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Cumulative effects of sex pheromone components in mate recognition of *Muscidifurax raptorellus*

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Abstract

In insects, chemical information is often crucial for mate recognition. The chemical signal may be perceived already from a distance or only at close range, depending on the volatility of the sex pheromone compounds. In many species, close-range mate recognition is mediated by compounds of low volatility that originate from the insects' cuticular lipid layer. The most prominent constituents are usually non-polar hydrocarbons, but other, more polar compounds can be present as well. Upon detection, these lipids can elicit courtship behaviour. We studied mate recognition in the parasitoid wasp *Muscidifurax raptorellus* Kogan & Legner (Hymenoptera: Pteromalidae). In this species, courtship behaviour starts with bouts of wing fanning (high frequency wing beats while standing or walking). Once the male has mounted the female, he shows stereotypical antennal movements (antennal courtship) that lead to female receptivity signalling and copulation. We investigated the role of chemical compounds for mate recognition by evaluating the reaction of male *M. raptorellus* towards live conspecifics and dummies treated with whole-body extracts or with extract fractions (non-polar, intermediately polar, and polar compounds) in bioassays. Our results indicate that despite the sex specificity of cuticular hydrocarbon profiles, these compounds alone are not sufficient for mate recognition. Rather, we found that cuticular lipids of all three fractions seem to constitute the mate recognition signal, with a ternary mixture eliciting wing fanning significantly more often than single fractions. Overall, our results suggest that the three lipid fractions contribute cumulatively to the mate recognition signal.

KEYWORDS
parasitoid wasp, cuticular lipids, cuticular hydrocarbons, close-range mate recognition, courtship behaviour, Hymenoptera, Pteromalidae, *Muscidifurax raptorellus*, chemical signal, information, polarity

INTRODUCTION

Chemical communication in insects can govern many crucial aspects of their lives, from complex signals that regulate interactions in social colonies (Leonhardt et al., 2016) to the most basic fitness prerequisite for all sexually reproducing species: the finding and acquisition of a mate (Cardé & Haynes, 2004; Howard & Blomquist, 2005). Potential mates can be detected already from a distance if volatile pheromones are employed (e.g., in many Lepidoptera; Cardé & Haynes, 2004). For close-range recognition, less volatile compounds can come into play. In many cases, these

are compounds of the cuticular lipid layer (Blomquist & Bagnères, 2010). The primary function of such lipids is thought to be the prevention of desiccation (Ramsey, 1935; Gibbs & Rajpurohit, 2010). However, qualitative and quantitative lipid composition—which compounds are produced in what relative amounts—is commonly species-specific, and they often serve additional, communicative purposes (Blomquist & Bagnères, 2010; Leonhard et al., 2016). Whereas the most ubiquitous constituents of the cuticular lipid layer are hydrocarbons, compounds of several other chemical classes may be present as well, e.g., alcohols, aldehydes, or wax esters (Lockey, 1985; Blomquist & Bagnères, 2010).

In several species of parasitoid wasps, cuticular lipids have been shown to enable mate recognition and to trigger male courtship behaviour (Ruther, 2013). Often, the cuticular hydrocarbon (CHC) profiles of males and females differ clearly, based on the relative amounts of shared compounds and/or the presence of sex-specific compounds. Investigations of the female pheromones that elicit male courtship have shown that in a number of species, CHCs alone constitute the chemical signal (Steiner et al., 2006; Ruther et al., 2011; Ablard et al., 2012; Weiss et al., 2015; Böttinger et al., 2020; Würf et al., 2020; Jungwirth et al., 2021). In other cases, however, CHCs must be present in combination with other cuticular lipids or glandular compounds (Kühbandner et al., 2012; Stökl et al., 2014; Weiss et al., 2015). Even within a genus, species can differ regarding the combination of compounds and compound classes that mediate mate recognition. For example, in the genus *Nasonia*, mate recognition by male wasps is predominantly mediated by female cuticular lipids (Steiner et al., 2006; Buellesbach et al., 2013, 2018; Mair et al., 2017). Males of *Nasonia vitripennis* Walker react to female CHCs (Steiner et al., 2006; Mair et al., 2017), whereas *Nasonia giraulti* Darling males seem to require more polar cuticular lipids in addition to the CHCs to recognize conspecific females (Mair et al., 2017). Wasps of the genus *Leptopilina* produce iridoids for use as defensive compounds. In some cases, they additionally function as pheromones, and different species of *Leptopilina* use either iridoids, CHCs, or a combination of both as their sex pheromones (Weiss et al., 2015; Böttinger et al., 2020).

