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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.*

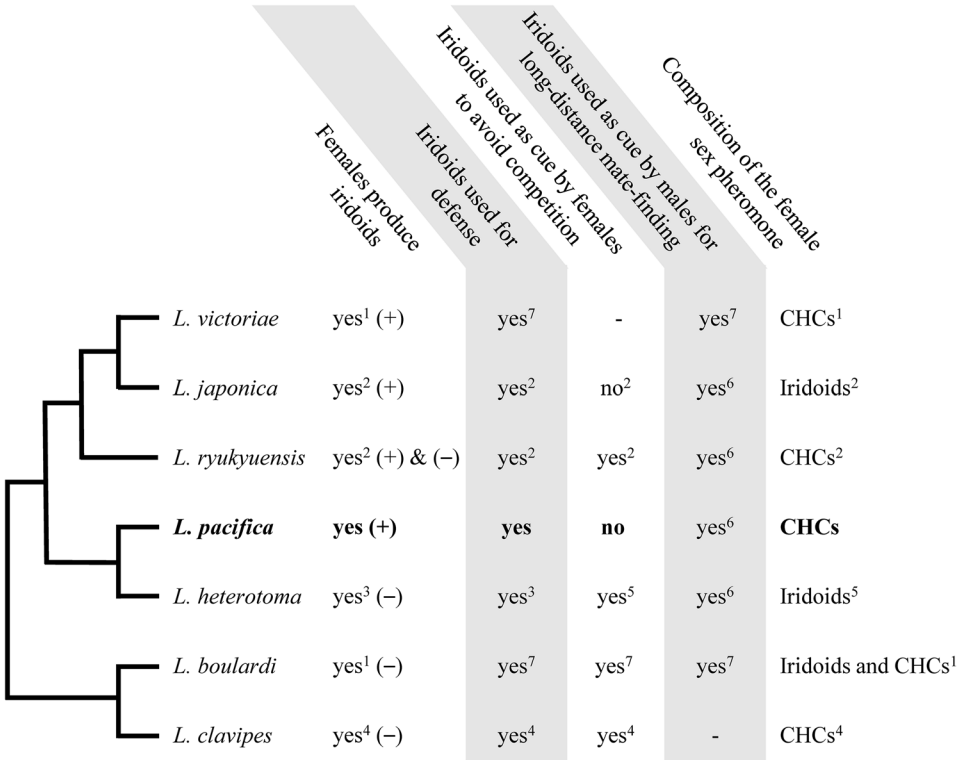

Fig. 1 Phylogenetic relationship and current knowledge on the production and use of iridoids by the nine species of *Leptopilina* studied so far. (+) indicates that (+)-iridomyrmecin is produced (among other iridoid compounds), and (–), that (–)-iridomyrmecin is produced. Hyphens indicate that this function 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).

