Biological Journal of the Linnean Society, 2025, **145**, blae069
https://doi.org/10.1093/biolinnean/blae069
Advance access publication 7 August 2024
Original Article

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Analysing the information content of the multimodal courtship display of a parasitoid wasp

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ABSTRACT

Courtship displays are generally composed of multiple signal components that are transmitted and perceived through different sensory modalities. However, previous studies on multimodal courtship displays have focused mainly on individual signals, thus failing to provide a full explanation of why these displays evolved despite the added costs involved. Therefore, it is essential to identify the purpose of each display component and link them to prevailing hypotheses on multimodal display function. Here, we study the multimodal courtship display of males of the parasitoid wasp *Leptopilina heterotoma*, which consists of wing fanning and a putative antennal pheromone, by determining the information conveyed about male quality through these two modalities. We found that a high wing-fanning frequency and a large body size led to a higher mating success, whereas we found no correlation of the pheromone composition with mating success. We conclude that the wing-fanning component conveys mate-assessment information, whereas the chemical display component, found to be species specific in a previous study, functions as a species-recognition mechanism but does not reflect the quality of a male. Altogether, our study suggests that the multimodal courtship display of *L. heterotoma* fits the multiple message hypothesis and might thus be a useful model for further studies.

Keywords: mate choice; insect communication; signal function; signal evolution; sexual selection

INTRODUCTION

Courtship is a distinct set of behaviours exhibited by an individual to attract and subsequently mate with a conspecific of the opposite sex. It is displayed primarily by males towards females but can also be displayed by females towards males or involve interactions between both sexes (Bastock 1967). Courtship displays have several functions, including the transmission of information on the sex and species of the sender (West-Eberhard 1983). They can also mediate the competition for mating partners by providing information on another mating-relevant trait of the sender, such as body size, and can thus be highly complex in terms of variety and composition (Candolin 2003). Most courtship displays contain multiple components, which are transmitted and perceived through different sensory channels (Hebets and Papaj 2005, Mitoyen *et al.* 2019). Such multimodal courtship displays are generally associated with increased costs for both sender and receiver, mainly owing to the production and perception of the signals and increased predation risk (Partan and Marler 2005).

The concept that animals typically communicate with multimodal displays was expressed by Darwin (1872). However, since then, most studies on multimodal courtship displays have focused on analysing the most salient courtship component, often the visual or auditory component (Candolin 2003, Mitoyen *et al.* 2019). At the same time, studies on multimodal displays in general have focused mainly on signals in isolation or signal specialists (Hebets and Papaj 2005). In recent years, however, there has been a growing interest in determining why multimodal displays evolved despite the additional costs involved (Partan and Marler 2005, Mitoyen *et al.* 2019). The first evolutionary frameworks on multimodal displays and multimodal courtship displays were published in the 1990s, and since then, a variety of hypotheses have been proposed and categorized (Partan and Marler 1999, Rowe 1999, Candolin 2003, Hebets and Papaj 2005, Higham and Hebets 2013). Most hypotheses on the evolutionary mechanisms of multimodal displays are based on two types of selection pressures: content-based and efficacy-based selection (Guilford and Dawkins 1991, Hebets and Papaj 2005). In

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Received 28 February 2024; revised 12 June 2024; accepted 4 July 2024

content-based hypotheses, both modalities can convey the same information for increased accuracy ('redundant signal hypothesis'). For example, the courtship display of several *Drosophila* species consists of visual, acoustic, chemical, and tactile elements. Experimental data suggest signal redundancy in *Drosophila*, because suppression of one modality does not alter copulation success (Colyott *et al.* 2016, Belkina *et al.* 2021). Alternatively, the modalities can convey different information ('multiple messages hypothesis'). These can include different quality aspects of the sender, or one modality can convey the quality of the signaller while another modality conveys species identity.

Recent reviews on multimodal (courtship) signals list several examples for the multiple message hypothesis (Hebets and Papaj 2005, Mitoyen *et al.* 2019). However, in most of those examples the two or more signal components studied were not from different modalities, but from the same modality. In only two of the examples, the information content of signals with components occurring in different modalities was analysed in more detail. In the pygmy swordtail fish, *Xiphophorus pygmaeus*, there is indirect evidence that chemicals mediate species recognition, whereas visual displays mediate conspecific assessment (Hebets and Papaj 2005). Probably the best-studied examples are wolf and peacock spiders. Males of both use a combination of visual and vibratory signalling during courtship, and each modality is important for mating success (Girard *et al.* 2015, Uetz *et al.* 2016, Mitoyen *et al.* 2019). However, although different components of the visual part can be attributed to different qualities of the males, no connection of the vibratory signal to a certain quality of the males was found (Girard *et al.* 2015). Consequently, although courtship displays have been studied intensively over recent years, the role of multiple messages in the evolution of multimodal courtship signals remains unclear.