A further genus of parasitoid wasps that is readily available for studies on mating behaviour (van den Assem & Povel, 1973) and chemical analyses of CHCs (Bernier et al., 1998) is *Muscidifurax* (Hymenoptera: Pteromalidae). It comprises nine species that are parasitoids of fly pupae (Xiao et al., 2018; Noyes, 2021). Up to now, there has been only a single study linking mating behaviour and chemical signals used in mate recognition for one species of this genus, *Muscidifurax uniraptor* Kogan & Legner (Buellesbach et al., 2018). This species is parthenogenetic, with irreversible, *Wolbachia*-induced thelytoky (Gottlieb & Zchori-Fein, 2001). Results suggested that the males, which are rare and infertile (van den Assem, 1976), react to female cuticular lipids, as courtship was shown towards dummies treated with crude female extract (Buellesbach et al., 2018). This indicates that cuticular lipids functioned as the mate recognition signal of the species before the transition to exclusively thelytokous reproduction. Although CHCs differed between the sexes (Bernier et al., 1998; Buellesbach et al., 2018), the CHC pattern of *M. uniraptor* females was considered to be in a state of transition back to the simpler composition typical for male *Muscidifurax*. It was suggested that this could be based on a lack of selection pressure to produce the CHC patterns typical for females of other, arrhenotokous *Muscidifurax* species, in which they might be involved in mate recognition (Bernier et al., 1998). However, whether other species actually use cuticular lipids or specifically CHCs as mate recognition signals has yet to be shown.

Here, we studied the chemical mate recognition of *Muscidifurax raptorellus* Kogan & Legner, which is likely the closest relative of *M. uniraptor* (van den Assem, 1976; Taylor et al., 1997; Bernier et al., 1998). *Muscidifurax raptorellus* is an arrhenotokous ectoparasitoid of the pupal stage of filth flies (Geden & Moon, 2009), and has a generation time of about 15 days at 25 °C (Lysyk, 2001). Most populations are gregarious and although originally from Chile, they are now widespread due to their use as biocontrol agent (Antolin et al., 1996; Geden & Moon 2009). Male courtship behaviour follows a stereotypic sequence: upon detection of a female, a *M. raptorellus* male fans his wings (very rapid wing movements with wings spread, similar to when in flight) and approaches her. Once the female is arrested, the male mounts and begins antennal courtship, and if the female signals acceptance by opening the genital orifice, copulation ensues (van den Assem & Povel, 1973). Chemical analysis of the CHCs has revealed marked differences between the sexes (Bernier et al., 1998), but the role of CHCs in the courtship behaviour of *M. raptorellus* is unknown. To evaluate whether cuticular lipids elicit male courtship behaviour, and to determine the bioactive lipid class(es), we exposed male *M. raptorellus* to live conspecifics or to dummies treated with cuticular lipid extracts or fractions thereof.

MATERIALS AND METHODS

Wasps

The starter culture of *M. raptorellus* was kindly provided by Dr. E. Verhulst (University of Wageningen, The Netherlands). Wasps were bred on freeze-killed pupae of *Lucilia caesar* L. (Diptera: Calliphoridae) at 25 °C and L16:D8 photoperiod. Wasp pupae were dissected from the hosts 2 days pre-emergence, and kept singly in 1.5-ml reaction tubes at 25 °C. The tubes were checked twice per day for eclosed wasps, which were kept in their tubes until used for bioassays. Males tested for their reactions were always aged 24–42 h (1 day) post-eclosion.

Bioassays

All bioassays (n = 20 for each tested setup) were carried out in an acrylic glass arena (a hole of 1.5 cm diameter in a

fluorescent acrylic glass pane: 3 mm high, 3.7 cm wide, and 4.9 cm long), which was placed on a pane of glass and covered with a glass microscope slide. The whole setup was positioned on top of a piece of aluminium foil. The arena was lit by cold light sources (KL 200 LED with a Puravis flexible light guide pair; Schott, Mainz, Germany) from two sides above, with the reflection from the aluminium foil providing a more even lighting throughout the arena. Bioassays were conducted at room temperature between 10:00 and 18:00 hours. Each confrontation was video-recorded from directly above (EOS M equipped with a Tamron 90 mm F/2.8 macro lens; Canon, Tokyo, Japan) and videos were later analysed using The Observer XT 11 scientific software (Noldus Information Technology, Wageningen, The Netherlands). The arena was cleaned with ethanol between repetitions. Each male was tested only once.

