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Ecology and Behavior

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# Tapping for love: courtship, mating, and behavioral asymmetries in two aphid parasitoids, *Aphidius ervi* and *Aphidius matricariae*

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Understanding parasitoid biology and ecology might have direct implications for their evaluation as biological control agents, as well as for the development and implementation of mass-rearing techniques. Nonetheless, our current knowledge of the possible influence of lateralized displays (i.e., the asymmetric expression of cognitive functions) on their reproductive behavior is scarce. Herein, we characterized the behavioral elements involved in courtship, and quantified their durations of 2 commercial aphid parasitoids, *Aphidius ervi* Haliday and *Aphidius matricariae* Haliday (Hymenoptera: Braconidae: Aphidiinae). We quantified the main indicators of copulation and examined the occurrence of lateralized traits at the population level. Results indicated that *A. matricariae* exhibited longer durations of wing fanning and antennal tapping and the overall precopula and copula phases compared to *A. ervi*. Postcopulatory behavior was observed only in *A. matricariae*. Unlike other parasitoid species, the duration of wing fanning, chasing, and antennal tapping did not affect the success of the mating of male *A. ervi* and *A. matricariae*. Both species exhibited a right-biased female kicking behavior at the population level during the precopula. Our study provides insights into the fundamental biology of aphidiine parasitoids and reports the presence of population-level lateralized mating displays which can serve as useful benchmarks to evaluate the quality of mass-rearing systems.

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AQ9 **Key words:** biological control, Braconidae, lateralization, mass rearing, parasitic wasp, reproductive behavior

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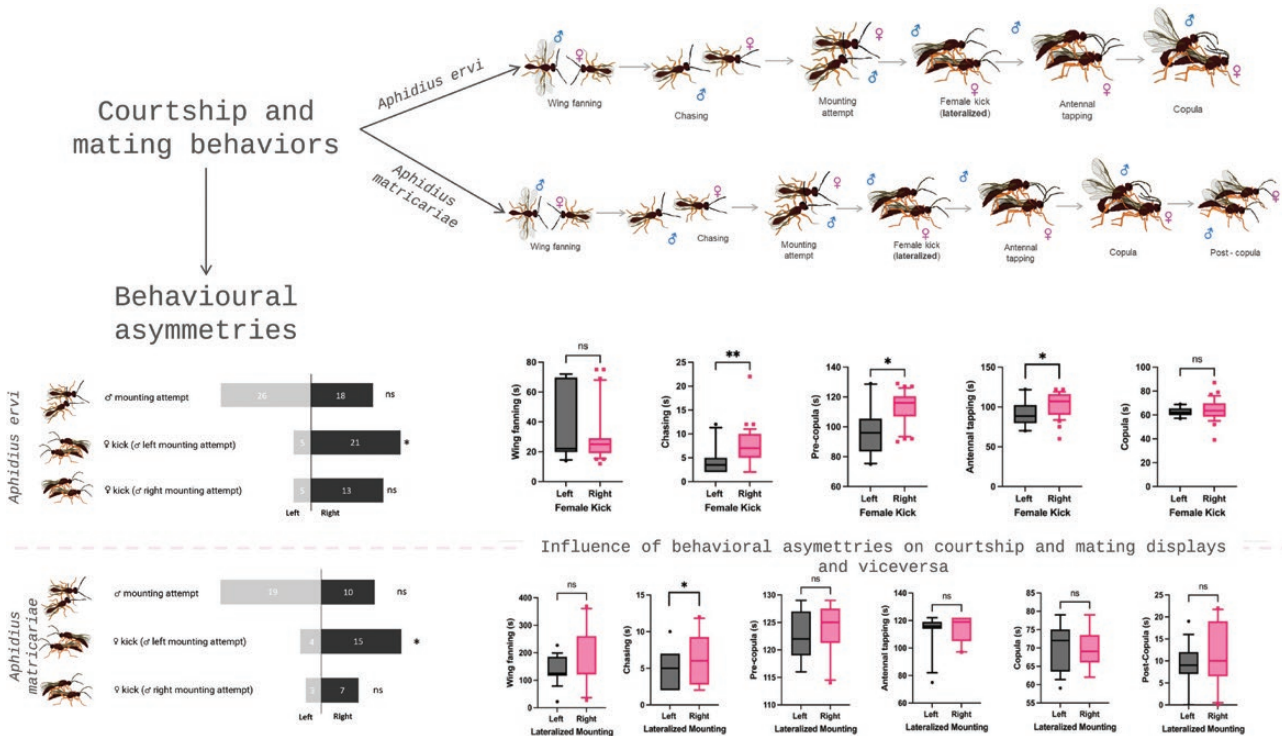
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## Graphical Abstract



## Introduction

Lateralization is defined as an aptitude to utilize 1 side of the brain for certain activities and is seen in both vertebrate and invertebrate species (Frasnelli and Vallortigara 2018, Niven and Frasnelli 2018, Güntürkün et al. 2020). Lateralization results in the performance of behavioral asymmetries which may occur at the individual level, ranging from 1 individual to another regardless of the common bias of the population, or at the population level, where all individuals within a group can consistently display a bias (Niven and Frasnelli 2018). Many instances of behavioral lateralization have been discovered in invertebrates, including insects (Niven and Frasnelli 2018), where the kind of asymmetry may be driven by their social context (Rogers et al. 2013a). Insects demonstrate lateralization in a variety of behavioral domains, including locomotion, foraging, hostile encounters, and mating (Vallortigara and Rogers 2005, Frasnelli et al. 2012, Rogers et al. 2013b, 2016, Hunt et al. 2014, 2018, Benelli et al. 2015a, 2015b, 2015c). For example, certain insects such as honeybees, bumblebees, and fruit flies show asymmetries in how they process olfactory information (Letzkus et al. 2006, Duistermars et al. 2009, Anfora et al. 2010, 2011, Frasnelli et al. 2010, Rigosi et al. 2015). Similarly, the Australian bush cricket, *Requena verticalis* (Walker) (Orthoptera: Tettigoniidae) demonstrates the same principle when it comes to its auditory sense (Bailey et al. 2022). There have been recorded cases of lateralized features found in several insects courting and mating activities. At the population level, for example, some insects demonstrate a left-biased approach, with most males preferring to approach possible mates from the left side (Benelli et al. 2015a, 2017a, Romano et al. 2020). Also, some parasitic wasps showed behavioral asymmetries at the population level, which may play a role in mate detection and sexual interactions (Romano et al. 2016, 2018, Benelli et al. 2020). However, a fundamental understanding of the occurrence and roles of behavioral asymmetries in parasitoids is still scarce. Additional research is required to shed

