	1	1	3	N/A	0	2
84	L_het	0	0	N/A	0	0	0	N/A	0	1	1	N/A	0	0	1	N/A	0	1	0	N/A	0	1	0
85	L_het	0	0	N/A	0	2	1	N/A	3	7	4	N/A	4	2	1	N/A	3	0	4	N/A	2	2	1
86	L_het	0	0	0	N/A	0	0	1	N/A	3	1	0	N/A	3	2	0	N/A	3	0	0	N/A	1	0
87	L_het	0	0	N/A	0	0	0	N/A	3	0	0	N/A	4	0	0	N/A	6	1	0	N/A	2	0	3
88	L_het	0	0	N/A	0	0	0	N/A	2	0	0	N/A	0	0	0	N/A	1	3	0	N/A	0	1	0
89	L_het	0	0	N/A	0	0	0	N/A	1	0	0	N/A	0	0	1	N/A	1	0	0	N/A	1	0	1
90	L_het	0	0	0	N/A	0	3	2	N/A	5	7	3	N/A	2	9	4	N/A	2	5	6	N/A	1	N/A

11/16 Numbers of female wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_het = *L. heterotoma.* Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t102	t96	t108	t114	t120	t126
61	L_het	0	0	0	N/A	0	0	1	N/A	2	5	5	N/A	7	11	11	N/A	19	19	19	N/A	12	12
62	L_het	0	0	0	N/A	0	0	0	N/A	0	11	10	N/A	11	24	24	N/A	17	17	17	N/A	27	27
63	L_het	0	0	0	N/A	0	3	3	N/A	5	19	19	N/A	25	25	25	N/A	27	27	27	N/A	48	48
64	L_het	0	0	0	N/A	0	2	2	N/A	4	11	11	N/A	17	17	17	N/A	11	11	11	N/A	27	27
65	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	5	5	5	N/A	3	3	3	N/A	12	12	12
66	L_het	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	2	2	2	N/A	12	12	12	N/A	11	11
67	L_het	0	0	0	N/A	0	0	0	N/A	2	7	6	N/A	18	15	15	N/A	18	18	18	N/A	18	18
68	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	3	3	N/A	5	5	5	N/A	12	12	12
69	L_het	0	0	0	N/A	0	0	0	N/A	5	9	13	N/A	20	19	19	N/A	24	24	24	N/A	20	20
70	L_het	0	0	N/A	0	0	1	N/A	1	1	1	N/A	3	3	3	N/A	3	3	3	N/A	5	5	5
71	L_het	0	0	0	N/A	0	1	1	N/A	1	1	1	N/A	4	4	4	N/A	4	4	4	N/A	6	6
72	L_het	0	0	0	N/A	1	1	2	N/A	5	25	25	N/A	25	25	25	N/A	23	23	23	N/A	19	19
73	L_het	0	0	0	N/A	0	1	1	N/A	1	10	10	N/A	15	15	15	N/A	22	22	22	N/A	28	28
74	L_het	0	0	0	N/A	0	0	0	N/A	0	8	5	N/A	3	15	15	N/A	12	12	12	N/A	13	13
75	L_het	0	0	0	N/A	0	0	1	N/A	5	20	8	N/A	13	19	19	N/A	21	21	21	N/A	16	16
76	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	11	11	N/A	11	11	11	N/A	23	23	23
77	L_het	0	0	0	N/A	0	0	1	N/A	1	16	15	N/A	15	20	20	N/A	20	20	20	N/A	15	15
78	L_het	0	0	0	N/A	0	7	4	N/A	7	10	10	N/A	20	20	20	N/A	12	12	12	N/A	18	18
79	L_het	0	0	0	N/A	0	1	1	N/A	10	10	10	N/A	10	10	10	N/A	9	9	9	N/A	27	27
80	L_het	0	0	0	N/A	0	0	0	N/A	1	2	2	N/A	3	3	3	N/A	11	11	11	N/A	14	14
81	L_het	0	0	0	N/A	0	0	1	N/A	4	7	9	N/A	22	30	25	N/A	20	19	19	N/A	11	N/A
82	L_het	0	0	0	N/A	0	0	0	N/A	2	0	3	N/A	6	11	9	N/A	14	16	16	N/A	17	17
83	L_het	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	6	2	12	N/A	12	34
84	L_het	0	0	N/A	0	0	0	N/A	3	0	3	N/A	7	13	13	N/A	18	20	19	N/A	22	21	20
85	L_het	0	0	N/A	0	0	0	N/A	2	2	3	N/A	5	21	20	N/A	26	32	28	N/A	26	21	15
86	L_het	0	0	0	N/A	0	0	0	N/A	2	0	2	N/A	7	19	15	N/A	36	36	28	N/A	18	16
87	L_het	0	0	N/A	0	1	1	N/A	5	2	6	N/A	12	14	14	N/A	18	21	20	N/A	17	17	17
88	L_het	0	0	N/A	0	0	0	N/A	1	1	5	N/A	9	9	8	N/A	13	31	30	N/A	32	29	28
89	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	2	3	N/A	5	12	11	N/A	8	9	9
90	L_het	0	0	0	N/A	0	0	0	N/A	3	12	12	N/A	22	11	21	N/A	18	22	13	N/A	15	N/A