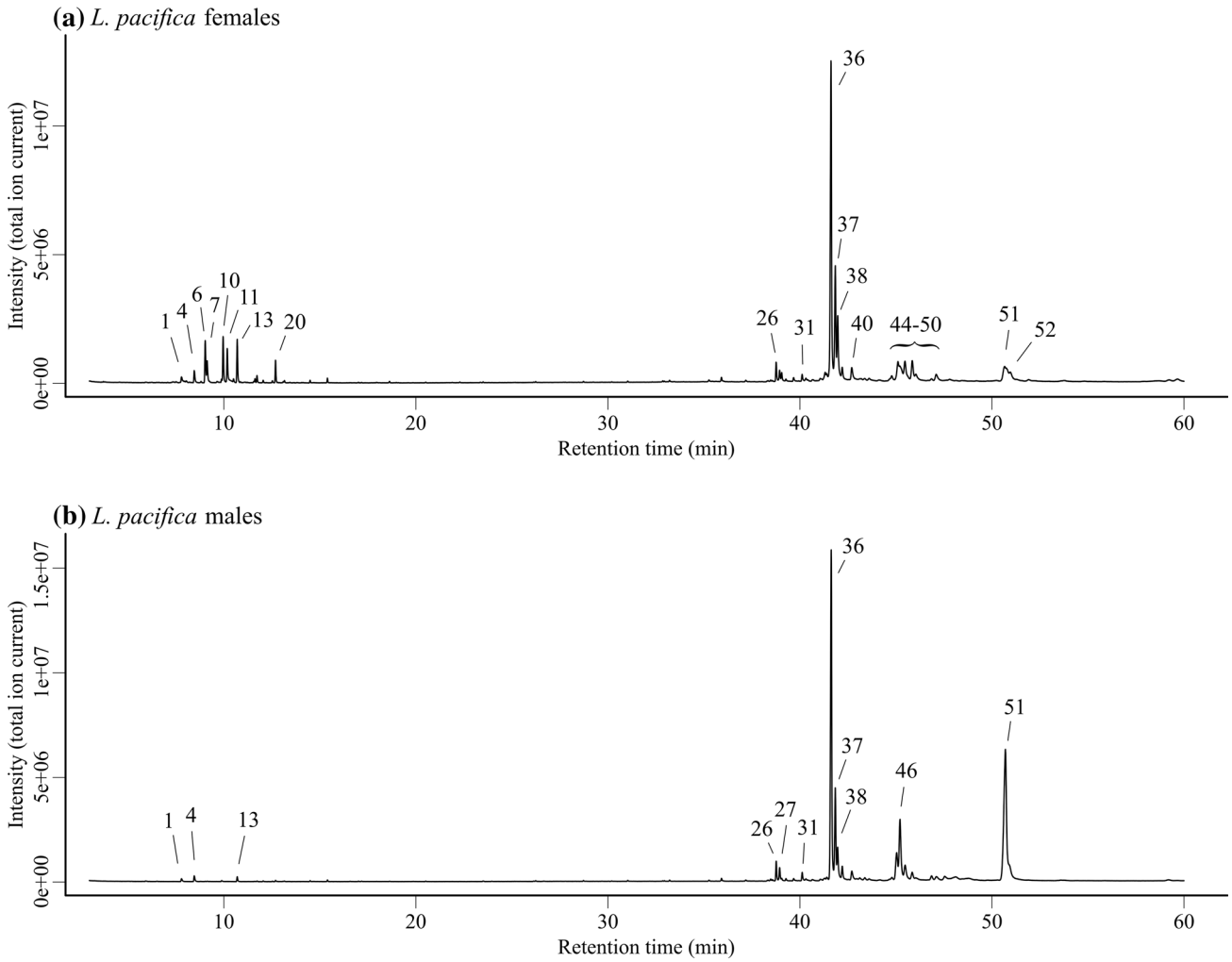

Fig. 2 Chemical compounds produced by *L. pacifica* wasps. Total ion current chromatograms of extracts of **a** *L. pacifica* females and **b** *L. pacifica* males, analyzed on a non-polar DB-5 GC column. Peak numbers correspond to Table 1

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

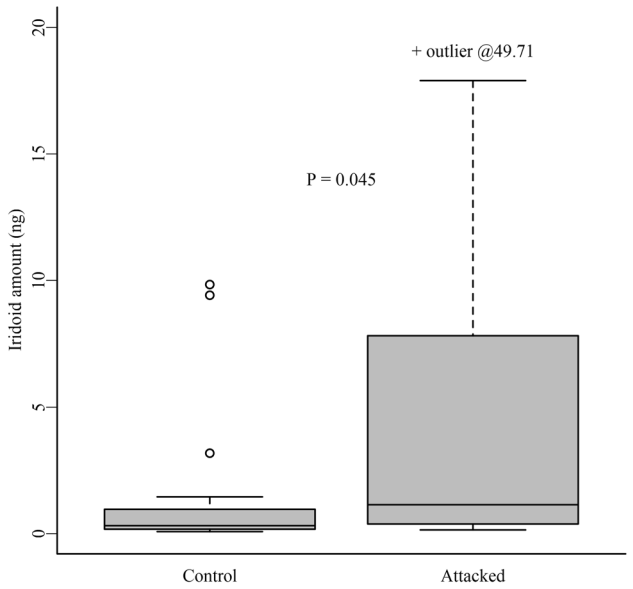

Fig. 3 Iridoids for defense released upon attack. Box-and-whisker plots showing median (horizontal line), inter-quartile range (box), outliers (unfilled circles), and maximum/minimum of the 1.5 × inter-quartile range (whiskers) of the released iridoid amounts (ng) of two females of *L. pacifica* in a 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 $N = 15$