In this study, we aim to expand the functional and evolutionary understanding of multimodal courtship displays by determining the information about male quality conveyed by the two modalities of the courtship display of the parasitoid wasp *Leptopilina heterotoma* Thomson, 1862. This solitary larval parasitoid is specialized on the model fly genus *Drosophila* (Jenni 1951) and has therefore been studied extensively in recent years (Fleury *et al.* 2009, Wertheim 2022, Quicray *et al.* 2023). The courtship of this species incorporates a two-component multimodal display of the male, which is an essential prerequisite to elicit female receptivity (Jenni 1951, van den Assem 1969). On the one hand, males display a high-frequency wing vibration during courtship, known as wing fanning (Jenni 1951, van den Assem 1969). On the other hand, a putative male antennal pheromone is transferred from the male to the female antennae during an act referred to as antennal stroking (Isidoro *et al.* 1999, Weiss 2015). Once the male has detected the female, courtship starts by displaying wing fanning in front of the female and, subsequently, the male attempts to mount the female. If mounting is successful, the male performs antennal stroking and immediately starts wing fanning on the back of the female (Jenni 1951, van den Assem 1969, Isidoro *et al.* 1999). If the female accepts the courting male, she will indicate receptivity by bringing her antennae into a forward position and opening the genital pouch. The male will then stop the courtship display and mate with the female (van den Assem 1969). Importantly, a mate-choice trial conducted by Weiss (2015) indicated that females are not only monandrous, but also appear to choose their mating partner. The first encounter of a naïve juvenile female with a likewise conspecific of the opposite sex resulted in mating in only one-sixth of all mating trials (Weiss 2015). The combination of the obligatory multimodal courtship display and this observed choosiness suggests the presence of a female mate-choice process. This selection process is most likely to be based on mate-assessment information transmitted in the multimodal courtship display of the male. Thereby, the information on mate quality, which is potentially encoded in the male courtship display, could provide indirect fitness benefits for the female wasp (Godfray 1994).

To investigate which component of the multimodal courtship display of *L. heterotoma* influences female mate choice, we conducted mate-choice trials, during which we measured the wing-fanning signal and, subsequently, conducted a quantified analysis of the putative male antennal pheromone. Although the putative male antennal pheromone of *L. heterotoma* has previously been found to be species specific (Weiss *et al.* 2015) and thus most probably mediates species recognition, it is currently not known whether it also carries mate-assessment information, as has been found for the sex pheromones of several insect species (Steiger and Stökl 2014). Nevertheless, based on the previous study (Weiss *et al.* 2015) we predicted that the chemical profiles of successful and unsuccessful males do not differ significantly. Furthermore, we analysed the wing-fanning frequency and other wing-fanning parameters, because some of these factors have previously been found to influence mate choice in various Hymenoptera taxa. For example, mate quality of some parasitoid wasps was found to be encoded in high wing-fanning frequency (Benelli *et al.* 2013, 2016b, 2020). Thus, we predicted that male mating success is influenced by the wing-fanning signal, with a higher wing-fanning frequency in successful males. Finally, given that male body size also often affects mating success in insects (e.g. Simmons 1986, Blaul and Ruther 2012, Benelli *et al.* 2016a), we also included this parameter in our analysis and predicted that it affects mating success.

MATERIALS AND METHODS

Experimental animals

The established wasp laboratory strain of *L. heterotoma* originates from a collection in Leiden, The Netherlands. The wasps were reared on larvae of *Drosophila melanogaster* as described previously (Böttinger and Stökl 2020). Approximately 30 *D. melanogaster* were put into a plastic jar (8 cm in height, 3.5 cm in diameter) with corn-based substrate [ingredients: 1 L water, 75 g sugar, 50 g cornmeal, 50 g wheat germ, 40 g yeast, 5 g agar, 2.5 mL propanoic acid, and 10 mL nipagin (10%)]. The jars were closed with foam plugs and stored in a climate-controlled chamber to maintain a temperature of 25 °C, ~60% humidity, and a 16 h–8 h light–dark cycle. Forty-eight hours after placing the flies on the substrate, flies were removed from the jar, and five to seven female wasps and three male wasps were added to it. Two to five days before hatching of *L. heterotoma*, pupae were removed from the jar and separated into 1.5 mL microcentrifuge tubes to remain unmated and naïve. Every tube was equipped with two pieces of filter paper (0.5 cm in diameter), one soaked in 50:50 honey water solution and one soaked in water, to provide nutrition for the hatched wasp. Hatching of the isolated wasps was tracked once a day to determine the approximate age of the individuals. All experimental animals were 1–7 days old and were used only once for experiments.

Arena mating trials

All mating trials were performed in the afternoon (12:30–18:00 h) from November 2021 to February 2022. For every trial, a random pair of one male and one female wasp were placed in an open Petri dish (4 cm in diameter; Supporting Information, Fig. S1). The 5–15 min observation time per couple was recorded with a video camera (Canon EOS 70D, 100 mm macro-objective; Supporting Information, Fig. S1) for later video analysis. The trial was terminated after the end of successful mating or after a maximum of 15 min of continuous courtship behaviour in the form of male wing fanning and mounting attempts. A mating was defined as successful if the female and male abdomens were connected continuously and the male individual was suspended in mid-air behind the female for >30 s (Supporting Information, Video S1 and S2; Jenni 1951, van den Assem 1969, Isidoro *et al.* 1999). If the experimental animals tried to escape the arena more than twice, the trial was terminated earlier. After the end of the trials, male individuals were assigned to two groups (successful males and unsuccessful males) according to their observed mating success during the trials. They were stored at −80°C to preserve the male antennal pheromone and structural integrity of the bodies for later chemical analysis and dissection. We observed the courtship of 234 pairs, of which 41 pairs mated successfully and 193 mating trials ended in rejection of the male. Owing to insufficient video or audio material, 6 of the 41 successful matings were excluded from the statistical analysis. To even up the two groups, 35 of the 193 unsuccessful matings were chosen randomly for statistical analysis with the online randomizing software RESEARCH RANDOMIZER (randomizer.org).