12/16 Numbers of female wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_het = *L. heterotoma.* Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
61	L_het	0	0	0	N/A	0	0	0	N/A	0	0	2	N/A	1	1	4	N/A	3	3	2	N/A	2	2
62	L_het	0	0	0	N/A	0	0	0	N/A	0	0	5	N/A	1	4	5	N/A	2	5	2	N/A	1	2
63	L_het	0	0	0	N/A	0	0	0	N/A	0	2	0	N/A	0	2	0	N/A	3	2	2	N/A	1	2
64	L_het	0	0	0	N/A	0	0	2	N/A	1	0	1	N/A	1	0	3	N/A	4	5	4	N/A	5	3
65	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	2	0	N/A	0	0	0	N/A	1	2	0
66	L_het	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	3	3	5	N/A	1	2
67	L_het	0	0	0	N/A	0	0	0	N/A	0	1	4	N/A	4	6	7	N/A	1	5	2	N/A	7	4
68	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	1	0	0	N/A	2	2	1
69	L_het	0	0	0	N/A	0	0	0	N/A	0	1	4	N/A	7	5	2	N/A	7	2	6	N/A	3	6
70	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	2	2	1	N/A	1	1	0
71	L_het	0	0	0	N/A	0	1	1	N/A	0	0	0	N/A	0	1	0	N/A	0	1	0	N/A	1	0
72	L_het	0	0	0	N/A	0	0	2	N/A	0	3	3	N/A	4	5	1	N/A	2	4	4	N/A	2	2
73	L_het	0	0	0	N/A	0	0	0	N/A	0	1	2	N/A	1	1	3	N/A	2	1	3	N/A	0	1
74	L_het	0	0	0	N/A	0	0	0	N/A	0	1	5	N/A	3	4	2	N/A	3	3	2	N/A	8	8
75	L_het	0	0	0	N/A	0	0	1	N/A	0	0	12	N/A	1	2	2	N/A	1	2	2	N/A	0	1
76	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	3	0	3	N/A	4	8	6
77	L_het	0	0	0	N/A	0	0	0	N/A	0	2	8	N/A	4	9	10	N/A	3	4	8	N/A	2	4
78	L_het	0	0	0	N/A	0	0	3	N/A	0	4	3	N/A	5	3	3	N/A	3	3	3	N/A	2	1
79	L_het	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	0	N/A	0	1	0	N/A	1	1
80	L_het	0	0	0	N/A	0	0	0	N/A	0	1	0	N/A	1	0	1	N/A	2	1	5	N/A	6	7
81	L_het	0	0	0	N/A	0	0	0	N/A	1	0	1	N/A	0	0	5	N/A	0	1	0	N/A	0	N/A
82	L_het	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	3	N/A	2	1	0	N/A	0	0
83	L_het	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	2	N/A	1	6
84	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	1	N/A	0	2	1	N/A	0	1	1
85	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	6	1	N/A	4	4	4	N/A	4	5	6
86	L_het	0	0	0	N/A	0	0	0	N/A	0	1	2	N/A	1	2	5	N/A	2	4	8	N/A	3	2
87	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	1	N/A	1	2	1	N/A	1	0	0
88	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	1	N/A	0	1	1	N/A	1	3	1
89	L_het	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	1	0
90	L_het	0	0	0	N/A	0	0	1	N/A	0	2	0	N/A	3	11	17	N/A	10	2	9	N/A	10	N/A

13/16 Numbers of male wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_pac = *L. pacifica*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
91	L_pac	9	9	N/A	12	6	6	N/A	6	6	2	N/A	1	1	1	N/A	1	1	1	N/A	1	1	1
92	L_pac	7	7	N/A	7	14	14	N/A	0	1	1	N/A	3	5	6	N/A	3	4	4	N/A	4	4	4
93	L_pac	2	12	13	N/A	12	13	13	N/A	13	13	13	N/A	12	11	13	N/A	13	1	1	N/A	5	5
94	L_pac	8	9	N/A	11	19	19	N/A	19	18	18	N/A	16	16	14	N/A	14	14	14	N/A	12	12	12
95	L_pac	13	13	N/A	17	43	40	N/A	40	38	38	N/A	37	28	24	N/A	22	22	21	N/A	14	12	12
96	L_pac	2	2	N/A	3	19	20	N/A	20	24	25	N/A	25	24	22	N/A	21	19	18	N/A	21	21	20
97	L_pac	1	7	7	N/A	7	12	11	N/A	11	14	14	N/A	14	14	14	N/A	14	13	12	N/A	5	5
98	L_pac	1	1	N/A	1	5	5	N/A	13	16	15	N/A	15	13	10	N/A	10	8	8	N/A	2	1	1
99	L_pac	7	8	N/A	14	24	31	N/A	33	33	31	N/A	31	28	27	N/A	29	27	27	N/A	32	29	28
100	L_pac	2	17	17	N/A	17	34	34	N/A	34	31	29	N/A	27	23	23	N/A	21	17	16	N/A	15	13
101	L_pac	1	1	N/A	1	13	14	N/A	19	23	28	N/A	30	27	27	N/A	27	26	22	N/A	25	21	20
102	L_pac	1	1	1	N/A	1	1	1	N/A	1	1	1	N/A	1	1	1	N/A	0	0	0	N/A	1	1
103	L_pac	1	1	N/A	2	3	3	N/A	1	1	1	N/A	0	0	0	N/A	0	0	0	N/A	0	N/A	N/A
104	L_pac	1	8	10	N/A	10	12	12	N/A	11	8	8	N/A	8	8	8	N/A	8	8	8	N/A	8	8
105	L_pac	1	2	7	N/A	8	26	28	N/A	26	25	23	N/A	23	23	14	N/A	13	13	12	N/A	12	11
106	L_pac	1	4	10	N/A	12	45	43	N/A	45	47	49	N/A	49	41	64	N/A	61	49	39	N/A	29	21
107	L_pac	4	N/A	4	14	15	N/A	16	25	24	N/A	23	23	27	N/A	25	22	16	N/A	10	9	8	N/A
108	L_pac	2	N/A	4	27	27	N/A	24	23	21	N/A	20	20	18	N/A	26	26	24	N/A	23	21	20	N/A
109	L_pac	1	2	N/A	2	13	13	N/A	10	24	27	N/A	24	23	27	N/A	38	37	31	N/A	29	14	10
110	L_pac	3	9	N/A	11	28	27	N/A	28	26	28	N/A	26	24	20	N/A	28	25	22	N/A	21	20	20
111	L_pac	4	N/A	5	8	7	N/A	7	14	14	N/A	10	11	9	N/A	15	12	12	N/A	7	7	3	N/A
112	L_pac	2	N/A	4	15	14	N/A	14	15	10	N/A	10	10	10	N/A	8	6	5	N/A	7	7	7	N/A
113	L_pac	2	7	N/A	8	23	24	N/A	23	33	33	N/A	32	32	39	N/A	35	34	33	N/A	22	16	13
114	L_pac	1	4	8	N/A	12	15	19	N/A	14	29	27	N/A	25	23	21	N/A	20	20	19	N/A	17	15
115	L_pac	2	7	15	N/A	15	23	23	N/A	21	24	26	N/A	21	21	14	N/A	14	14	14	N/A	14	12
116	L_pac	7	N/A	9	29	28	N/A	26	43	41	N/A	35	33	23	N/A	18	18	16	N/A	13	12	10	N/A
117	L_pac	3	N/A	3	35	38	N/A	35	36	37	N/A	34	32	22	N/A	28	28	26	N/A	25	22	21	N/A
118	L_pac	1	3	17	N/A	19	36	35	N/A	34	38	39	N/A	32	31	30	N/A	26	26	26	N/A	25	23
119	L_pac	3	N/A	3	15	17	N/A	17	34	36	N/A	35	35	29	N/A	38	38	34	N/A	29	28	25	N/A
120	L_pac	1	7	7	N/A	6	12	9	N/A	9	21	19	N/A	17	11	5	N/A	8	7	7	N/A	9	6