light on the scope and relevance of these asymmetries in parasitoids. Overall, the study of their courting and mating behavior has practical implications for biological control programs (Benelli et al. 2012a, 2017). Indeed, understanding sexually selected displays and lateralized behaviors can also aid in the selection of parasitoid strains with higher reproductive success, improve mass-rearing processes, and reduce mating failures, all of which can impede the overall success of biological control programs (Rodríguez et al. 2002, Joyce et al. 2010). Furthermore, determining lateralized behavior could help in the process of introducing new genetic material into laboratory-reared populations.

In this scenario, the purpose of the present research is to investigate the courtship and mating behaviors of 2 wasps, *Aphidius ervi* Haliday and *Aphidius matricariae* Haliday (Hymenoptera: Braconidae: Aphidiinae). These parasitoids are solitary, cosmopolitan species used in biological control of many aphids of economic importance, such as *Myzus persicae* (Sulzer), *Aphis craccivora* Koch, and *Macrosiphum rosae* (L.) (Hemiptera: Aphididae) (McClure et al. 2007, Giri et al. 2012, Rakhshani et al. 2019, Gadallah et al. 2022). Although several studies have been conducted on *A. ervi* and *A. matricariae* host-parasitoid interactions (Tahriri et al. 2007, Nyabuga et al. 2010, He et al. 2011, Rezaei et al. 2019), little is known about their courting and mating behavior (McClure et al. 2007, Bourdais and Hance, 2009). In this circumstance, the research presents a characterization of the *A. ervi* and *A. matricariae* courting and mating behavior, revealing insight into the major mating displays and overall mating success of both species. The knowledge of the reproductive patterns of parasitoids is essential for assessing their viability as BCAs, as well as developing and implementing mass-rearing systems. Since recent research has shown that lateralized traits (Vallortigara and Rogers, 2005, Rogers and Vallortigara, 2015, Frasnelli 2018) have a significant impact on parasitoid mating performances (Romano et al. 2016, 2018), herein we investigated



the occurrence of population-level behavioral asymmetries during the sexual interactions of *A. ervi* and *A. matricariae*.

## Materials and Methods

### Insect Collection and Rearing

Fifty colonies of aphids were collected live and mummified from *Medicago sativa* L. (Fabaceae) in Athens in April and *Antirrhinum majus* L. (Plantaginaceae) in Kifissia in May. The material was inserted in plastic containers covered with fine elastic gauze and transferred to the Laboratory of Agricultural Zoology and Entomology (Agricultural University of Athens, Greece) for aphid identification following the keys of Blackman and Eastop (2000, 2006). *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae) on *M. sativa* and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) on *A. majus* were the only aphids found. Voucher aphid individuals were kept inside a solution of 90/75% ethanol/lactic acid (Eastop and van Emden 1972). The plastic containers were carefully closed and transferred into boxes bearing holes for sufficient aeration of their content. Subsequently, the boxes were mailed to the BioRobotics Institute (Italy) with a high-speed courier that delivers items within 24 h. The plant-aphid-mummy material remained at 16 h:8 h photoperiod,  $22 \pm 1^\circ\text{C}$ ,  $55 \pm 5\%$  relative humidity. Per plant, each mummy was very carefully transferred, with a small piece of the plant that the mummy was stuck, into gelatin capsules by using a brush. The capsules were inspected daily for parasitoid emergence. Adult parasitoids were identified at the species level relying on the taxonomic keys of Kavallieratos et al. (2013) and Ghaliow et al. (2018) with the use of a Leica ES2 (Wetzlar, Germany) stereomicroscope. The identification revealed 2 species: *A. ervi* and *A. matricariae* parasitizing *A. pisum* on *M. sativa* and *A. matricariae* on

*A. majus*, respectively. The emerged parasitoids were kept for 48 h in Petri dishes ( $\phi = 5$  cm,  $h = 1$  cm) to become sexually matured and maintained on honey mixed with bee-collected pollen and water ad libitum, which were provided on a 1 cm diameter filter paper (Benelli et al. 2014).

### General Observations

Behavioral tests were conducted in a room lighted by daylight fluorescent tubes ( $22 \pm 1^\circ\text{C}$ ;  $55 \pm 5\%$  RH). Between 10:00 and 16:30, trials were held in an arena ( $\phi = 60$  mm). The tested parasitoids were 2–4 days old. New parasitoids of the same age were introduced for each replication.

### Courtship and Mating Behavior

A virgin male ( $\delta$ ) and a virgin female ( $\text{♀}$ ) were carefully moved to the testing arena using a glass vial to evaluate the courting and mating displays of *A. ervi* and *A. matricariae*. A Leica S9E stereomicroscope was used to observe male behavior for 20 min (or until the mating was completed). We recorded the duration (i.e., how long a given display last) of the following phases for each replica: (i) wing fanning (time spent by  $\delta$  fluttering his wings towards  $\text{♀}$ ) (Benelli et al. 2020); (ii) chasing (time spent by  $\delta$  following  $\text{♀}$ ); (iii) precopula (time spent by  $\delta$  mounting  $\text{♀}$ , until genital contact); (iv) antennal tapping (time spent by  $\delta$  palpating  $\text{♀}$  body with his antennae); (v) copula (from  $\delta$  insertion of the aedeagus into  $\text{♀}$  genital chamber until genital disengagement); (vi) postcopula phase (time spent by  $\delta$  motionless on the substrate close to  $\text{♀}$ , after genital disengagement) (Figs. 1 and 2).