Recording of male wing-fanning vibrations

The wing-fanning vibrations produced by males while mounting the female during courtship were recorded with a laser Doppler vibrometer (Polytech PDV-100, Waldbronn, Germany) with a velocity measurement rate of 20 mm/s and low-pass filter at 22 kHz (Supporting Information, Fig. S1). The laser was connected to a laptop with a 64-bit sound card, and RAVEN PRO v.1.6.4 sound-editing software (Cornell Lab of Ornithology, Ithaca, NY, USA) was used for the audio recordings with a sampling rate of 44.1 kHz. Reflector foil was placed on the arena ground to amplify the laser-beam reflection. The laser was positioned ~50 cm above the arena, with the laser beam pointing at the arena floor. This resulted in a maximal distance of 4 cm between the experimental animal and the laser beam (Supporting Information, Fig. S1).

Sound analysis

Sound analysis was performed in RAVEN PRO v.1.6.4 and included 35 wing-fanning events of successful males and 35 of unsuccessful males. Wing-fanning events were defined as periods during which the male individual showed wing fanning while mounting the female. If more than one such wing-fanning event was recorded, we analysed the wing-fanning event resulting in female acceptance (successful males) or chose the wing-fanning event randomly (unsuccessful males). We assessed wing-fanning duration (in seconds), peak frequency (in herz), fundamental frequency (in herz), and delta frequency (in herz) at the beginning and end of wing-fanning sequences (Fig. 1). The fundamental frequency represents the frequency of the wing movement, whereas the peak frequency is the frequency with the highest intensity in the recording and can be the same as the fundamental frequency. To determine the fundamental frequency, the algorithm calculating peak frequency was used while selecting the lowest visible frequency only. Delta frequency gives the increase and decrease of the wing-fanning frequency at the beginning and end of a wing-fanning event. Delta frequencies

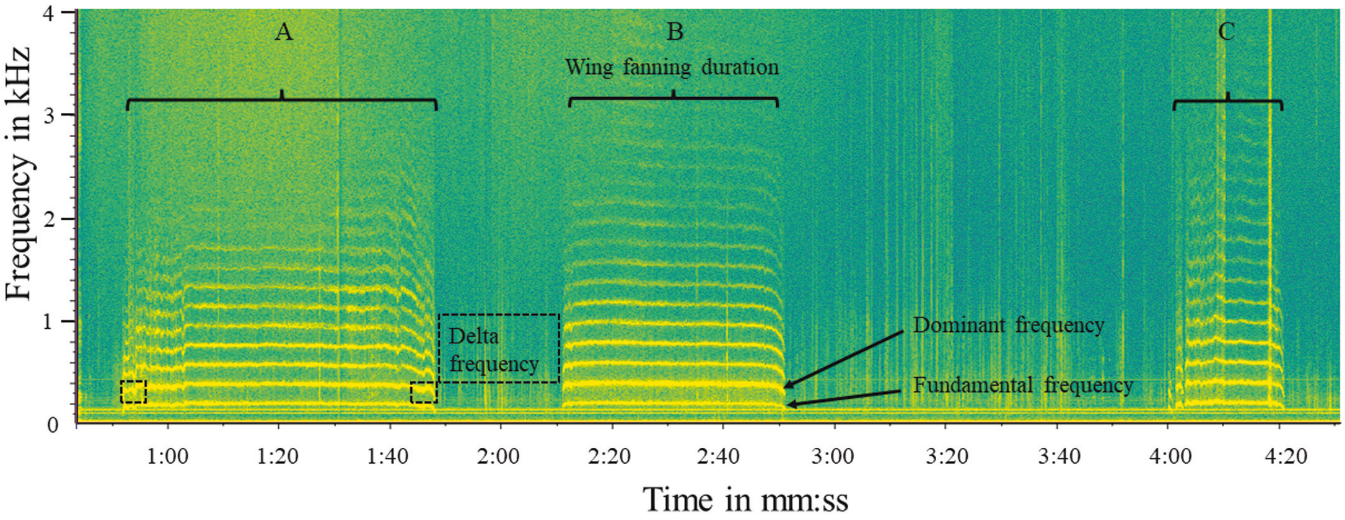

Figure 1. Example spectrogram from sound analysis of *Leptopilina heterotoma* wing fanning. A–C, individual wing-fanning sequences from the same individual. The braces indicate wing-fanning duration, the dashed boxes are delta frequency at the beginning and end of the wing-fanning sequence, and the arrows point out the dominant frequency and the fundamental frequency.

were measured by selecting the rising initial part and decreasing end part of the sound sequences.

Chemical analysis

Male wasps were defrosted for 5–10 min before extracting the individual antennal pheromone for 10 min in 15 µL *n*-hexane (Roth, Karlsruhe, Germany) with 5 ng/µL Octadecan C18 (Sigma Aldrich, Taufkirchen, Germany) as the internal standard. The samples were analysed with a coupled gas chromatograph (GC2030) and mass spectrometer (QP2020NX, Shimadzu, Duisburg, Germany). The gas chromatograph was equipped with a non-polar capillary column (SH Rxi-5 Sil MS, 30 m in length, .25 mm inner diameter, .25 µm film thickness, Shimadzu, Duisburg, Germany). Helium was used as the carrier gas (50 cm/s linear velocity). The oven programme started at 80°C, and temperature was raised by 5°C/min to 280°C. The putative male antennae pheromone of *L. heterotoma* has been identified by Weiss *et al.* (2015). It consists of 22 cuticular hydrocarbons but is dominated by 9,19-pentatriacontadiene, which is found only in males and makes up almost 50% of all compounds found in the pheromone (Weiss *et al.* 2015). In our study, we quantified the 20 most frequent cuticular hydrocarbons with the GCMS POSTRUN ANALYSIS software (Shimadzu, Duisburg, Germany).