14/16 Numbers of male wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_pac = *L. pacifica*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
91	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0
92	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0
93	L_pac	0	0	0	N/A	1	3	0	N/A	0	1	0	N/A	1	1	1	N/A	0	3	0	N/A	0	0
94	L_pac	0	0	N/A	0	0	0	N/A	0	1	0	N/A	2	1	2	N/A	0	0	0	N/A	2	0	0
95	L_pac	0	0	N/A	0	3	3	N/A	0	2	0	N/A	1	9	4	N/A	0	0	2	N/A	1	2	0
96	L_pac	0	0	N/A	0	0	2	N/A	0	1	0	N/A	0	1	2	N/A	1	2	1	N/A	4	0	1
97	L_pac	0	0	0	N/A	0	0	1	N/A	0	1	0	N/A	0	0	0	N/A	0	1	1	N/A	2	0
98	L_pac	0	0	N/A	0	0	0	N/A	0	1	1	N/A	0	0	0	N/A	0	3	0	N/A	0	1	0
99	L_pac	0	0	N/A	0	0	4	N/A	1	1	2	N/A	0	3	1	N/A	0	2	0	N/A	0	3	1
100	L_pac	0	0	0	N/A	1	2	0	N/A	0	5	2	N/A	2	4	0	N/A	2	7	1	N/A	1	2
101	L_pac	0	0	N/A	0	0	0	N/A	0	1	3	N/A	4	3	0	N/A	0	1	4	N/A	1	4	1
102	L_pac	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
103	L_pac	0	0	N/A	0	1	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	N/A	N/A
104	L_pac	0	0	0	N/A	1	0	0	N/A	0	3	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
105	L_pac	0	0	0	N/A	0	0	0	N/A	4	1	2	N/A	1	0	0	N/A	0	1	3	N/A	0	1
106	L_pac	0	0	0	N/A	0	0	3	N/A	1	1	2	N/A	0	7	0	N/A	7	12	10	N/A	7	8
107	L_pac	0	N/A	0	0	0	N/A	1	5	1	N/A	0	0	0	N/A	0	3	9	N/A	6	1	1	N/A
108	L_pac	0	N/A	1	1	0	N/A	3	3	3	N/A	1	0	0	N/A	0	1	2	N/A	1	2	1	N/A
109	L_pac	0	0	N/A	0	0	0	N/A	4	3	2	N/A	1	1	0	N/A	0	1	6	N/A	2	15	4
110	L_pac	0	0	N/A	0	0	1	N/A	2	3	4	N/A	2	2	0	N/A	0	4	3	N/A	1	1	0
111	L_pac	0	N/A	0	0	2	N/A	0	1	0	N/A	0	1	0	N/A	0	3	0	N/A	2	0	6	N/A
112	L_pac	0	N/A	0	1	1	N/A	0	6	5	N/A	0	0	2	N/A	1	3	1	N/A	2	0	0	N/A
113	L_pac	0	0	N/A	0	0	0	N/A	1	1	0	N/A	1	0	0	N/A	0	1	1	N/A	1	6	3
114	L_pac	0	0	0	N/A	0	1	1	N/A	5	1	2	N/A	0	1	0	N/A	0	0	1	N/A	2	2
115	L_pac	0	0	0	N/A	1	2	0	N/A	2	1	1	N/A	0	0	0	N/A	0	1	0	N/A	0	2
116	L_pac	0	N/A	0	0	5	N/A	5	1	3	N/A	1	5	0	N/A	2	2	2	N/A	3	1	2	N/A
117	L_pac	0	N/A	0	1	1	N/A	3	1	3	N/A	1	2	0	N/A	1	0	2	N/A	1	3	1	N/A
118	L_pac	0	0	0	N/A	0	0	2	N/A	2	1	2	N/A	1	2	0	N/A	0	0	0	N/A	1	2
119	L_pac	0	N/A	0	0	0	N/A	0	4	0	N/A	0	1	0	N/A	1	0	4	N/A	5	1	3	N/A
120	L_pac	0	0	0	N/A	2	3	3	N/A	0	2	0	N/A	1	8	6	N/A	1	1	0	N/A	0	1