Furthermore, we looked at the occurrence of behavioral asymmetries in both species at the population level. To accomplish this, we analyze either the preferred side from which the male mounts the female or the female's kicking behavior during precopula. Lastly,

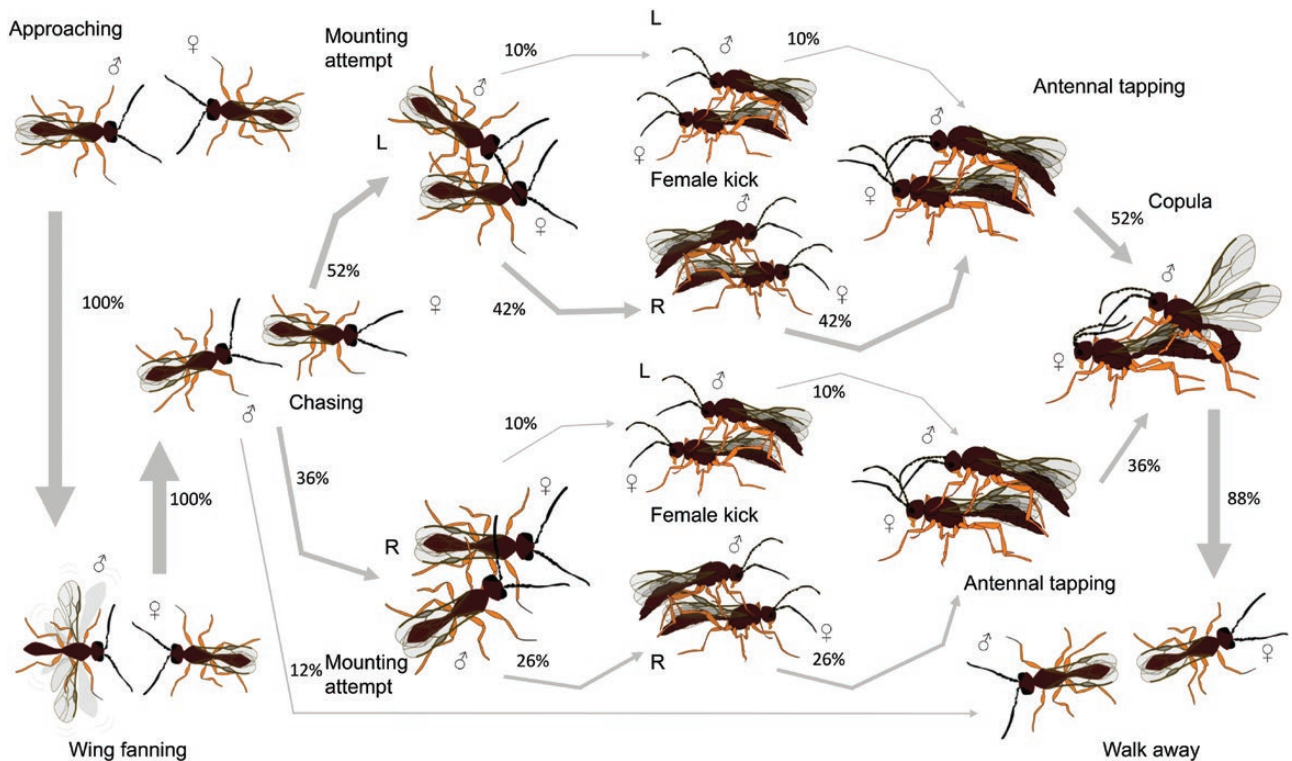
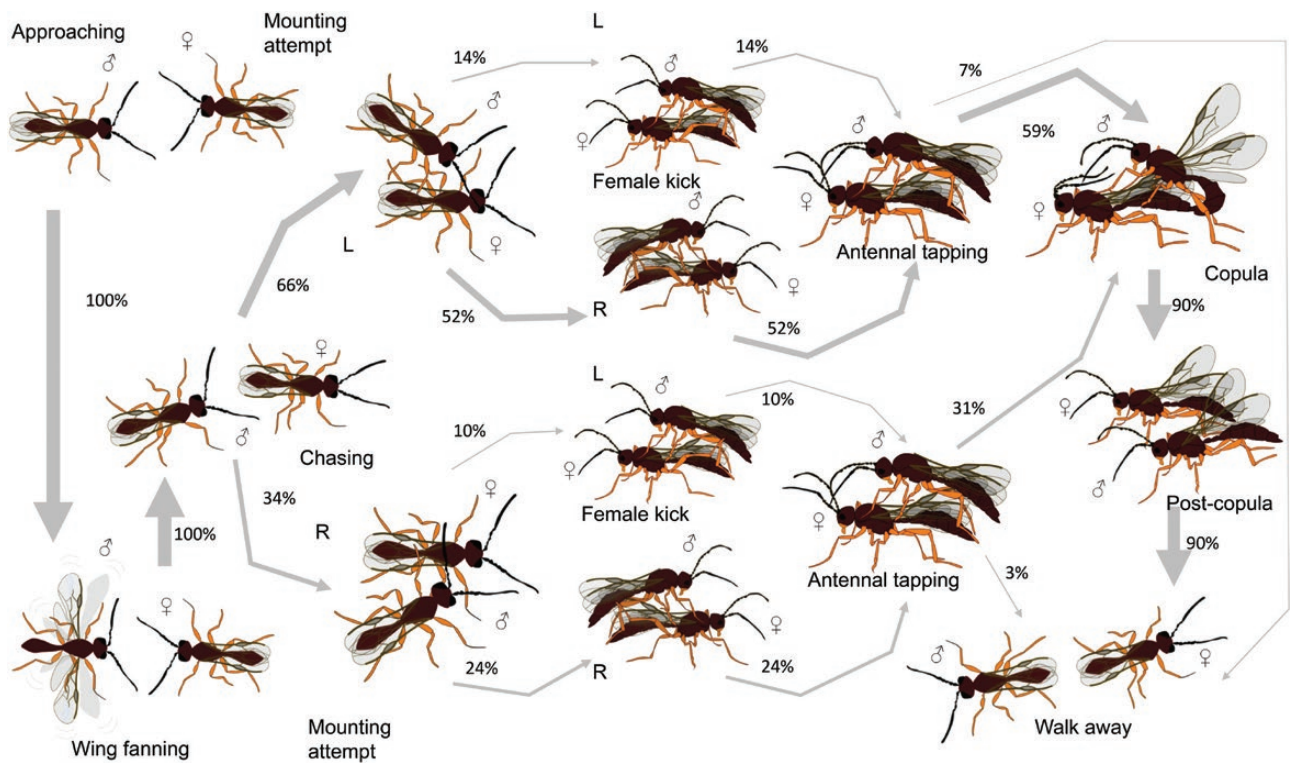