For each repetition a new stimulus was used. This was either a live conspecific wasp or a black glass dummy (2.5 mm long, 2 mm wide, 2 mm high, made in-house) treated with either pure solvent (control) or an extract/fraction (described below). Glass dummies were placed in the centre of a small circle (5 mm diameter) that was drawn on the aluminium foil below the pane of glass. The arena was then placed on the glass so that the circle outline (and thus the stimulus) was off centre. For testing, each male was introduced into the arena at the opposite side of the test stimulus, after which the arena was covered with the glass slide and video recording was started. Male behaviour was filmed for a minimum of 5 min. Video analyses covered the first 5 min after the male had been introduced, and included the evaluation of whether the test male had encountered the test stimulus (an encounter being defined as when the live animals met, or when the tip of one antenna of the test male reached into the circle around the dummy). In one case, the video analysis was extended for 20 s, as the encounter of the dummy had only taken place within the last half a second of the standard observation time. If a tested male failed to come close enough to the test stimulus within the observation time, the test was repeated with a new male and setup. Only those repetitions in which males had encountered the test stimulus were analysed further.

The antennal courtship behaviour and copulation attempts were only very rarely (5×) shown in experiments using extract fractions or mixtures thereof. Wing fanning behaviour, however, was shown by males in all tested setups. In general, wing fanning behaviour was very variable between males (in response to live females: total number 0–32, total duration 0–69.7 s, average duration per wing fanning bout 0.48–15.2 s), and small-scale changes might be masked by the overall variability. Thus, behavioural analyses focused on whether wing fanning behaviour had been shown or not. As an additional measure of the 'interest' males showed towards the stimuli applied to the glass dummies, the total time spent antennating (repeated, intermittent antennal contact with the dummy) the tested stimulus was documented.

First, we evaluated the reaction of 1-day-old males towards live conspecific males and females of different ages (time after eclosion from the pupal exuvia): 0-18 h (0 days), 24–42 h (1 day), and 48–66 h (2 days). Based on the results, the age of wasps to be used for extraction in all subsequent bioassays was standardized to 0 days for males and 1 day for females. We tested male reactions towards dummies treated with crude extracts of either males, females, or the pure solvent (control). In the following test, dummies were treated with the control or the recombined ternary mixture of all three extract fractions from males or females. To narrow down candidate fractions containing the female sex pheromone, we subsequently tested male reactions to binary mixtures, as well as to single fractions in comparison to solvent controls. All bioassays were conducted in no-choice setups. Dummies were always treated with two animal equivalents or the same amount of pure solvent for control trials in order to compensate for the larger size of the glass dummies compared to the wasps.

Wing fanning results were analysed by χ^2 tests with Bonferroni–Holm correction for pairwise comparisons, and Monte Carlo simulation (2000 replicates) for cases when cells had an expected frequency of <5 (in such cases the degrees of freedom are reported as 'NA', not applicable). The total duration of antennation was tested using Kruskal–Wallis tests for comparisons of several samples, and Bonferroni–Holm corrected Mann–Whitney U tests for pairwise comparisons. Analyses were conducted using R v.3.6.3 (R Core Team, 2020).

Extraction and fractionation of extracts

To obtain crude extracts, 0-day males and 1-day females were killed by freezing at –21 °C. For the bioassay with crude extracts, 50 individuals of the same sex and age were extracted together, whereas 2 × 100 individuals of the same sex were always used for extracts that would be subjected to fractionation (see below). The wasps (50 or 100) were placed in a 2-ml glass vial and extracted with 15 µl of dichloromethane (DCM) per individual for 30 min. Thereafter, the extract was transferred to a new vial and placed under a gentle stream of nitrogen to reduce the volume. The wasps were rinsed twice (5 min each time) with another 5 µl of DCM per individual, subsequently adding the rinses to the extract. Crude extracts for bioassays were then evaporated and refilled with 50 µl DCM (1 µl per individual). For fractionation, the two extracts of 100 individuals of the same sex were concentrated to about 500 µl under nitrogen and then combined. Thereafter, the solvent was evaporated, and the extract was re-dissolved in 400 µl of hexane (2 µl per individual). For fractionation, we prepared silica gel cartridges (Chromabond 100 mg; Macherey & Nagel, Düren, Germany) and fractionated aliquots of 100 µl of extract as described in Jungwirth et al. (2021), eluting the compounds successively with 1 ml of each hexane, DCM, and methanol.