15/16 Numbers of female wasps that had emerged/were present in each jar per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_pac = *L. pacifica*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t102	t96	t108	t114	t120	t126
91	L_pac	0	0	N/A	0	0	0	N/A	0	9	9	N/A	17	9	16	N/A	16	16	16	N/A	20	20	19
92	L_pac	0	0	N/A	0	0	0	N/A	0	13	12	N/A	13	20	19	N/A	22	19	21	N/A	24	21	21
93	L_pac	0	0	0	N/A	0	5	5	N/A	6	17	27	N/A	27	26	26	N/A	32	26	32	N/A	36	36
94	L_pac	0	0	N/A	0	1	1	N/A	1	4	16	N/A	18	21	25	N/A	37	29	37	N/A	37	37	37
95	L_pac	0	0	N/A	0	0	0	N/A	2	0	2	N/A	6	23	21	N/A	32	31	31	N/A	43	41	41
96	L_pac	0	0	N/A	0	0	0	N/A	0	1	5	N/A	5	19	22	N/A	27	22	27	N/A	30	29	29
97	L_pac	0	0	0	N/A	0	5	5	N/A	6	20	18	N/A	18	28	27	N/A	26	26	25	N/A	23	20
98	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	6	7	N/A	12	11	12	N/A	14	14	14
99	L_pac	0	0	N/A	0	0	0	N/A	0	11	13	N/A	27	16	27	N/A	41	41	40	N/A	43	43	43
100	L_pac	0	0	0	N/A	0	4	4	N/A	5	8	19	N/A	19	21	24	N/A	27	27	27	N/A	27	27
101	L_pac	0	0	N/A	0	0	0	N/A	0	0	1	N/A	3	14	26	N/A	32	24	29	N/A	27	25	21
102	L_pac	0	0	0	N/A	0	3	3	N/A	3	3	3	N/A	3	4	4	N/A	4	4	4	N/A	6	6
103	L_pac	2	2	N/A	3	2	3	N/A	3	3	3	N/A	3	3	3	N/A	3	2	2	N/A	1	N/A	N/A
104	L_pac	0	0	0	N/A	0	5	3	N/A	4	5	17	N/A	31	19	30	N/A	35	34	34	N/A	37	36
105	L_pac	0	0	0	N/A	0	2	2	N/A	5	13	14	N/A	13	13	41	N/A	39	41	39	N/A	42	42
106	L_pac	0	0	0	N/A	0	2	3	N/A	3	9	10	N/A	10	8	20	N/A	33	19	32	N/A	36	36
107	L_pac	0	N/A	0	1	1	N/A	1	6	5	N/A	6	6	9	N/A	28	11	28	N/A	28	28	28	N/A
108	L_pac	0	N/A	0	1	2	N/A	2	18	11	N/A	16	16	38	N/A	46	46	44	N/A	41	41	40	N/A
109	L_pac	0	0	N/A	0	2	3	N/A	4	22	24	N/A	18	18	18	N/A	34	31	33	N/A	38	38	34
110	L_pac	0	0	N/A	0	0	1	N/A	1	12	13	N/A	15	15	32	N/A	39	39	39	N/A	37	36	36
111	L_pac	0	N/A	0	0	0	N/A	0	15	15	N/A	11	11	31	N/A	26	30	25	N/A	29	29	29	N/A
112	L_pac	0	N/A	0	0	0	N/A	0	17	15	N/A	14	13	31	N/A	39	30	39	N/A	48	48	48	N/A
113	L_pac	0	0	N/A	0	1	3	N/A	3	14	12	N/A	11	11	24	N/A	24	24	24	N/A	22	22	22
114	L_pac	0	0	0	N/A	0	4	5	N/A	6	10	8	N/A	10	12	37	N/A	33	35	33	N/A	32	31
115	L_pac	0	0	0	N/A	1	5	10	N/A	10	21	35	N/A	35	35	37	N/A	37	37	37	N/A	37	37
116	L_pac	0	N/A	0	2	5	N/A	5	11	17	N/A	15	15	36	N/A	41	41	41	N/A	41	41	41	N/A
117	L_pac	0	N/A	0	0	4	N/A	3	4	12	N/A	12	12	24	N/A	25	25	23	N/A	23	22	22	N/A
118	L_pac	0	0	0	N/A	1	3	6	N/A	6	7	17	N/A	17	17	27	N/A	29	29	29	N/A	27	27
119	L_pac	0	N/A	0	0	1	N/A	1	2	7	N/A	7	10	17	N/A	21	21	21	N/A	21	21	21	N/A
120	L_pac	0	0	0	N/A	0	13	13	N/A	13	13	21	N/A	44	45	39	N/A	39	34	32	N/A	45	45

16/16 Numbers of female wasps that had dispersed per observation time point (t), starting from the first emergence of wasps in a jar (t0). *N* = 30, L_pac = *L. pacifica*. Corresponds to Fig. 1 - 5.

ID	Species	t0	t6	t12	t18	t24	t30	t36	t42	t48	t54	t60	t66	t72	t78	t84	t90	t96	t102	t108	t114	t120	t126
91	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	0	0	1
92	L_pac	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	1	N/A	0	2	1	N/A	0	3	0
93	L_pac	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	1	N/A	0	1	0	N/A	0	0
94	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	1	0	N/A	0	0	0	N/A	0	0	0
95	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	1	1	2	N/A	6	1	0	N/A	0	2	0
96	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	1	0
97	L_pac	0	0	0	N/A	0	0	0	N/A	1	0	2	N/A	0	0	1	N/A	1	0	1	N/A	2	3
98	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	2	0	N/A	0	0	0
99	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	1	0	0
100	L_pac	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	4	1	0	N/A	1	0
101	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	3	2	3	N/A	5	2	4
102	L_pac	0	0	0	N/A	0	1	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0
103	L_pac	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	0	N/A	0	N/A	N/A
104	L_pac	0	0	0	N/A	0	0	2	N/A	0	1	1	N/A	0	3	1	N/A	1	1	0	N/A	0	1
105	L_pac	0	0	0	N/A	0	0	0	N/A	0	1	5	N/A	1	0	0	N/A	0	2	0	N/A	5	0
106	L_pac	0	0	0	N/A	0	0	0	N/A	0	1	0	N/A	0	2	2	N/A	0	1	1	N/A	2	0
107	L_pac	0	N/A	0	0	0	N/A	0	4	1	N/A	0	0	1	N/A	2	0	0	N/A	0	0	0	N/A
108	L_pac	0	N/A	0	0	0	N/A	0	0	4	N/A	2	1	0	N/A	0	0	2	N/A	3	0	1	N/A
109	L_pac	0	0	N/A	0	0	0	N/A	0	3	1	N/A	3	0	0	N/A	2	0	1	N/A	2	0	4
110	L_pac	0	0	N/A	0	0	0	N/A	0	0	1	N/A	1	0	0	N/A	0	2	0	N/A	3	1	0
111	L_pac	0	N/A	0	0	0	N/A	0	0	0	N/A	1	0	0	N/A	1	4	1	N/A	0	0	0	N/A
112	L_pac	0	N/A	0	0	0	N/A	0	1	2	N/A	0	0	0	N/A	1	1	0	N/A	1	0	0	N/A
113	L_pac	0	0	N/A	0	0	0	N/A	0	1	2	N/A	0	0	0	N/A	1	0	0	N/A	2	0	0
114	L_pac	0	0	0	N/A	0	0	1	N/A	0	0	3	N/A	0	0	0	N/A	1	4	0	N/A	1	1
115	L_pac	0	0	0	N/A	0	0	0	N/A	0	0	2	N/A	0	0	0	N/A	1	0	0	N/A	0	0
116	L_pac	0	N/A	0	0	1	N/A	0	0	2	N/A	0	1	0	N/A	0	0	0	N/A	0	0	0	N/A
117	L_pac	0	N/A	0	0	2	N/A	1	0	0	N/A	0	0	1	N/A	1	0	2	N/A	0	1	0	N/A
118	L_pac	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	1	0	0	N/A	2	0
119	L_pac	0	N/A	0	0	0	N/A	0	0	0	N/A	0	0	1	N/A	0	0	0	N/A	0	0	0	N/A
120	L_pac	0	0	0	N/A	0	0	1	N/A	0	1	0	N/A	0	1	5	N/A	2	5	2	N/A	1	0