Fig. 1. Ethogram showing courtship and mating behavior in *Aphidius ervi*. The thickness of each arrow indicates the proportion of wasps displaying each behavior ( $n = 50$  pairs).



**Fig. 2.** Ethogram showing courtship and mating behavior in *Aphidius matricariae*. The thickness of each arrow indicates the proportion of wasps displaying each behavior ( $n = 29$  analyzed pairs).

we investigated whether these behavioral asymmetries had any effect on courtship and mating displays. Mating attempts that were successful and unsuccessful were also recorded. Males and females who did not participate in any courting approach or remained motionless for more than 30 min were discarded. A total of 29 pairs of *A. matricariae* and 50 pairs of *A. ervi* have been observed, but only 44 pairs of *A. ervi* were considered for statistical analysis.

### Statistical Analysis

A  $\chi^2$  test with Yates' correction was used to analyze the number of *A. ervi* and *A. matricariae* males with side-biased mounting attempts and females with side-based kicks during the precopula phase (Sokal and Rohlf 1981).

The inter-specific differences in terms of durations of each display have been analyzed using a Generalized Linear Mixed Model. The 'glmmTMB' package (Brooks et al. 2017) was used to fit each model with a  $t$  distribution for the duration of wing fanning, chasing, and copula models. Also, the antennal tapping model follows a  $t$  distribution, but data have been previously log-transformed. The Gaussian distribution was utilized in the precopula duration model. We utilized species as a predictor factor and parasitoid ID as a random factor. The 'DHARMa' package (Hartig 2022) was used to evaluate model fit, followed by the 'car' package (Fox and Weisberg 2019) to determine the significant impact of model components on the dependent variable. Next, we utilized the 'emmeans' package (Lenth 2022) to conduct a post hoc analysis. This involved estimating marginal means and applying Bonferroni correction to examine the statistical variances among the experimental groups (see Supplementary Materials 1).

Furthermore, the potential association of lateralized traits on the duration of wing fanning, chasing, precopula, antennal tapping, and copula behaviors of each species was analyzed using a Generalized

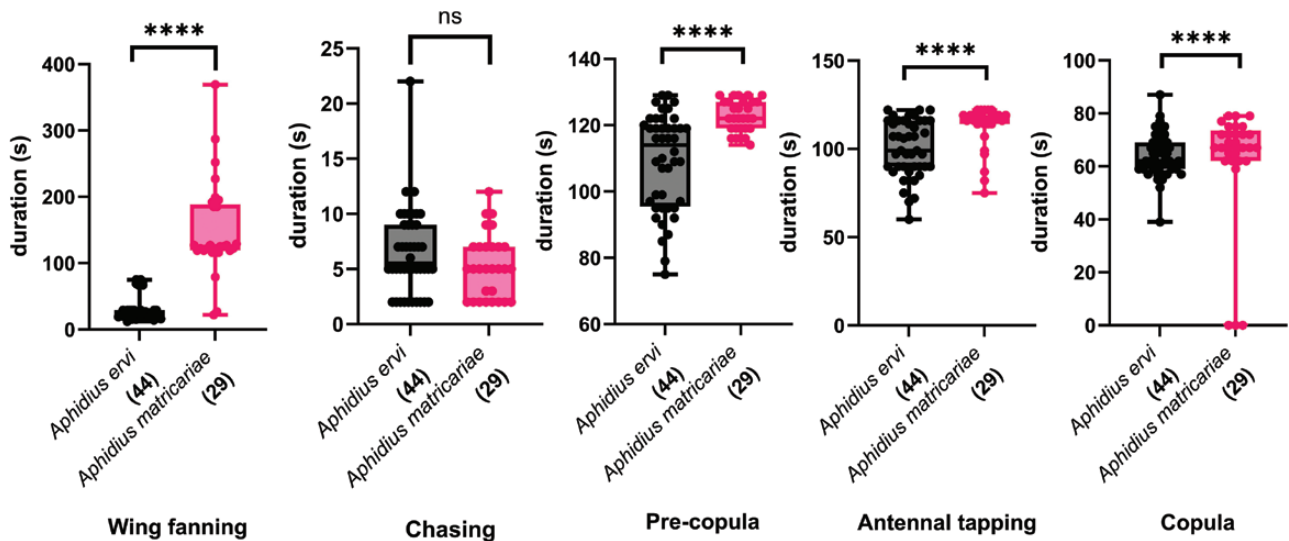
Linear Mixed Model. The 'glmmTMB' package (Brooks et al. 2017) was used to fit each model. The Poisson distribution was used to analyze all *A. ervi* displays, whereas wing fanning, chasing, precopula, and antennal tapping models were analyzed using a Gaussian distribution in *A. matricariae*. The zero-inflated Poisson distribution for the copula and postcopula models was used. We utilized lateralized male mounting attempt and lateralized female kick as predictor factors; the parasitoid ID was used as a random factor. The 'DHARMa' package (Hartig 2022) was used to evaluate model fit, followed by the 'car' package (Fox and Weisberg 2019) to determine the significant impact of model components on the dependent variable. Next, we utilized the 'emmeans' package (Lenth 2022) to conduct a post hoc analysis (see Supplementary Materials 2). This involved estimating marginal means and applying Bonferroni correction to examine the statistical variances among the experimental groups. The statistical analyses were conducted using R 4.2.1 (R Development Core Team 2008).