Body size analysis

We measured the wing length, tibia length, and femur length of all males, because these factors are typically used as indicators of body size in parasitoid wasps (Burton-Chellew *et al.* 2007, Avila *et al.* 2017). To account for potential bilateral body asymmetry, we dissected the right wing and right hind leg of every animal [except for one male with a damaged right wing, for which we used the left wing instead; leg (right) $N = 70$, wing (right) $N = 69$, and wing (left) $N = 1$] under a binocular microscope (Zeiss Stemi 305) and fixed them on flash cards with adhesive tape. We measured wing length, femur length, and tibia length under a digital macroscope (DvM6M, Leica, Wetzlar, Germany) with ×100 optical zoom.

Statistical analysis

All calculations were performed in R v.4.3.2 (R Core Team 2023). Plots were built with the R packages *ggplot2* (v.3.3.6), *ggsignif* (v.0.6.3), *jtools* (v.2.2.0), *ggpubr* (0.6.0), and *interactions* (v.1.1.5). We used the Wilcoxon test to compare the total duration of wing-fanning events between groups. Furthermore, we calculated the Pearson correlation coefficient of all three body-size indicators to determine their relationship. Given that they exhibited highly significant correlations (see Results), we chose wing length as the body-size indicator, because it is the organ producing the vibrations. Subsequently, we calculated a regression plot of the chosen body-size indicator (wing length) and fundamental frequency to determine the relationship of these two factors.

The effect of wing-fanning frequency parameters and body size on mating success was assessed with a binomial generalized linear model (GLM) and a subsequent ANOVA using scaled values. In this analysis, we used male success as the explanatory variable, with the response variables fundamental frequency, peak frequency, delta frequency start/end, and wing length. The model was checked using the *Dharma* package (v.0.4.6). Additionally, we visualized the interactive effect of body size and fundamental frequency on male mating success, based on this binomial GLM, with the packages *jtools* (v.2.2.0) and *interactions* (v.1.1.5).

To determine whether frequency and body size might mutually influence mate choice, we calculated wing acceleration during wing fanning using the following formula: (fundamental frequency)^2 × (wing length). We performed a binomial GLM with mating success as the explanatory variable and wing acceleration as the only response variable. This calculation was followed by an ANOVA.

To test for significant differences in the compositional data of the male antennal pheromone between groups, we performed distance-based analysis based on centred log-ratio (clr)-transformed data according to Hervé *et al.* (2018). Initially, we clr-transformed the compositional data with the *compositions* (v.2.0.6) R package, then performed a non-metric dimensional scaling (NMDS) ordination with Euclidean distance with the R packages *vegan* (v.2.6-2), *lattice* (v.0.20-45), and *permute* (v.0.9-7). We also performed a principal component analysis and a one-way analysis of similarity based on the Bray–Curtis distance with 9999 permutations using the *vegan* (v.2.6-2) package. A complete R script is provided in the Supporting Information (Lang *et al* R-Skript v3.Rmd).

RESULTS

Wing-fanning duration

Wing-fanning duration was significantly shorter in successful courtship compared with unsuccessful courtship (Fig. 2; Wilcoxon test, $W = 177$, $P < .001$). Successful wing fanning lasted, on average, 22.17 ± 7.17 s (mean ± SD), whereas unsuccessful wing fanning took, on average, 36.99 ± 11.91 s.

Wing fanning and body-size parameters

The three body-size parameters (femur length, tibia length, and wing length) exhibited highly significant correlations (Supporting Information, Fig. S2; $R > 0.85$, $P < .001$). Fundamental frequency and wing length were negatively correlated (Fig. 3; $R = -0.41$, $P < .001$); therefore, higher frequency was significantly correlated with smaller body size. We found a significant effect of fundamental frequency on mating success ($\chi^2_{1,\ 63} = 6.1113$, $P = .0134$) and of wing length on mating success ($\chi^2_{1,\ 63} = 7.3927$, $P = .0065$) in the GLM containing all frequency parameters and body size (Table 1). Accordingly, males with higher fundamental frequency were more successful in obtaining mating than males with lower fundamental frequency (Fig. 4; Supporting Information, Fig. S3), and larger males were more successful in courtship than smaller males (Fig. 4). At the same fundamental frequency, big males (+1 SD) had a ~30% higher chance to succeed in mating compared with small males (−1 SD). This difference in mating success was true for all observed fundamental frequencies but was lowest at high frequencies (20%, >220 Hz) and strongest at medium-high frequencies (40%, 180–210 Hz). Additionally, small males (−1 SD) had a very low to zero chance of mating if they also vibrated at very low frequencies (Fig. 4; 160 Hz). In contrast,