Supplementary Table 2 Attraction of male wasps of the species *L. ryukuyensis* (**A**), *L. japonica* (**B**), *L. heterotoma* (**C**), and *L. pacifica* (**D**) to conspecific females in olfactometer experiments. Males had the choice between a host patch or a host patch occuppied with 20 conspecific females. Experiments lasted until 30 wasps had crossed a decision line, each within 5 min. Corresponds to Fig. 6A

A

Nr	Side of females	Decision	Time [s]	Attraction to females?
1	left	left	225	yes
2	right	right	73	yes
3	right	right	260	yes
4	left	left	16	yes
5	left	left	20	yes
6	right	right	48	yes
7	right	right	232	yes
8	left	left	70	yes
9	left	left	176	yes
10	right	right	245	yes
11	right	0	>5 min	
12	right	right	134	yes
13	left	left	283	yes
14	right	0	>5 min	
15	right	right	162	yes
16	left	left	41	yes
17	right	right	29	yes
18	left	left	43	yes
19	left	0	>5 min	
20	left	left	23	yes
21	right	right	53	yes
22	right	left	82	no
23	left	left	22	yes
24	right	right	73	yes
25	left	left	36	yes
26	left	left	104	yes
27	right	left	194	no
28	right	right	38	yes
29	left	0	>5 min	
30	left	right	46	no
31	left	left	81	yes
32	right	right	20	yes
33	left	right	215	no
34	right	0	>5 min	
35	left	left	91	yes

B

Nr	Side of females	Decision	Time [s]	Attraction to females?
1	left	left	149	yes
2	right	right	255	yes
3	left	left	28	yes
4	right	right	22	yes
5	left	left	258	yes
6	right	0	>5 min	
7	right	right	171	yes
8	left	left	25	yes
9	right	right	88	yes
10	left	right	36	no
11	right	left	98	no
12	left	left	208	yes
13	right	right	39	yes
14	left	left	17	yes
15	right	right	51	yes
16	left	left	15	yes
17	right	left	23	no
18	left	left	46	yes
19	right	0	>5 min	
20	right	right	14	yes
21	left	left	24	yes
22	right	right	21	yes
23	left	left	19	yes
24	right	right	25	yes
25	left	left	17	yes
26	left	left	24	yes
27	right	right	76	yes
28	left	left	72	yes
29	right	right	57	yes
30	left	left	79	yes
31	right	right	56	yes
32	left	left	169	yes

C

Nr	Side of females	Decision	Time [s]	Attraction to females?
1	left	left	152	yes
2	right	right	55	yes
3	left	left	30	yes
4	right	right	12	yes
5	left	left	275	yes
6	right	right	89	yes
7	left	0	>5 min	
8	left	0	>5 min	
9	right	right	29	yes
10	left	left	22	yes
11	right	left	272	no
12	left	right	68	no
13	left	left	52	yes
14	right	right	85	yes
15	left	left	283	yes
16	right	right	49	yes
17	left	left	46	yes
18	right	right	24	yes
19	left	0	>5 min	
20	left	right	42	no
21	right	right	12	yes
22	left	right	122	no
23	right	right	76	yes
24	left	left	24	yes
25	right	right	179	yes
26	left	left	37	yes
27	right	right	29	yes
28	left	left	18	yes
29	right	right	44	yes
30	left	left	20	yes
31	right	right	13	yes
32	left	left	68	yes
33	right	right	61	yes

D

Nr	Side of females	Decision	Time [s]	Attraction to females?
1	right	left	21	no
2	left	right	18	no
3	right	left	13	no
4	left	right	19	no
5	right	right	81	yes
6	left	left	14	yes
7	right	right	59	yes
8	left	left	18	yes
9	right	right	48	yes
10	left	right	20	no
11	right	right	15	yes
12	left	left	11	yes
13	right	left	64	no
14	left	left	14	yes
15	right	right	19	yes
16	left	left	17	yes
17	right	left	31	no
18	left	left	14	yes
19	right	right	32	yes
20	left	left	22	yes
21	right	right	39	yes
22	left	left	40	yes
23	right	right	65	yes
24	left	left	13	yes
25	right	right	16	yes
26	left	left	29	yes
27	right	right	35	yes
28	left	left	19	yes
29	right	left	41	no
30	left	left	146	yes

Supplementary Table 3 Attraction of male wasps of the species *L. ryukuyensis* (**A**), *L. japonica* (**B**), *L. heterotoma* (**C**), and *L. pacifica* (**D**) to extracts of conspecific females in olfactometer experiments. Males had the choice between the odor of a host patch and 2 µl of Dichloromethane (DCM), or the odor of a host patch and 2 µl of an extract of conspecific females (10 µl DCM per female). Experiments lasted until 30 wasps had crossed a decision line, each within 5 min. Corresponds to Fig. 6B

A

Nr	Side females' extract	Decision	Time [s]	Attraction to females' extract?
1	left	left	68	yes
2	right	0	>5 min	
3	right	left	178	no
4	right	0	>5 min	
5	right	0	>5 min	
6	left	0	>5 min	
7	right	right	16	yes
8	left	right	61	no
9	left	0	>5 min	
10	left	0	>5 min	
11	right	left	35	no
12	left	right	20	no
13	right	0	>5 min	
14	left	left	122	yes
15	right	0	>5 min	
16	left	0	>5 min	
17	right	0	>5 min	
18	left	0	>5 min	
19	right	0	>5 min	
20	left	right	145	no
21	right	0	>5 min	
22	left	0	>5 min	
23	right	left	231	no
24	left	left	67	yes
25	left	0	>5 min	
26	right	0	>5 min	
27	left	left	124	yes
28	right	0	>5 min	
29	left	left	28	yes
30	right	right	95	yes
31	left	left	210	yes
32	right	right	183	yes
33	left	0	>5 min	
34	right	right	123	yes
35	left	0	>5 min	
36	left	0	>5 min	
37	left	0	>5 min	
38	right	0	>5 min	
39	left	left	82	yes
40	right	right	101	yes
41	left	0	>5 min	
42	right	0	>5 min	
43	left	left	98	yes
44	right	right	94	yes
45	left	left	193	yes
46	right	right	71	yes
47	left	right	52	no
48	right	right	101	yes
49	left	left	174	yes
50	right	0	>5 min	
51	left	left	129	yes
52	right	right	83	yes
53	left	left	181	yes
54	right	left	209	no
55	left	left	219	yes