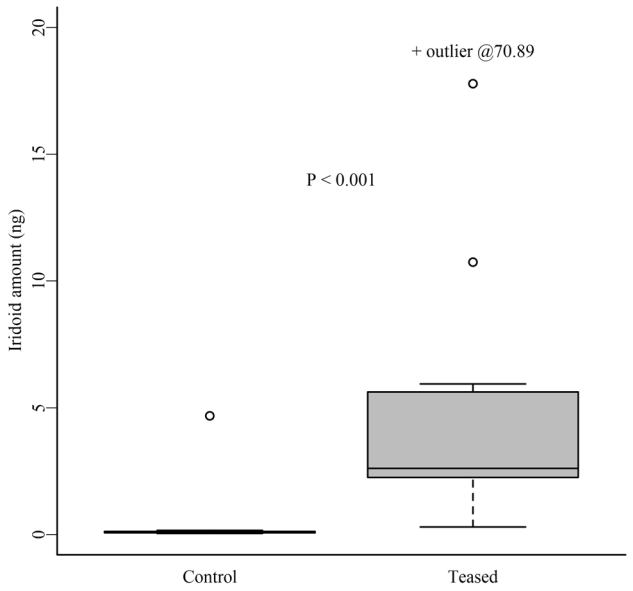

Fig. 4 Iridoids for defense released upon teasing. Box-and-whisker plots showing median (horizontal line), inter-quartile range (box), outliers (unfilled circles), and maximum/minimum of the 1.5 × inter-quartile range (whiskers) of the released iridoid amounts (ng) of two females of *L. pacifica* in 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 $N = 15$

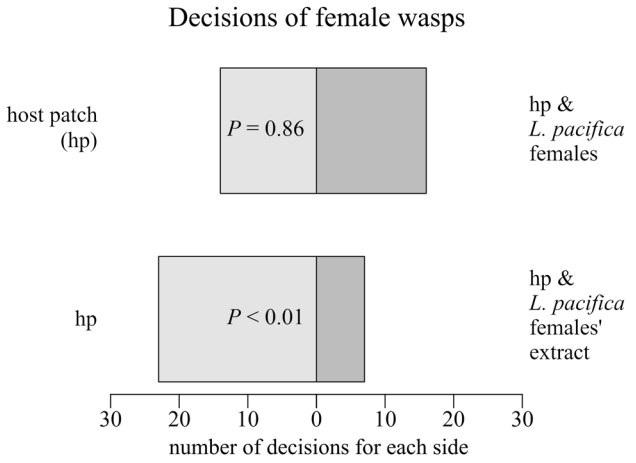

Fig. 5 Competition avoidance of females. Frequency of decision for control (light grey bars) or sample (dark grey bars) of females of *L. pacifica* in a Y-tube experiment when choosing between the odor of unexploited host patches (hp) or host patches with either 20 conspecific females or the extract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

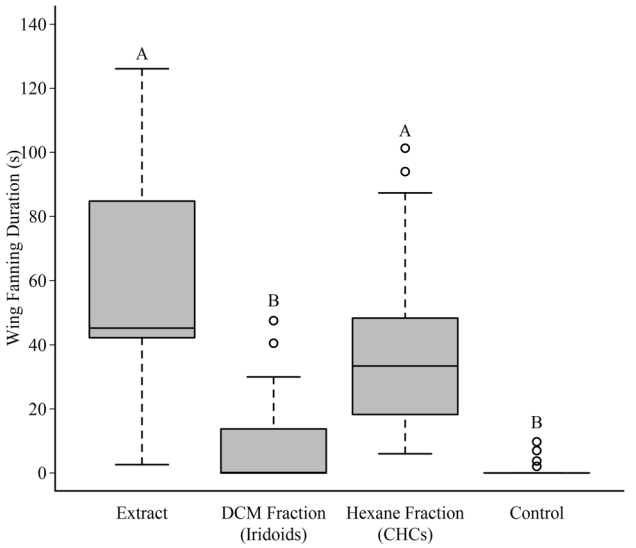

Fig. 6 Female sex pheromone. Box-and-whisker plots showing median (horizontal line), inter-quartile range (box), outliers (unfilled circles), and maximum/minimum of the 1.5 × inter-quartile range (whiskers) of the duration of courtship behavior (i.e., wing fanning) displayed by males of *L. pacifica* 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. 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.

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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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