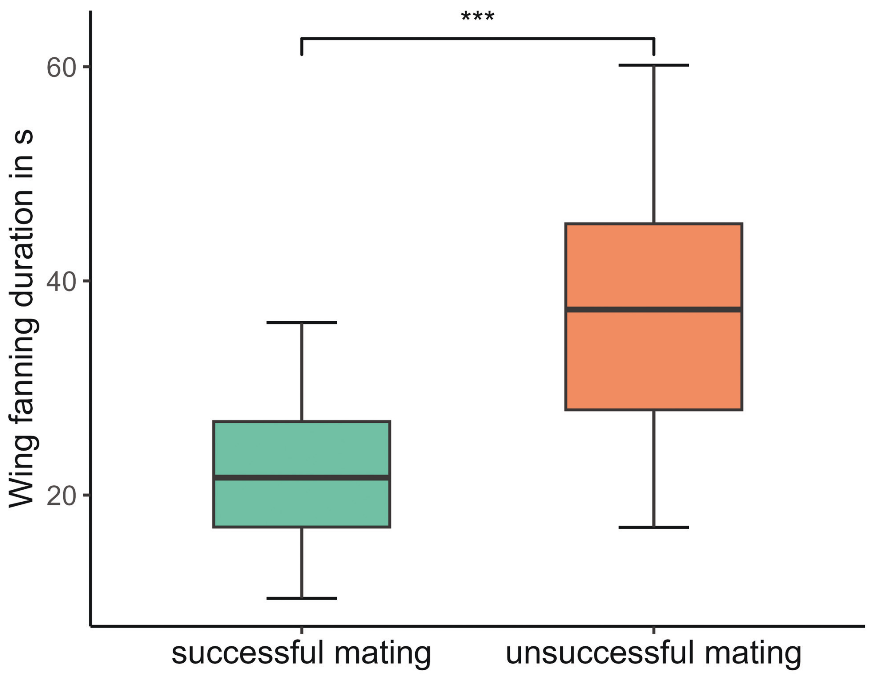

Figure 2. Boxplots showing the observed wing-fanning duration (in seconds) of successful ($N = 35$) and unsuccessful ($N = 35$) courtships of *Leptopilina heterotoma* males. ***Significant difference (Wilcoxon test, $W = 177$, $P < .001$).

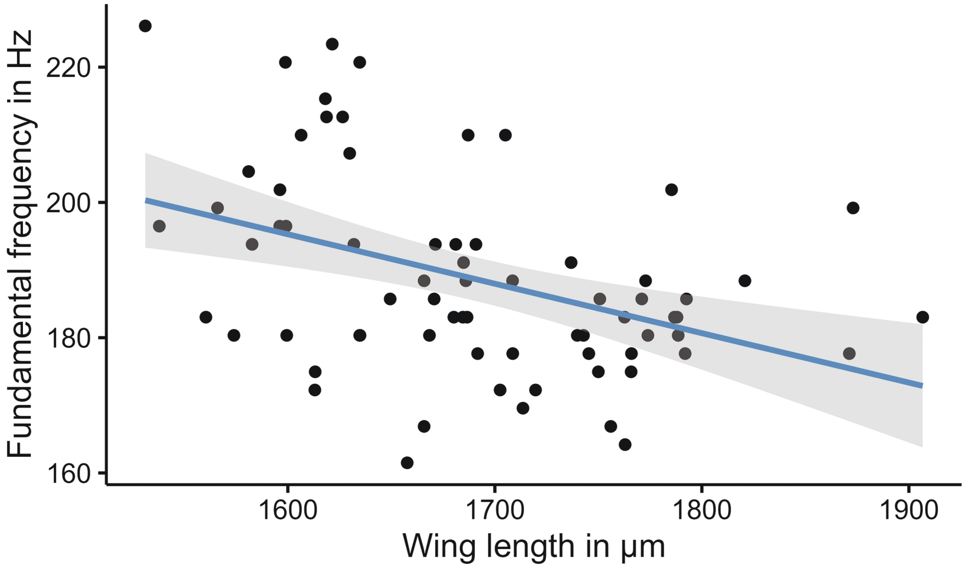

Figure 3. Scatterplot of the correlation of fundamental frequency (in herz) and wing length (in micrometres) of all 70 analysed *Leptopilina heterotoma* males. The black dots represent individual males. Linear regression: $R = -0.41$, $P < .001$. The grey area indicates the 95% confidence interval of the regression line.

large males (+1 SD) that used very high frequencies (>220 Hz) were accepted for mating with >75% chance (Fig. 4). Wing acceleration had a significant effect on male mating success (GLM, $\chi^2_{1,\,68} = 4.1915$, $P = .04063$). Successful males had significantly higher wing acceleration than unsuccessful males (Fig. 5).

Chemical analysis

The statistical analysis of the quantified putative antennal pheromone data showed a complete overlap of successful and unsuccessful males (Fig. 6; NMDS stress = 0.065). The analysis of similarity confirmed that there was no significant difference between the chemical profiles of successful and unsuccessful males ($R = 0.023$, $P = .11$).

DISCUSSION

We found no significant differences in the chemical profiles of successful and unsuccessful males (Fig. 6). Thus, our study provided no evidence that this olfactory signal transmits mate-quality information in addition to the information on species identity that it carries. Furthermore, the analysis of the wing-fanning display revealed significantly shorter wing-fanning durations of successful males (Fig. 2), suggesting that this part of the courtship display also does not carry information on male quality. However, we found significant differences in the fundamental frequency of the wing-fanning display and the body size of successful and unsuccessful males (Table 1; Fig. 4), demonstrating that the wing-fanning frequency and male body size influence female mate choice. Thus, our study provided evidence for our prediction that the multimodal courtship display of *L. heterotoma* contains multiple messages to mediate mate choice and species recognition (Pfennig 1998, Hebets and Papaj 2005).

The chemical analysis of the pheromone of the males used in the mating trials did not show a significant difference between successful and unsuccessful males. A previous study found significant differences in the chemical composition of the putative pheromone between species and revealed that males could elicit female receptiveness only in conspecific females, suggesting that the pheromone functions as a mate-recognition signal (Weiss *et al.* 2015). This conclusion is supported by our finding that the putative male antennal pheromone had no significant effect on mating success and thus does not transmit mate-assessment information.