B

Nr	Side females' extract	Decision	Time [s]	Attraction to females' extract?
1	left	left	33	yes
2	right	right	36	yes
3	left	left	179	yes
4	right	right	41	yes
5	left	left	297	yes
6	right	right	73	yes
7	left	left	230	yes
8	right	right	28	yes
9	left	0	>5 min	0
10	left	left	125	yes
11	right	right	168	yes
12	left	right	38	no
13	right	right	38	yes
14	left	0	>5 min	0
15	left	left	75	yes
16	right	right	107	yes
17	left	left	68	yes
18	right	right	14	yes
19	left	left	243	yes
20	right	right	73	yes
21	left	left	43	yes
22	right	right	19	yes
23	left	right	281	no
24	right	left	79	no
25	left	left	32	yes
26	right	right	20	yes
27	left	left	163	yes
28	right	right	19	yes
29	left	left	98	yes
30	right	0	>5 min	0
31	left	left	45	yes
32	right	left	69	no
33	right	right	100	yes

C

Nr	Side females' extract	Decision	Time [s]	Attraction to females' extract?
1	left	left	131	yes
2	right	right	45	yes
3	left	left	20	yes
4	right	right	31	yes
5	left	left	19	yes
6	right	right	30	yes
7	left	left	31	yes
8	right	right	68	yes
9	left	left	56	yes
10	right	right	20	yes
11	left	right	29	no
12	right	right	17	yes
13	left	left	15	yes
14	right	right	8	yes
15	left	right	44	no
16	right	right	22	yes
17	left	left	59	yes
18	right	right	46	yes
19	left	left	42	yes
20	right	right	21	yes
21	left	left	150	yes
22	right	right	55	yes
23	left	left	63	yes
24	right	right	13	yes
25	left	left	170	yes
26	right	right	183	yes
27	left	right	28	no
28	right	right	34	yes
29	left	left	25	yes
30	right	right	82	yes

D

Nr	Side females' extract	Decision	Time [s]	Attraction to females' extract?
1	right	right	13	yes
2	left	left	9	yes
3	right	right	15	yes
4	left	left	14	yes
5	right	left	27	no
6	left	left	21	yes
7	right	right	22	yes
8	left	left	11	yes
9	right	right	19	yes
10	left	left	11	yes
11	right	left	55	no
12	left	left	21	yes
13	right	right	12	yes
14	left	left	12	yes
15	right	left	190	no
16	left	left	14	yes
17	right	right	14	yes
18	left	left	13	yes
19	right	right	36	yes
20	left	left	15	yes
21	right	right	12	yes
22	left	left	12	yes
23	right	right	35	yes
24	left	left	25	yes
25	right	right	10	yes
26	left	left	35	yes
27	right	left	105	no
28	left	left	21	yes
29	right	right	14	yes
30	left	left	18	yes

Supplementary Table 4 Attraction of male wasps of the species *L. ryukuyensis* (**A**), *L. japonica* (**B**), *L. heterotoma* (**C**), *and L. pacifica* (**D**) to host patches in olfactometer experiments. Males had the choice between a host patch or clean humidified air. Experiments lasted until 30 wasps had crossed a decision line, each within 10 min. Corresponds to Fig. 6C

A

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	right	22	no
2	right	right	31	yes
3	left	left	32	yes
4	right	left	140	no
5	left	0	>10 min	0
6	left	right	41	no
7	right	left	102	no
8	left	left	11	yes
9	right	right	118	yes
10	left	right	425	no
11	right	0	>10 min	0
12	right	right	54	yes
13	right	right	66	yes
14	left	left	16	yes
15	left	right	22	no
16	right	left	36	no
17	left	left	31	yes
18	right	right	47	yes
19	right	left	217	no
20	left	right	46	no
21	left	right	105	no
22	right	left	73	no
23	left	left	335	yes
24	right	left	53	no
25	right	right	43	yes
26	left	left	51	yes
27	left	left	100	yes
28	right	left	214	no
29	right	right	45	yes
30	left	left	71	yes
31	left	left	99	yes
32	right	right	61	yes

B

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	right	right	470	yes
2	left	left	100	yes
3	right	left	27	no
4	left	right	27	no
5	left	right	121	no
6	right	left	36	no
7	right	left	540	no
8	left	right	32	no
9	right	left	31	no
10	left	right	21	no
11	left	left	106	yes
12	right	right	34	yes
13	left	left	145	yes
14	right	right	26	yes
15	right	right	142	yes
16	left	right	23	no
17	left	left	116	yes
18	right	right	81	yes
19	left	right	41	no
20	right	right	175	yes
21	right	right	25	yes
22	left	right	252	no
23	left	right	280	no
24	right	left	129	no
25	right	right	179	yes
26	left	right	105	no
27	right	left	308	no
28	left	right	19	no
29	left	right	31	no
30	right	left	25	no

C

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	left	22	yes
2	right	right	44	yes
3	left	right	17	no
4	right	left	23	no
5	left	left	12	yes
6	right	right	37	yes
7	left	right	180	no
8	right	right	25	yes
9	left	left	187	yes
10	right	left	222	no
11	left	right	45	no
12	right	left	93	no
13	left	right	24	no
14	right	left	224	no
15	left	right	46	no
16	right	right	13	yes
17	left	left	414	yes
18	right	left	274	no
19	left	right	44	no
20	right	right	185	yes
21	left	right	27	no
22	right	left	28	no
23	left	left	30	yes
24	left	left	22	yes
25	right	right	468	yes
26	left	right	469	no
27	right	left	294	no
28	left	right	290	no
29	right	left	74	no
30	left	right	40	no

D

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	left	13	yes
2	right		>10 min	
3	right	left	481	no
4	left	left	11	yes
5	right	left	69	no
6	left	left	22	yes
7	right	left	14	no
8	left	right	47	no
9	right	right	94	yes
10	left	left	22	yes
11	right	left	26	no
12	left	right	367	no
13	right		>10 min	
14	right	left	99	no
15	left	left	20	yes
16	right	left	249	no
17	left	right	35	no
18	right		>10 min	
19	right	right	13	yes
20	left	left	39	yes
21	right	left	79	no
22	left	left	216	yes
23	right	right	56	yes
24	left	right	94	no
25	right	left	181	no
26	left	left	21	yes
27	right	right	235	yes
28	left	right	21	no
29	right	left	214	no
30	left	right	109	no
31	left	left	81	yes
32	left	left	56	yes
33	right	left	37	no

Supplementary Table 5 Attraction of female wasps of the species *L. ryukuyensis* (**A**), *L. japonica* (**B**), *L. heterotoma* (**C**), and *L. pacifica* (**D**) to conspecific males in olfactometer experiments. Females had the choice between a host patch or a host patch occuppied with 20 conspecific males. Experiments lasted until 30 wasps had crossed a decision line, each within 5 min. Corresponds to Fig. 7A