The procedure was repeated with all remaining extract aliquots, after which the equivalent fractions of each fractionation were combined. This led to three fractions: (1) a non-polar fraction (H), eluted with hexane (containing the CHCs), (2) a fraction containing compounds of intermediate polarity (D), eluted with DCM, and (3) a polar fraction (M), eluted with methanol. The solvent of each fraction was evaporated under nitrogen, subsequently re-dissolving the compounds in 200 µl of the respective solvent (1 µl per individual). To create fraction mixtures (ternary or binary), the respective fractions were combined in equal ratios (to combine the immiscible fractions H and M, the aliquot of H was carefully evaporated under nitrogen and re-dissolved with DCM), and the solvent amount was adjusted to a concentration of one animal equivalent per microliter. To be able to conduct more bioassays to replace tests in which the tested male had not approached the stimulus closely enough to be considered as having encountered it, a second batch of 2 × 100 females was extracted, fractionated, and combined as described above.

RESULTS

Males showed wing fanning significantly more often towards live females than towards males, irrespective of the age of the stimulus wasp (overall: $\chi^2 = 73.167$, d.f. = 5, P<0.001; no differences within sexes, all comparisons between female and male age groups significant after Bonferroni-Holm correction; Figure 1, Table S1). In confrontations with glass dummies, males showed wing fanning more often towards dummies treated with female extract than towards dummies treated with male extract or the solvent control (overall: $\chi^2 = 35.966$, d.f. = 2, P<0.001; pairwise with Bonferroni–Holm correction, solvent vs. female extract: $\chi^2 = 20.417$, d.f. = 1, P<0.001, female vs. male extract: $\chi^2 = 24$, d.f. = 1, P<0.001, solvent vs. male extract: $\chi^2 = 1.027$, d.f. = 1, P = 1; Figure 2A). Male reactions were similar if dummies were treated with reconstituted extracts (ternary mixture of the three fractions), with wing fanning responses shown more often towards the female-derived fraction mixture (overall: $\chi^2 = 26.121$, d.f. = NA, P<0.001; pairwise with Bonferroni–Holm correction, solvent vs. female HDM: $\chi^2 = 13.789$, d.f. = 1, P<0.001, female vs. male HDM: $\chi^2 = 17.143$, d.f. = 1, P<0.001, solvent vs. male HDM: $\chi^2 = 1.027$, d.f. = NA, P = 1; Figure 2B).

The same patterns were observed for the duration of antennation of the dummies (female crude extracts or female-derived ternary fraction mixtures were antennated significantly longer than controls or male-derived stimuli, Table S2, Figure S1). In tests of binary mixtures, male reactions towards the four treatments differed overall ($\chi^2 = 12.080$, d.f. = 3, P = 0.007; Figure 3A), but there was no difference in wing fanning behaviour towards the three female-derived binary mixtures (HD vs. HM: $\chi^2 = 0.440$, d.f. = 1, P = 0.51; HD vs. DM: $\chi^2 = 0.902$, d.f. = 1, P = 0.68; HM vs. DM: $\chi^2 = 2.558$, d.f. = 1, P = 0.33; Figure 3A). When compared to the solvent control, wing fanning was shown more often towards dummies treated with the DM combination ($\chi^2 = 11.905$, d.f. = 1, P = 0.003; Figure 3A). Although males showed some wing fanning behaviour in response to the other two combinations, responses did not differ from the control (solvent vs. HD: $\chi^2 = 7.025$, d.f. = NA, P = 0.075; solvent vs. HM: $\chi^2 = 4.329$, d.f. = NA, P = 0.39; Figure 3A). The duration of antennation of the dummies did not differ between any of the tested stimuli in this setup, including the solvent control (Kruskal–Wallis overall: $\chi^2 = 2.572$, d.f. = 3, P = 0.46; Table S2, Figure S2A). When tested singly, none of the female-derived extract fractions elicited more wing fanning behaviour than pure solvent (overall: $\chi^2 = 3.466$, d.f. = NA, P = 0.35; Figure 3B). Males spent less time antennating dummies treated with the H fraction than dummies treated with M fraction (Kruskal–Wallis overall: $\chi^2 = 11.032$, d.f. = 3, P = 0.012; pairwise H vs. M: W = 80, P = 0.007; Figure S2B), whereas the other comparisons were not significant (Table S2).