Table 1. Effect of frequency parameters and body size on male mating success of *Leptopilina heterotoma*. All estimates were calculated with a generalized linear model with binomial distribution and a subsequent ANOVA. Significant values are indicated in bold.

Parameter	χ^2	d.f.	*P*-value
Fundamental frequency	6.1113	1	**.0134**
Wing length	7.3927	1	**.0065**
Peak frequency	2.9977	1	.0833
Delta frequency start	0.2059	1	.6500
Delta frequency end	1.9771	1	.1597

Heterospecific matings impose high fitness losses on *L. heterotoma* females because of their monandry (Jenni 1951, van den Assem 1969, Weiss *et al.* 2015) and the *Wolbachia*-induced cytoplasmic incompatibility (Fleury *et al.* 2000). Thus, there is a high selective pressure on the prevention of heterospecific matings. Females of *L. heterotoma* produce a volatile sex pheromone to attract males and induce courtship (Weiss *et al.* 2013). However, mate attraction is not species specific in *L. heterotoma*, because the sex pheromone attracts not only conspecific males but also heterospecific males from the closely related species *Leptopilina boulardi* (Weiss *et al.* 2013). The potential spatial proximity of heterospecific individuals during the precourtship stage presents a high risk of heterospecific matings, which could necessitate a reliable downstream mate-recognition system. Furthermore, there is a high level of cryptic morphological variation among *Leptopilina* species (Lue *et al.* 2016); therefore, there are no clear visual cues ensuring mate recognition in this genus. In such cases of morphologically very similar animals, detection of conspecifics can be mediated via several courtship signals (Mitoyen *et al.* 2019). There are examples of multiple mate-recognition signals in other species, e.g. in *Heliconus* butterflies, which produce olfactory and visual signals that are thought to ensure reproductive isolation (Southcott and Kronforst 2018). Therefore, it is not surprising that another species-recognition mechanism is implemented in the last step before mating takes place. This could explain why there was no additional mate-assessment information encoded in the species-recognition

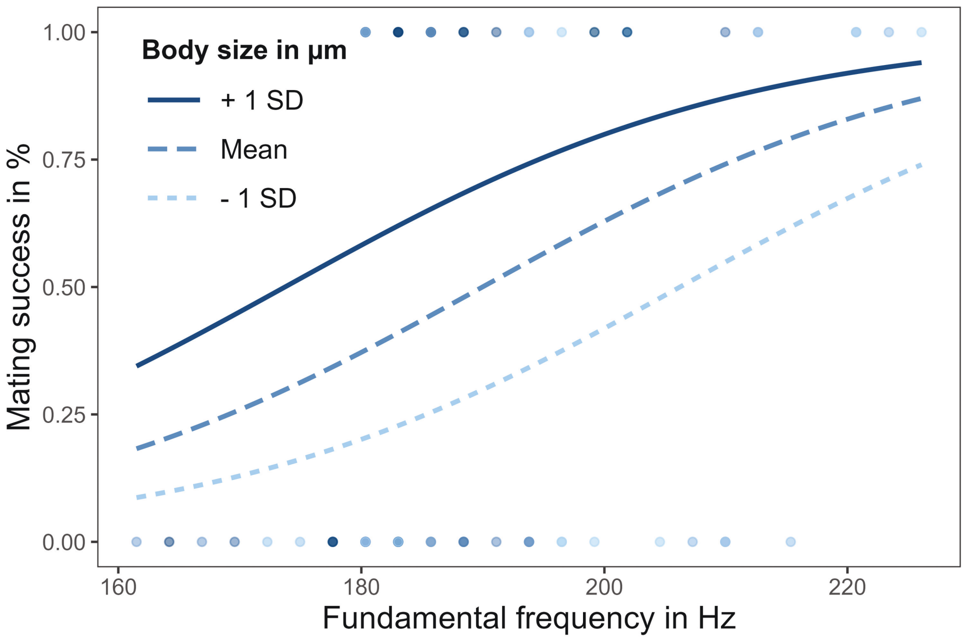

Figure 4. Interactive effect of male body size and fundamental frequency on male mating success of *Leptopilina heterotoma* males. The interaction is demonstrated by regression lines for the mean chance of mating success of small (−1 SD; light blue/dotted line), medium (middle blue/dashed line), and large (+1 SD; dark blue/solid line) males. The dots represent individual males, with the colour coded as for the lines.