## Results

### Courtship and Mating Behavior

Figs. 1 and 2 provide quantitative information on the courting and mating sequences of *A. ervi* and *A. matricariae*. After female detection, the male began to quickly fan their wings (wing fanning, mean duration  $\pm$  SE—*A. ervi*:  $31.23 \pm 2.93$  s; *A. matricariae*:  $148.07 \pm 13.21$  s), started following the female (chasing, mean duration  $\pm$  SE—*A. ervi*:  $6.57 \pm 0.59$  s; *A. matricariae*:  $5.31 \pm 0.53$  s), approached the female and tries to mount her from 1 side (precopula, mean duration  $\pm$  SE—*A. ervi*:  $109.14 \pm 2.18$  s; *A. matricariae*:  $122.76 \pm 0.88$  s). These behaviors were common to both species. However, in *A. matricariae*, all males observed in the study engaged in precopula behavior, whereas 88% of the males of *A. ervi* exhibited





**Fig. 3.** Comparison of the duration of courtship and mating behavioral traits between *Aphidius ervi* and *A. matricariae*. Each boxplot indicates the median and its range of dispersion (lower and upper quartiles and outliers). \*\*\*\* $P < 0.0001$ ; ns = not significant (GLMM, Bonferroni correction).

this behavior, and the remaining 12 % walked away (Fig. 1). During precopula, females of both species exhibited a lateralized kick on the male which was in both cases right-biased when the male's copulation attempt occurred from the left (*A. ervi*  $\chi^2 = 9.846$ ,  $P = 0.002$ ; *A. matricariae*  $\chi^2 = 6.368$ ,  $P = 0.012$ ) (see Fig. 4). In addition, we looked at how behavioral asymmetries were associated with courtship and mating displays (Figs. 5 and 6). For instance, more right-sided female kicking was associated with a longer period of chasing (left vs. right—GLMM, Bonferroni correction:  $SE = 0.121$ ;  $z.ratio = -2.684$ ;  $P = 0.0073$ ) and a longer precopula phase (left vs. right—GLMM, Bonferroni correction:  $SE = 0.0377$ ;  $z.ratio = -3.446$ ;  $P = 0.0006$ ) (Fig. 5). On the other hand, a longer wing fanning was associated with the interaction of the right male mounting attempt and the female left kick in *A. ervi* (lateralized kick = L, mounting left vs. right—GLMM, Bonferroni correction:  $SE = 0.138$ ;  $z.ratio = -2.613$ ;  $P = 0.0090$ ; lateralized kick = R, mounting left vs. right—GLMM, Bonferroni correction:  $SE = 0.175$ ;  $z.ratio = 0.091$ ;  $P = 0.927$ ). Though not lateralized at the population level, we also found an association between a longer duration of chasing and the male mounting attempt on the right in *A. matricariae*. This was also related to the sequent female right kick (left vs. right—GLMM, Bonferroni correction:  $SE = 1.64$ ;  $t.ratio = -2.995$ ;  $P = 0.0069$ ).

After all the precopula displays, the courting male started palpating the female with antennae which was primarily concentrated on the female antennae (antennal tapping, mean duration  $\pm SE$ —*A. ervi*:  $100.00 \pm 2.42$  s; *A. matricariae*:  $112.41 \pm 2.30$  s). As well as chasing and the overall precopula phase, a longer duration of male antennal tapping seems to be associated with more female right-sided kicks in *A. ervi* (left vs. right—GLMM, Bonferroni correction:  $SE = 0.0502$ ;  $z.ratio = -2.104$ ;  $P = 0.0354$ ), with a longer antennal tapping when females kick from the right (Fig. 5b). The antennal tapping was then followed by copula (mean duration  $\pm SE$ —*A. ervi*:  $63.93 \pm 1.23$  s; *A. matricariae*:  $62.38 \pm 4.13$  s). During copulation, females of both species may walk around, but most individuals maintain a stationary posture. All males of *A. ervi* engaged copula, whereas 10% ( $n = 3$ ) of *A. matricariae* did not engage copula and walked away. After mating, males and females disengaged their genitals and moved away from each other (see Figs. 1 and 2). Only *A. matricariae* exhibited postcopulatory behavior where both sexes, after male dismounting, remained closer,

and entered a condition of brief quiescence, staying immobile for a few seconds (postcopula, mean duration  $\pm SE$ —*A. matricariae*:  $9.48 \pm 41.06$  s). As highlighted in Fig. 3, *A. matricariae* displayed longer durations of wing fanning (*A. ervi* vs. *A. matricariae*—GLMM, Bonferroni correction:  $SE = 1.92$ ;  $z.ratio = -51.694$ ;  $P < 0.0001$ ), precopula (*A. ervi* vs. *A. matricariae*—GLMM, Bonferroni correction:  $SE = 2.83$ ;  $t.ratio = -5.007$ ;  $P < 0.0001$ ), antennal tapping (*A. ervi* vs. *A. matricariae*—GLMM, Bonferroni correction:  $SE = 0.028$ ;  $t.ratio = -4.256$ ;  $P < 0.0001$ ), and copula phases (*A. ervi* vs. *A. matricariae*—GLMM, Bonferroni correction:  $SE = 1.81$ ;  $z.ratio = -3.299$ ;  $P < 0.001$ ) compared to *A. ervi*.

## Discussion

The findings of this research reveal that both *A. ervi* and *A. matricariae* reproductive sequences involve the male wing fanning and chasing of the female and a precopula phase in which the male mounts the female and courts her until the copula happens. There were differences between the 2 species in terms of the duration of wing fanning, precopula, antennal tapping, and copula behaviors. These variations might be partly attributed to ecological conditions that can drive selection, resulting in the emergence of behavioral distinctions among closely related species. These variations aid in differentiating between species and decrease the likelihood of heterospecific matings (König et al. 2019). The differences found in the postcopula phase may suggest that females of *A. matricariae* may be more inclined to re-mate than *A. ervi*. Indeed, postcopulatory mate guarding is a frequent behavior that happens after insemination and prevents sperm competition with sperm from potential competitor males with whom the female may mate in the future (Parker 1970, Alcock 1994, Elias et al. 2014, Vellnow et al. 2020). Overall, the mating sequences of these parasitoids are in line with those reported by Boulton et al. (2015) and references therein.