As there were no significant differences between male responses (wing fanning and duration of antennation) to the three binary mixtures, nor to single fractions (for wing

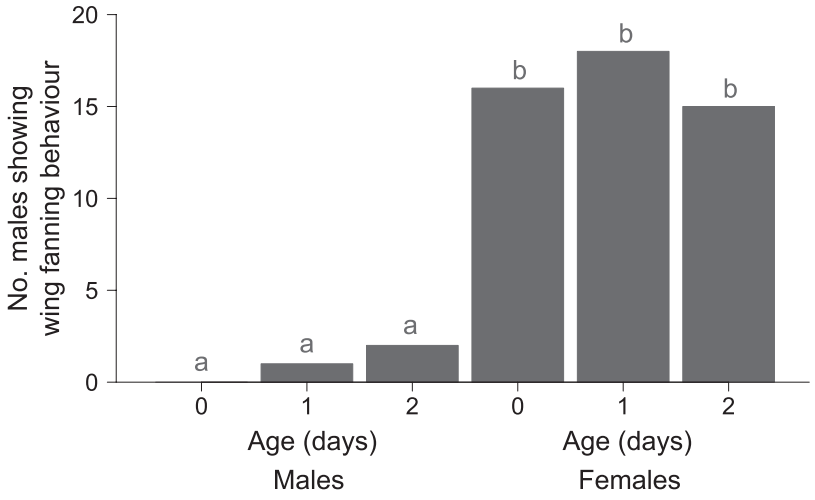

FIGURE 1 Frequency of wing fanning shown by 1-day-old *Muscidifurax raptorellus* males towards live male and female conspecifics of three ages: 0 days (0–18 h), 1 day (24–42 h), or 2 days (48–66 h) since eclosion from the pupal exuvia (n = 20 each). Different letters capping the columns indicate significant differences in male responses (χ^2 tests with Bonferroni-Holm correction: P<0.05)

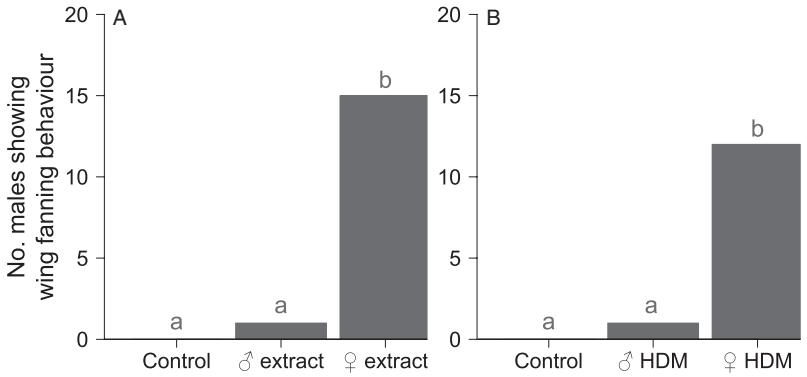

FIGURE 2 Frequency of wing fanning shown by *Muscidifurax raptorellus* males towards glass dummies treated with (A) pure solvent (control), crude male extract, or crude female extract, and (B) pure solvent (control) or ternary mixtures of the three fractions resulting from fractionation of the respective extracts: H, containing non-polar CHCs; D, containing compounds of intermediate polarity; and M, the polar fraction (n = 20 each). Different letters capping the columns within a panel indicate significant differences in male responses (χ^2 tests with Bonferroni-Holm correction: $P<0.05$)