A

Nr	Side of males	Decision	Time [s]	Attraction to males?
1	left	0	>5 min	
2	left	right	156	no
3	right	left	239	no
4	left	0	>5 min	
5	left	left	281	yes
6	right	left	297	no
7	left	right	146	no
8	right	right	148	yes
9	left	0	>5 min	
10	left	0	>5 min	
11	right	left	68	no
12	left	left	26	yes
13	left	left	141	yes
14	right	left	54	no
15	left	left	126	yes
16	right	right	87	yes
17	left	right	177	no
18	right	right	212	yes
19	left	right	43	no
20	right	right	146	yes
21	left	left	213	yes
22	right	left	31	no
23	left	right	134	no
24	right	right	219	yes
25	left	right	166	no
26	right	right	180	yes
27	left	right	267	no
28	right	right	264	yes
29	left	left	160	yes
30	right	right	75	yes
31	left	0	>5 min	
32	left	left	128	yes
33	left	right	109	no
34	right	0	>5 min	
35	right	left	51	no
36	right	left	42	no

B

Nr	Side of males	Decision	Time [s]	Attraction to males?
1	left	left	268	yes
2	right	right	180	yes
3	left	right	229	no
4	right	left	44	no
5	left	right	37	no
6	right	left	93	no
7	left	right	171	no
8	right	right	73	yes
9	left	left	170	yes
10	right	left	13	no
11	left	left	113	yes
12	right	left	120	no
13	left	right	50	no
14	right	right	9	yes
15	left	left	123	yes
16	right	left	252	no
17	left	left	126	yes
18	right	right	81	yes
19	left	left	68	yes
20	right	left	18	no
21	left	right	75	no
22	right	left	49	no
23	left	left	259	yes
24	left	left	184	yes
25	right	right	27	yes
26	left	right	179	no
27	right	left	39	no
28	left	left	89	yes
29	right	left	128	no
30	left	right	34	no

C

Nr	Side of males	Decision	Time [s]	Attraction to males?
1	right	right	149	yes
2	left	right	47	no
3	right	right	43	yes
4	left	left	51	yes
5	right	right	282	yes
6	left	right	209	no
7	right	left	22	no
8	left	0		
9	left	left	23	yes
10	right	left	124	no
11	left	right	212	no
12	right	right	131	yes
13	left	right	18	no
14	right	right	213	yes
15	left	left	173	yes
16	right	left	87	no
17	left	left	40	yes
18	right	left	83	no
19	left	right	229	no
20	right	right	67	yes
21	left	right	85	no
22	right	left	46	no
23	left	right	287	no
24	right	0		
25	right	left	28	no
26	left	right	150	no
27	right	left	257	no
28	left	right	87	no
29	right	right	178	yes
30	left	left	45	yes
31	right	right	155	yes
32	left	right	147	no

D

Nr	Side of males	Decision	Time [s]	Attraction to males?
1	right	left	11	no
2	left	right	33	no
3	right	left	62	no
4	left	right	13	no
5	right	right	10	yes
6	left	right	8	no
7	right	left	34	no
8	left	right	19	no
9	right	right	16	yes
10	left	left	33	yes
11	right	left	95	no
12	left	right	27	no
13	right	right	12	yes
14	left	right	26	no
15	right	right	20	yes
16	left	left	34	yes
17	right	right	33	yes
18	left	right	11	no
19	right	right	16	yes
20	left	right	31	no
21	right	left	29	no
22	left	right	42	no
23	right	left	52	no
24	left	right	42	no
25	right	right	44	yes
26	left	right	86	no
27	right	left	119	no
28	left	left	155	yes
29	right	left	189	no
30	right	left	81	no

Supplementary Table 6 Attraction of virgin female wasps of the species *L. ryukuyensis* (**A**), *L. japonica* (**B**), *L. heterotoma* (**C**), and *L. pacifica* (**D**) to host patches in olfactometer experiments. Females had the choice between a host patch or clean humidified air. Experiments lasted until 30 wasps had crossed a decision line, each within 10 min. Corresponds to Fig. 7B

A

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	right	0	>10 min	
2	left	0	>10 min	
3	right	right	130	yes
4	left	left	120	yes
5	left	left	104	yes
6	right	right	50	yes
7	left	left	137	yes
8	right	0	>10 min	
9	right	right	15	yes
10	left	left	27	yes
11	left	left	25	yes
12	right	left	62	no
13	right	right	223	yes
14	left	left	110	yes
15	left	0	>10 min	
16	left	0	>10 min	
17	right	0	>10 min	
18	right	left	72	no
19	left	0	>10 min	
20	left	left	209	yes
21	right	right	232	yes
22	left	0	>10 min	
23	left	left	224	yes
24	right	right	242	yes
25	right	0	>10 min	
26	right	right	77	yes
27	left	left	256	yes
28	right	right	275	yes
29	left	left	223	yes
30	left	left	265	yes
31	right	left	186	no
32	right	right	119	yes
33	left	0	>10 min	
34	right	0	>10 min	
35	left	right	207	no
36	left	left	244	yes
37	right	right	66	yes
38	left	left	94	yes
39	right	0	>10 min	
40	right	right	303	yes
41	right	right	216	yes
42	left	left	190	yes

B

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	right	right	58	yes
2	left	left	136	yes
3	right	left	102	no
4	right	right	328	yes
5	left	left	525	yes
6	right	left	51	no
7	left	left	406	yes
8	right	right	30	yes
9	left	left	14	yes
10	left	left	17	yes
11	right	left	562	no
12	right	right	168	yes
13	left	right	435	no
14	left	right	131	no
15	right	right	351	yes
16	left	left	184	yes
17	right	right	139	yes
18	right	left	13	no
19	left	left	104	yes
20	right	right	99	yes
21	left	left	43	yes
22	right	right	31	yes
23	left	left	35	yes
24	right	right	25	yes
25	right	right	45	yes
26	left	left	278	yes
27	right	right	288	yes
28	left	left	87	yes
29	right	right	65	yes
30	left	right	24	no