According to our results, the duration of wing fanning, chasing, and antennal tapping had no effect on the copula in *A. ervi* and *A. matricariae*, suggesting that other factors are more important in determining reproductive success in these species, such as the frequency of male wing fanning and antennal tapping (Benelli et al. 2014). For instance, male wing fanning is a key courtship display of many parasitic wasps (Boulton et al. 2015), some of which are

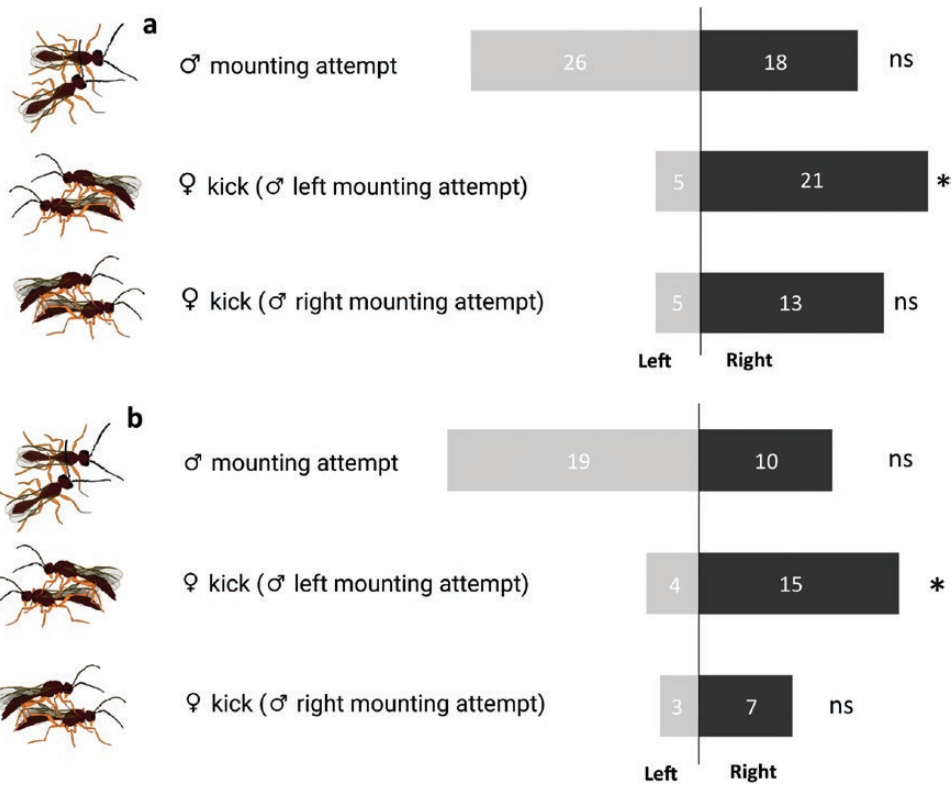


Fig. 4. Population-level lateralization of male mounting attempts and female kicks of a) *Aphidius ervi* and b) *Aphidius matricariae*. Within each display, the asterisk indicates a significant difference in the overall abundance of individuals performing side-biased acts ( $\chi^2$  test with Yates' correction,  $P < 0.05$ ).