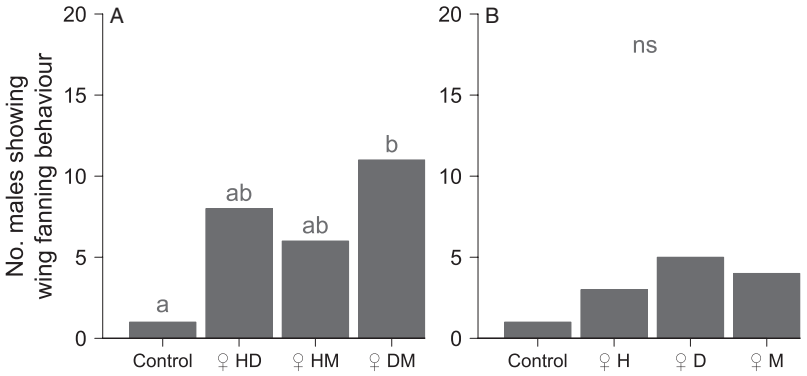

FIGURE 3 Frequency of *Muscidifurax raptorellus* male wing fanning behaviour elicited by female-derived extract fractions H (containing non-polar compounds), D (containing compounds of intermediate polarity), or M (polar fraction) on treated glass dummies: (A) pure solvent (control) or binary mixtures fractions, and (B) pure solvent (control) or single fractions (n = 20 each). Different letters capping the columns within a panel indicate significant differences in male responses (χ^2 tests with Bonferroni-Holm correction: $P<0.05$; ns, $P>0.05$)

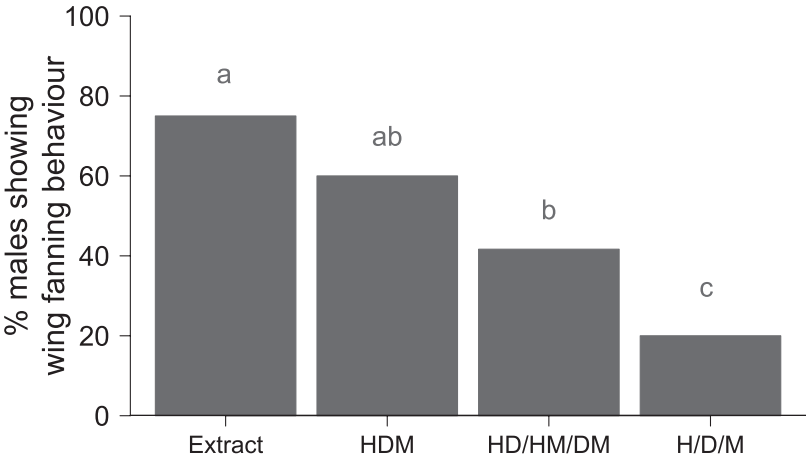

FIGURE 4 Overall frequency (%) of *Muscidifurax raptorellus* males displaying wing fanning behaviour towards glass dummies treated with either crude female extract (n = 20), the ternary mixture of all female-derived extract fractions (HDM, n = 20; H, containing non-polar compounds; D, containing intermediately polar compounds; M, containing polar compounds), binary fraction mixtures (HD/HM/DM, n = 60; results combined as there were no differences in male reactions between them), or single fractions (H/D/M, n = 60; results combined as there were no differences in male reactions between them). Different letters capping the columns indicate significant differences in male responses (χ^2 tests with Bonferroni–Holm correction: $P<0.05$)

fanning only; for antennation no significant differences between D and M fractions), the respective responses were combined for each behaviour (omitting the antennation responses to H fraction for the single fractions) to compare the overall reactions of wing fanning and antennation to female lipids in the crude extract, the ternary mixture, binary mixtures, and single fractions. Males showed wing fanning more often towards crude extract than towards binary fraction mixtures and single fractions (overall: $\chi^2 = 23.062$, d.f. = 3, P<0.001; extract vs. binary mixture: $\chi^2 = 6.667$, d.f. = 1, P = 0.039; extract vs. single fraction: $\chi^2 = 19.838$, d.f. = 1, P<0.001; Figure 4), but not towards the ternary fraction mixture ($\chi^2 = 1.026$, d.f. = 1, P = 0.31; Figure 4). In turn, the ternary fraction mixture elicited more male wing fanning reactions than single fractions ($\chi^2 = 11.109$, d.f. = 1, P = 0.004), but not than binary mixtures ($\chi^2 = 2.028$, d.f. = 1, P = 0.31; Figure 4). Males showed more wing fanning towards binary mixtures than to single fractions ($\chi^2 = 6.316$, d.f. = 1, P = 0.036; Figure 4). The duration of antennation elicited by dummies treated with crude extract was longer than when they were treated with a binary mixture or a single fraction (Kruskal–Wallis overall: $\chi^2 = 14.360$, d.f. = 3, P = 0.002; crude extract vs. binary mixture: W = 867, P = 0.018; crude extract vs. single fraction: W = 586, P = 0.018, Figure S3), but did not differ from the antennation of dummies treated with the ternary fraction mixture (W = 270, P = 0.12; Figure S3). Males did not differ in the duration of antennation of dummies treated with fraction mixtures or single fractions (ternary vs. binary mixture: W = 797.5, P = 0.086; ternary mixture vs. single fraction: W = 541, P = 0.11; binary mixtures vs. single fractions: W = 1148, P = 0.72; Figure S3).