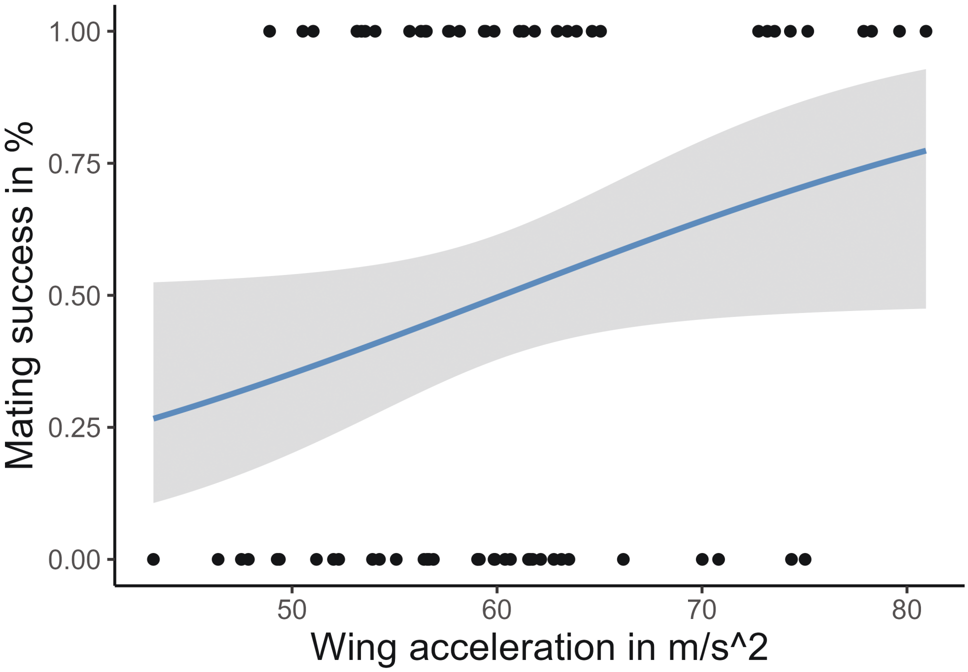

Figure 5. Relationship between mating success and wing acceleration of *Leptopilina heterotoma* males. The blue line represents the calculated regression line, and the grey area indicates the respective 95% confidence interval. The black dots represent individual males. The graphs are calculated with a generalized linear model, with success as the explanatory variable and with wing acceleration, calculated as (fundamental frequency)^2 × (wing length), as the response variable.

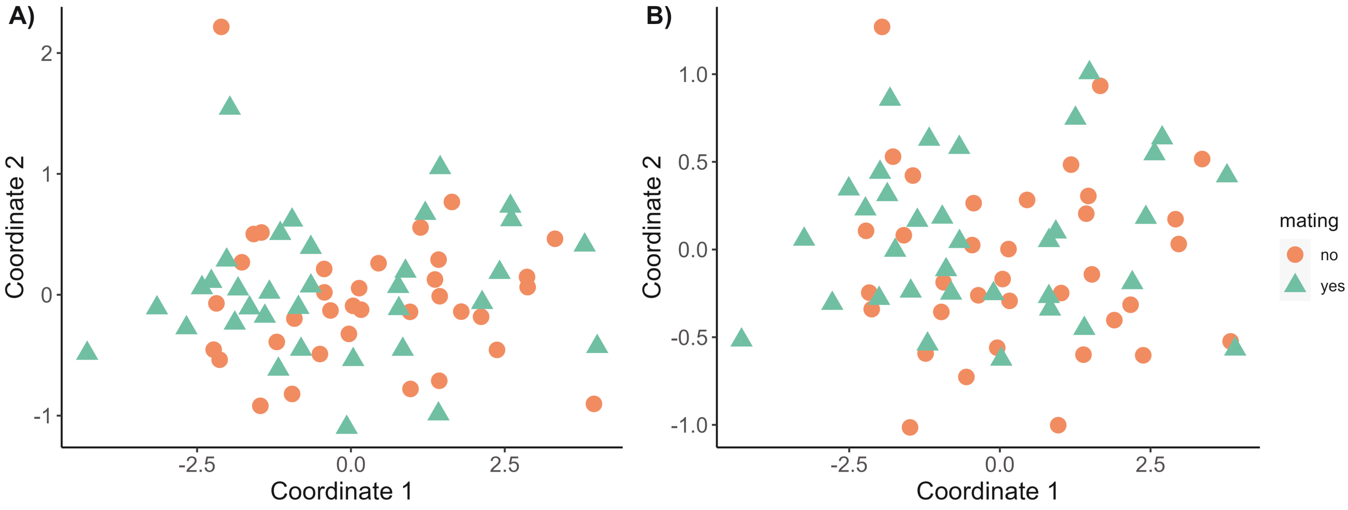

Figure 6. Analysis of chemical profiles of *Leptopilina heterotoma* males. Non-metric multidimensional scaling based on the Euclidean distances (stress = 0.065; A) and principal components analysis (B) of centred log-ratio-transformed relative proportions of putative male antennae pheromone components for unsuccessful (orange dots) and successful (green triangles) *L. heterotoma* males. Each dot represents an individual male (N = 70).

signal: if there is high stabilizing selective pressure on the species-specific information of the signal, there is not much capacity for variation encoding mate-quality information (West-Eberhard 1984). Additionally, it is assumed that the necessary transmission of various qualities always facilitates the evolution of multimodal signalling (Wilson *et al.* 2013, Mitoyen *et al.* 2019).

Antennal glands and courtship are not unique to the genus *Leptopilina*, but are found in several species of wasps from different families (Isidoro *et al.* 1999, Battaglia *et al.* 2002, Romani *et al.* 2008, Klopfstein *et al.* 2010), in addition to bees and ants (Romani *et al.* 2003, Heinze *et al.* 2021). However, the function of the antennal courtship behaviours in these taxa have not been elucidated comprehensively. It thus remains unclear whether antennal courtship always functions as an additional species-recognition mechanism or has evolved a different function in other taxa. Furthermore, the chemical identification of the pheromones involved in these courtship behaviours remains largely unexplored across the various species mentioned. The exact compounds responsible for signalling and their specific roles in the courtship rituals remain elusive.