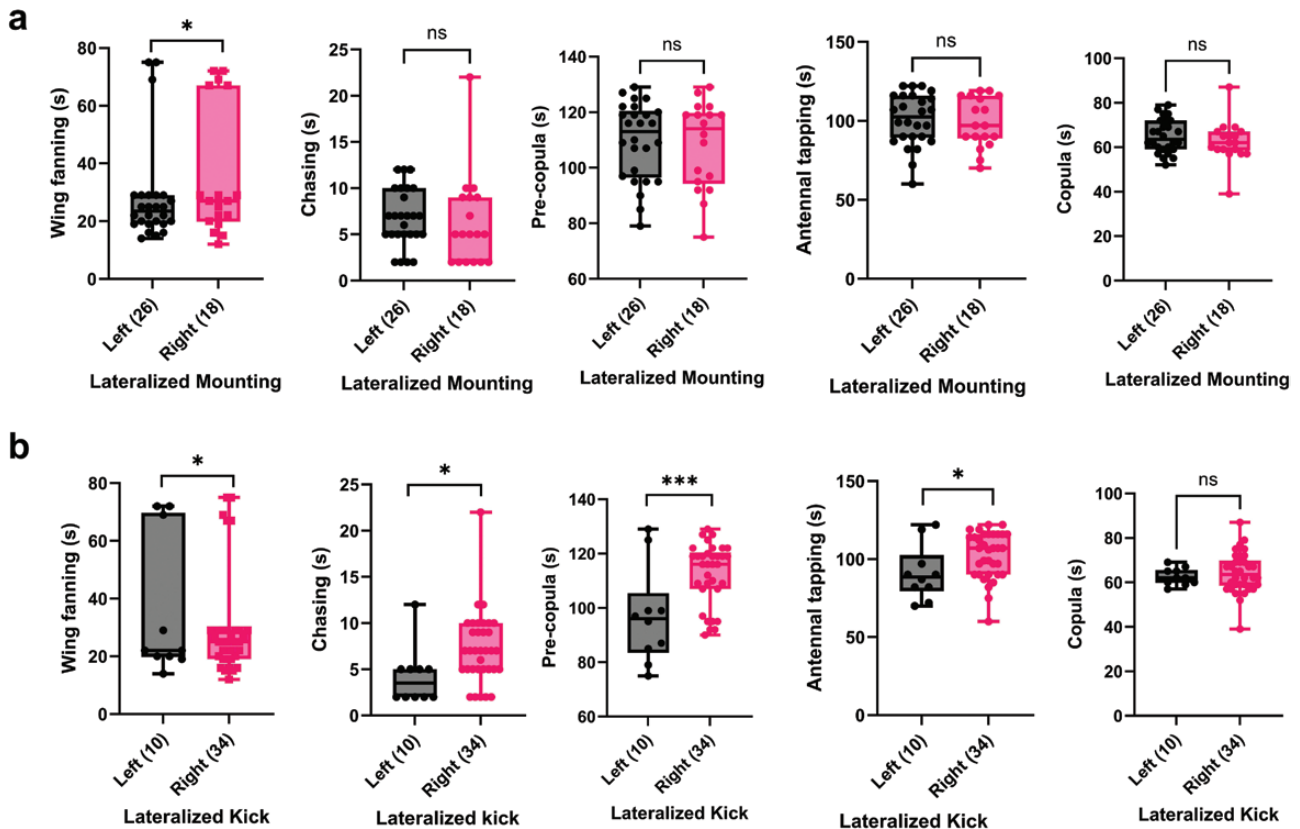
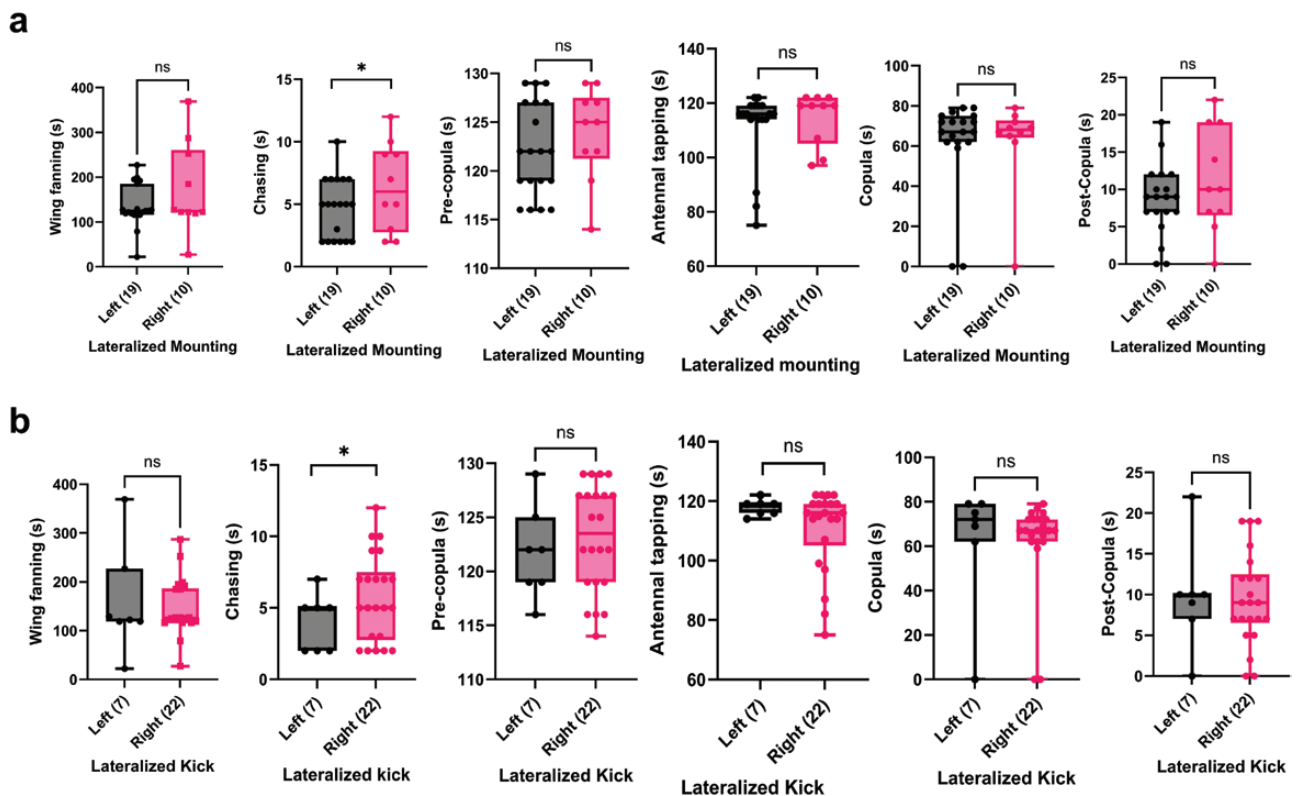


Fig. 5. a) Influence of lateralized male mounting attempt on *A. ervi* main features b) influence of lateralized female kick on *A. ervi* main features. Each boxplot indicates the median and its range of dispersion (lower and upper quartiles and outliers). \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ns = not significant (GLMM, Bonferroni correction).



**Fig. 6.** a) Influence of lateralized male mounting attempt on *A. matricariae* main features b) influence of lateralized female kick on *A. matricariae* main features. Each boxplot indicates the median and its range of dispersion (lower and upper quartiles and outliers). \* $P < 0.05$ ; ns = not significant (GLMM, Bonferroni correction).

also important in the context of biocontrol (Benelli et al. 2013, 2016, 2020, Romano et al. 2018). It is acknowledged that male wing fanning may help males recognize females by increasing airflow around the male antennae, allowing males to more effectively pick up female pheromones (Loudon and Koehl 2000), or the specific frequency of wing fanning may signal male fitness to females (Eichorn et al. 2017). Further studies will be undertaken to investigate the relationship between wing fanning and antennal tapping frequency and mating success. Here, we discovered 1 lateralized trait in *A. ervi* and *A. matricariae* after examining courting and mating data from both species. Both species demonstrated right-biased female kicking behaviors at the population level during the precopula phase. Female kicks during precopula may be associated with an aggressive female reaction to unwanted males. A similar display was also observed in mosquitoes (Benelli et al. 2015b). However, unlike mosquitoes, this display did not result in the effective displacement of unwanted partners (Benelli et al. 2015b).