DISCUSSION

Male *M. raptorellus* recognize prospective mates based on their cuticular lipids. Although the reactions to single lipid fractions and two of the binary fraction mixtures did not differ significantly from male wing fanning responses to the solvent controls, this was likely due to the low number of responding males rather than to a general 'unattractiveness' of single fractions/certain binary fraction mixtures. The emerging pattern of male responses to ternary/binary fraction mixtures and single fractions followed the pattern expected for a cumulative effect of the three lipid fractions. The fractions could function in a simple additive way, as the numbers resulting from our bioassays suggest, although this hypothesis will have to be verified with a larger sample size. Overall, the more 'complete' the tested female lipid profile was, the more males responded. A minimum of two female extract fractions was required to elicit more wing fanning responses than the control with our sample size, whereas only the ternary fraction mixture elicited longer antennation behaviour than the control. It did not seem to make a difference which specific extract fractions were combined, as there was no significant difference between the male reactions to each of the three binary mixtures, although this should ideally be tested in choice setups in future.

The results of our bioassays were unexpected. In *M. raptorellus*, compounds from all three tested lipid fractions contribute to the mate recognition signal, and thus contain components of the female close-range sex pheromone. However, neither of the fractions seemed to contain 'essential' compounds, without which male reactions would be reduced to zero or that synergistically increased male responses. Such a differentiated function of different cuticular lipid fractions was found, for example, in *Lariophagus distinguendus* Förster. In that species, the omission of all CHCs resulted in the absence of male responses. When dividing the CHCs into two fractions—one containing the key hydrocarbon 3-methylheptacosane (3-Me C27, the presence and amount of which correlates with the attractiveness of conspecific individuals; Steiner et al., 2005) and the remaining CHC background (most other CHCs with higher molecular masses)—the omission of the CHC background again led to a virtually complete loss of bioactivity despite 3-Me C27 being present. On the other hand, 3-Me C27 as well as non-volatile cuticular lipids, triacylglycerides, functioned synergistically when added to binary mixtures of the CHC background combined with the respective other compound(s), drastically increasing the duration and frequency of male responses (Kühbandner et al., 2013).

The lack of differentiated reactions of *M. raptorellus* males to the omission of either of the three female-derived extract fractions tested in the present study suggests that in this species, all three lipid fractions seem to be of more or less equal importance for the total signal, and that males may assess the 'completeness' of the signal. To our knowledge, such a cumulative function of the three lipid fractions of different polarities for mate recognition has not been shown before in parasitoid wasps. In most reported cases, female CHCs lead to males spending more time on the filter paper or a dummy treated with that lipid class (Steiner et al., 2006; Ruther et al., 2011), and either CHCs alone or CHCs in combination with only one other lipid fraction or glandular secretions constituted the mate recognition signal (Steiner et al., 2006; Ruther et al., 2011; Ablard et al., 2012; Kühbandner et al., 2012; Stökl et al., 2014; Weiss et al., 2015; Böttinger et al., 2020; Würf et al., 2020; Jungwirth et al., 2021). Our results suggest that although sex-specific CHC profiles are often considered to be an indication for the high potential of CHCs to function for mate recognition, this assumption should be taken with caution. The role of CHCs in chemical communication should be specifically tested by assessing the function of the different cuticular lipid classes using extract fractions rather than testing only crude extracts. Indeed, in our bioassays on binary fraction mixtures the combination omitting the CHCs (binary mixture of D and M fractions) actually elicited the highest number of male reactions—nearly as many as the ternary mixture of all three fractions did in the preceding assay. However,

whether this specific binary mixture really constitutes a 'complete' signal in *M. raptorellus* will need to be evaluated with a larger sample size and in direct comparison to the reactions elicited by the ternary mixture.