The significantly shorter wing-fanning duration of successful males (Fig. 2) indicates that this parameter did not convey mate-assessment information either. Other studies that investigated wing fanning in parasitoid wasps did not find any effect of courtship duration on mating success (Benelli *et al.* 2016b, 2020). Males of *L. heterotoma* stop wing fanning and antennal stroking as soon as the females expose their genitalia (van den Assem 1969). Therefore, the shorter wing-fanning duration of successful males can be explained by the fact that females mated with them, leading to a shorter courtship period, whereas unsuccessful males continued their efforts until they eventually gave up.

Our most important finding was that the fundamental frequency of the wing-fanning signal and male body size had a significant effect on male mating success (Table 1; Fig. 4). Previous studies on Hymenoptera also detected significant correlations between increased mating success and higher fundamental wing-fanning frequencies (Benelli *et al.* 2013, 2016b, 2020) or between increased mating success and larger body sizes (Teder 2005, Joyce *et al.* 2009, Avila *et al.* 2017). However, there is little research on multimodal courtship displays in insects that incorporate wing vibration and body-size parameters (Webb *et al.* 1984, Benelli *et al.* 2016a); therefore, there is no consistent theoretical framework on how these two quality indicators mutually influence mating success of Hymenoptera. Benelli *et al.* (2016a) found that in the olive fruit fly, *Bactrocera oleae*, wing vibrations displayed by successful large males had higher frequencies than those of smaller males and unsuccessful large males. They suggested that females select for larger males through the evaluation of wing-fanning frequency, and that larger males have advantages to produce higher wing-fanning frequencies owing to larger wing muscles (Benelli *et al.* 2016a). This interpretation indicates a positive correlation between body size and fundamental frequency. However, from a kinetics point of view, higher frequencies are generally associated with smaller body size (Byrne *et al.* 1988), because there is a strong supposed connection between the frequency of vibration and the natural resonant frequency of wing beats (Pringle 1957, De Luca *et al.* 2019). This kinetic view is supported by our study, in that we found a negative correlation of wing-fanning frequency and body size in *L. heterotoma* (Fig. 3), but also by the study of Webb *et al.*(1984) on the courtship of the Caribbean fruit fly, *Anastrepha suspensa*. In that species, larger males produce calling songs of lower fundamental frequency (Webb *et al.* 1984).

Therefore, we believe that there is potential for a contrasting interpretation of the interplay of wing-fanning frequency and body size. We hypothesize that fundamental frequency and body-size parameters are evaluated mutually, because owing to the negative correlation of wing-fanning frequency and body size, wing-fanning frequency is a good indicator of condition only if seen in relationship to body size. Reaching the same fundamental frequency requires a higher metabolic rate for bigger animals, because they must accelerate their longer wings faster to achieve the same frequency (Clark 2012, De Luca *et al.* 2019); therefore, from a metabolic perspective, a medium-high frequency can possibly be a great achievement for large males and a very weak performance for small individuals, because the former would require much higher wing acceleration. Our data show that females do indeed evaluate the wing-fanning frequency in relationship to body size. A wing-fanning frequency of 200 Hz, for example, resulted in a ~40% mating chance if produced by a small male, but in ~75% mating chance in a large male (Fig. 5). This scenario could result in a selection for large males with shorter wings to increase the wing-fanning frequency. However, we do not see such an effect in our correlation of leg length and wing length (Supporting Information, Fig. S2), probably because shorter wings deteriorate the flight abilities of males.

An alternative hypothesis is that body size and wing-fanning frequency could inform about two distinct qualities in males. In this scenario, wing-fanning frequency might be indicative of the physiological condition of the male, reflecting his current health and vigour, whereas body size might indicate a more general aspect of quality related to developmental history and long-term fitness. A larger body size is often associated with higher overall fitness, which has also been observed in parasitoid wasps. For instance, in *A. tabida*, the fecundity, fat reserves, and longevity of females were positively correlated with body size (Ellers *et al.* 1998). However, the body size of parasitoids is determined predominantly by the host size (Hails 1989, Cohen *et al.* 2005), which renders body size an unreliable indicator of the genetic quality of a male. In several insect species, a positive correlation between male body size and the number of sperm has been observed (e.g. Gage 1994, Sturm 2014). Therefore, it is plausible that male body size is indicative of a direct benefit (i.e. a high number of sperm) for the female in *L. heterotoma*. However, this hypothesis remains to be tested empirically.

CONCLUSION

In conclusion, our study showed that body size and wing-fanning performance had an effect on mating success, whereas the antennal pheromone did not. In combination with the previously discovered species specificity of the antennal pheromone, our data suggest that the two modalities of courtship convey different information: male quality in the vibrational modality and male species identity in the chemical modality. This finding aligns with the multiple messages hypothesis proposed by Hebets and Papaj (2005) and highlights the potential of *L. heterotoma* as a valuable model system for further research into the complex dynamics of multimodal communication.

SUPPLEMENTARY DATA

Supplementary data is available at *Biological Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

We would like to thank Dr Matthias Schott and the Department Animal Ecology I of the University of Bayreuth for the possibility of using their digital macroscope for the measurements of body size. We would also like to express thanks to Dr Magdalena Mair for her consultations with R coding and statistical analysis. We would also like to thank our technical assistants, Andreas Liehr and Andrea Kirpal, for their assistance in rearing the experimental animals.

CONFLICT OF INTEREST

None declared.

FUNDING

This work was supported by the German Research Foundation (DFG; grant number 520619503/STO 966/3-1 to J.S.) and the Open Access Publishing Fund of the University of Bayreuth.

DATA AVAILABILITY

All datafiles and the R-script are available in the Supporting Information for this article.

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