C

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	left	19	yes
2	right	right	229	yes
3	left	left	175	yes
4	right	left	52	no
5	left	left	115	yes
6	right	left	283	no
7	left	left	91	yes
8	right	right	235	yes
9	left	right	161	no
10	right	right	412	yes
11	left	right	180	no
12	right	right	228	yes
13	left	left	52	yes
14	right	left	252	no
15	left	0	>10 min	
16	left	left	318	yes
17	right	right	334	yes
18	left	left	220	yes
19	right	right	253	yes
20	left	left	71	yes
21	right	right	200	yes
22	left	0	>10 min	
23	left	0	>10 min	
24	right	right	209	yes
25	left	0	>10 min	
26	left	left	170	yes
27	right	right	222	yes
28	left	left	218	yes
29	right	left	584	no
30	left	right	152	no
31	right	right	294	yes
32	left	left	351	yes
33	right	right	71	yes
34	left	right	552	no

D

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	left	21	yes
2	right	right	24	yes
3	left	left	51	yes
4	right	right	12	yes
5	left	left	11	yes
6	right	right	40	yes
7	left	left	26	yes
8	right	right	71	yes
9	left	>10 min		
10	left	>10 min		
11	left	left	12	yes
12	right	right	16	yes
13	left	left	54	yes
14	right	right	70	yes
15	left	left	60	yes
16	right	right	82	yes
17	left	left	14	yes
18	right	right	19	yes
19	left	left	32	yes
20	right	right	76	yes
21	left	left	25	yes
22	right	right	16	yes
23	right	left	13	no
24	left	left	98	yes
25	right	left	14	no
26	left	left	12	yes
27	right	right	14	yes
28	left	left	23	yes
29	right	right	18	yes
30	left	left	21	yes
31	right	right	9	yes
32	left	right	19	no

Supplementary Table 7 Attraction of mated female wasps of the species *L. ryukuyensis* (**A**), *L. japonica* (**B**), *L. heterotoma* (**C**), and *L. pacifica* (**D**) to host patches in olfactometer experiments. Females had the choice between a host patch or clean humidified air. Experiments lasted until 30 wasps had crossed a decision line, each within 10 min. Corresponds to Fig. 7C

A

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	left	34	yes
2	right	right	12	yes
3	right	right	53	yes
4	left	left	31	yes
5	left	left	58	yes
6	right	right	134	yes
7	right	left	20	no
8	left	left	14	yes
9	left	left	122	yes
10	right	right	90	yes
11	right	right	12	yes
12	left	left	13	yes
13	left	left	15	yes
14	right	right	166	yes
15	right	left	27	no
16	left	left	78	yes
17	right	right	170	yes
18	left	left	62	yes
19	right	right	143	yes
20	left	left	205	yes
21	left	right	23	no
22	right	right	19	yes
23	right	right	320	yes
24	left	left	74	yes
25	right	right	176	yes
26	left	right	121	no
27	left	0	>10 min	
28	left	left	12	yes
29	right	right	171	yes
30	left	left	78	yes
31	right	left	10	no

B

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	right	579	no
2	right	left	21	no
3	right	right	204	yes
4	left	left	184	yes
5	right	right	133	yes
6	left	right	173	no
7	left	left	122	yes
8	right	right	55	yes
9	left	left	162	yes
10	right	right	154	yes
11	right	right	40	yes
12	left	left	18	yes
13	right	right	89	yes
14	left	left	14	yes
15	right	right	272	yes
16	left	left	514	yes
17	right	right	189	yes
18	left	left	478	yes
19	right	right	30	yes
20	left	left	13	yes
21	right	left	378	no
22	left	left	18	yes
23	right	left	404	no
24	left	left	96	yes
25	right	right	27	yes
26	left	left	62	yes
27	right	right	34	yes
28	left	left	13	yes
29	right	right	107	yes
30	left	left	39	yes

C

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	left	241	yes
2	right	right	310	yes
3	left	left	113	yes
4	right	right	143	yes
5	left	left	307	yes
6	right	left	16	no
7	left	left	14	yes
8	right	left	537	no
9	left	left	300	yes
10	right	right	34	yes
11	left	left	70	yes
12	right	right	21	yes
13	left	left	69	yes
14	right	right	193	yes
15	left	left	143	yes
16	right	right	77	yes
17	left	left	73	yes
18	right	right	168	yes
19	left	left	502	yes
20	right	right	95	yes
21	left	left	375	yes
22	right	left	120	no
23	left	right	376	no
24	right	left	285	no
25	left	left	78	yes
26	right	left	21	no
27	left	left	222	yes
28	right	left	519	no
29	right	right	15	yes
30	left	right	21	no

D

Nr	Side of hp	Decision	Time [s]	Attraction to hp?
1	left	left	20	yes
2	right	right	16	yes
3	left	left	22	yes
4	right	right	18	yes
5	left	left	69	yes
6	right	right	10	yes
7	left	left	53	yes
8	right	right	23	yes
9	left	left	48	yes
10	right	right	17	yes
11	left	left	61	yes
12	right	right	55	yes
13	left	left	25	yes
14	right	right	63	yes
15	left	left	112	yes
16	right	right	36	yes
17	left	right	33	no
18	right	right	75	yes
19	left	left	86	yes
20	right	right	32	yes
21	left	left	39	yes
22	right	right	179	yes
23	left	left	48	yes
24	right	right	17	yes
25	left	left	80	yes
26	right	right	22	yes
27	left	left	45	yes
28	right	right	11	yes
29	left	left	60	yes
30	right	right	58	yes

Publication Record

Record of all publications presented in this thesis:

Böttinger LC, Hüftlein F, Stökl J (2020) "Mate Attraction, chemical defense, and competition avoidance in the parasitoid wasp *Leptopilina pacifica*". *Chemoecology*.

Böttinger LC, Stökl J (2020) "Dispersal from Natal Patch Correlates with the Volatility of Female Sex Pheromones in Parasitoid Wasps". *Frontiers in Ecology and Evolution* 8:557527.

Böttinger LC, Hofferberth J, Ruther J, Stökl J (2019) "Semiochemicals Mediating Defense, Intraspecific Competition, and Mate Finding in *Leptopilina ryukyuensis* and *L. japonica* (Hymenoptera: Figitidae), Parasitoids of *Drosophila*". *Journal of Chemical Ecology* 45(3):241-252.

Record of further own publications not used in this thesis:

Stoeffler M, **Boettinger LC**, Tolasch T, Steidle JLM (2013) "The Tergal Gland Secretion of the Two Rare Myrmecophilous Species *Zyras collaris* and *Z. haworthi* (Coleoptera: Staphylinidae) and the Effect on *Lasius fuliginosus*". *Psyche: A Journal of Entomology*.

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Statutory declarations

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