One interesting finding of our study was that courtship behaviour beyond wing fanning was only very rarely shown towards dummies treated with single lipid fractions or combinations thereof (only five males responded with antennal courtship and copulation attempts). The number of males showing full courtship behaviour was much larger in response to full extracts (13 of 20 tested males). This may be partly due to the dummies used, as they do not provide any cues for male positioning for the courtship behaviour. Males spent time antennating the glass dummy while walking around on it, presumably to find the correct orientation. This behaviour, however, was hardly shown when positioning themselves on live females, where they usually took up the courtship position on the female very rapidly before directly starting with antennal courtship. Van den Assem & Povel (1973) noted that, when using dead individuals as stimuli ('models'), males would often take up their courtship position oriented towards the rear end of the dead female (and sometimes towards a protruding wing), which rarely occurred with live individuals. This could have been due to males using orientation cues for positioning themselves on live females, perhaps previous movement direction and the female antennae. As the glass dummies used in the present study provide even fewer orientation cues than dead females would, this may cause males to cease courtship behaviour after initial wing fanning, especially for cases in which the chemical signal is not 'complete'. However, this would not explain the almost complete absence of further courtship behaviours to the recombined ternary fraction mixture. It could be that fractionation and the associated solvent evaporation steps led to a partial loss of more volatile compounds. This could have changed the relative ratios of compounds relevant to the mate recognition signal. A reduction in the extent of male responses could ensue if full courtship behaviour would be elicited mostly in response to the right compounds in the right ratios.

It will be interesting to study whether a cumulative function (more than one lipid class being required to elicit courtship reactions in as many males as crude female extract) of different cuticular lipid classes is present in other *Muscidifurax* species. The only other species of *Muscidifurax* for which the signal function of cuticular lipids has been investigated is *M. uniraptor* (Buellesbach et al., 2018). However, this study tested only crude female extract, and it remains unknown which compound class(es) the males actually responded to. Results of a more detailed future study could clarify this, and thereby shed light on the question whether the CHC profile of the parthenogenetic *M. uniraptor* females really represents a reversion to a simpler male-like profile due to a lack of selection pressure to produce a mate recognition signal, as suggested by Bernier et al. (1998). This still remains a viable hypothesis, since different species of *Muscidifurax* might rely on different combinations of cuticular lipid classes for sexual communication, as seems to be the case in *Nasonia* (Steiner et al., 2006; Mair et al., 2017) or as has been shown for the mate recognition function of ididoids, CHCs, or the combination thereof in *Leptopilina* (Weiss et al., 2015; Böttinger et al., 2020).

Although we could show that the chemical signal is vital for mate recognition in *M. raptorellus*, visual cues seem to play a role as well. In all experiments using the black glass dummies, one of the 20 tested males reacted with wing fanning to the dummy even if it was treated only with pure solvent. When crude female extract (two female equivalents as in the other bioassays) was applied to a white glass dummy of the same size, it failed to elicit male wing fanning behaviour (n = 10, data not shown), whereas the same amount of crude female extract applied to black glass dummies was clearly bioactive (wing fanning shown by 15 of 20 males). Similarly, the visual aspect (dark object) and/or tactile cues increased arrestment of *N. vitripennis* males when confronted with female extract applied to beetle elytra or dead conspecifics in comparison to female extract presented on filter paper discs (Steiner et al., 2006).

The study of chemical mate recognition in parasitoid wasps has shown that cuticular lipids are often used for this purpose. However, the assumption that the easily detectable and determinable CHCs are the cuticular lipids responsible for signal function must be taken cautiously, as several studies have revealed that it can be a combination of different cuticular lipid classes or other signals and cues instead. The comparative study of congeneric species could be an especially interesting venue for future research. Several congeneric species seem to use different combinations of chemical compounds for mate recognition, and future studies might evaluate whether sympatric species use different combinations of available compounds for mate recognition more often than allopatric ones.

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AUTHOR CONTRIBUTIONS

Tamara Pokorny:Conceptualization (lead); Formal analysis (equal); Investigation (equal); Supervision (lead); Visualization (lead); Writing—original draft (lead); Writing—review & editing (lead). Katharina Bogenberger: Formal analysis (equal); Investigation (equal); Writing—review & editing (supporting). Joachim Ruther: Conceptualization (supporting); Resources (lead); Supervision (supporting); Writing—review & editing (supporting).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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