The female kicking behavior could be linked to the release by the male of sex pheromones during antennation. As reported by Romani et al. (2008), in other Hymenoptera, males secrete sex pheromones from glands in their antennae, which are then applied to females during the precopula phase. In *A. ervi*, males also produce sex pheromones in antennal glands that modify female behavior courtship (Battaglia et al. 2002), which might be an explanation for why female aggravation induces antennation from males. Overall, the population-level lateralization of this behavior in both species may be associated with a faster response rate of the right leg compared to the left (Benelli et al. 2015a, 2015b), as well as differences in nerve innervation between the legs (Vallortigara and Rogers 2005, Pflüger et al. 2011, Knebel and Rigosi 2021). More research is required

at morphological and neurophysiological levels to understand the processes causing the right-biased kick in these 2 species.

These insights into the courtship and mating processes of these 2 commercially available parasitoids can boost biocontrol programs and mass-rearing practices. Continuous mass-rearing has been shown to generate major changes in parasitoid courtship and mating behavior as a result of inbreeding (Joyce et al. 2010). Understanding sexually chosen displays (e.g., wing fanning and antennal tapping) and lateralized behaviors may aid in the selection of parasitoid strains with greater reproductive success, improving mass-rearing operations, and reducing mating failures, which can impede biological control efforts. According to Joyce et al. (2010), the mate mating success rate and the mean copula duration are key benchmarks to successfully monitor *A. ervi* and *A. matricariae* reproduction in laboratory settings throughout time. Indeed, if the length of the copula diminishes and female rejection of approaching mates rises, it may signal that wild individuals should be introduced into the rearing process to avoid fitness reduction. Similarly, male wing fanning may be a valuable measure for tracking male courting abilities over time. However, further research is required to determine the frequency of wing fanning and its possible relation to mating success.

## Author Contributions

V. Z. (Conceptualization [Equal], Data curation [Equal], Investigation [Equal], Writing—original draft [Equal]), D. R. (Conceptualization [Equal], Data curation [Equal], Funding acquisition [Equal], Investigation [Equal], Writing—original draft [Equal]), N. K. (Data curation [Equal], Investigation [Equal], Writing—original draft [Equal]), C. S. (Funding acquisition [Equal], Writing—review &

- 8.5 editing [Equal]), A. L. (Data curation [Equal], Writing—review & editing [Equal]), A. C. (Data curation [Equal], Writing—review & editing [Equal]), and G. B. (Conceptualization [Equal], Data curation [Equal], Funding acquisition [Equal], Investigation [Equal], Writing—original draft [Equal])
- 8.10
- AQ17 **Supplementary Material**
- Supplementary material is available at *Journal of Economic Entomology* online.
- 8.15
- References**
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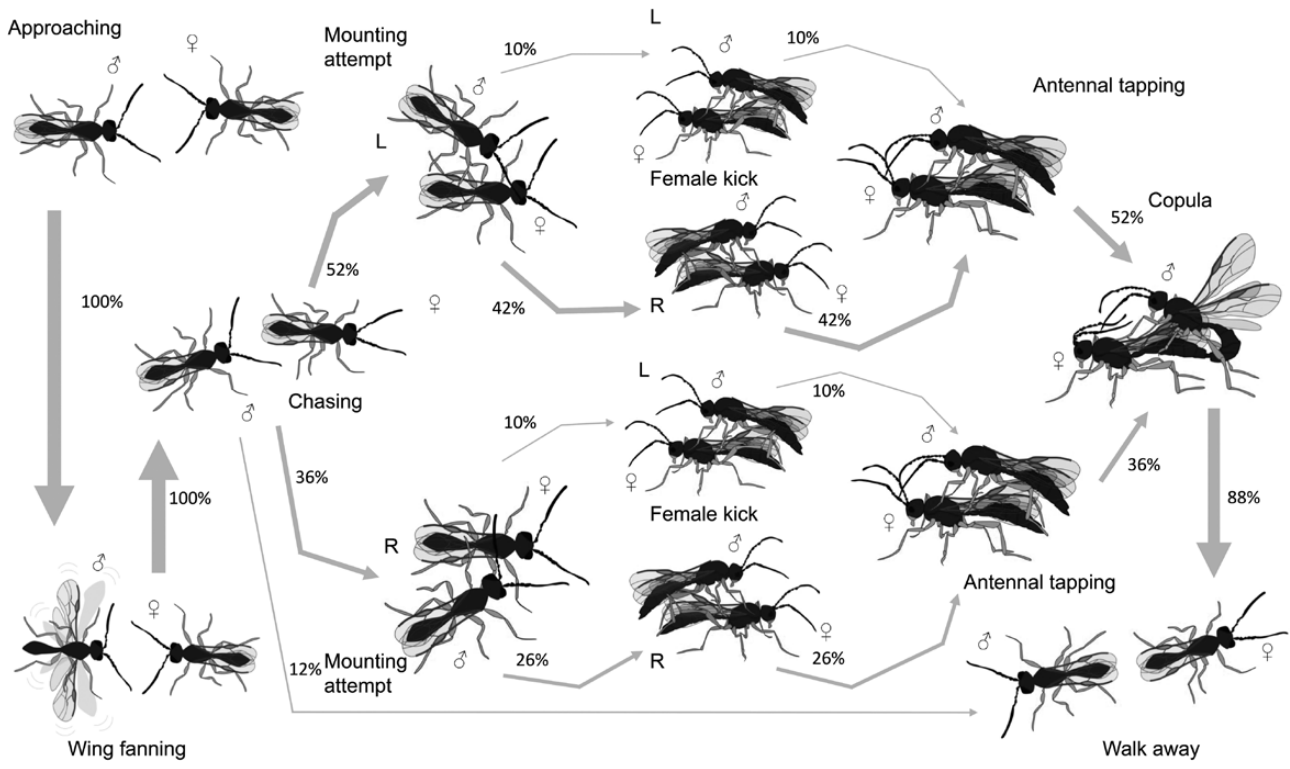


Figure 1

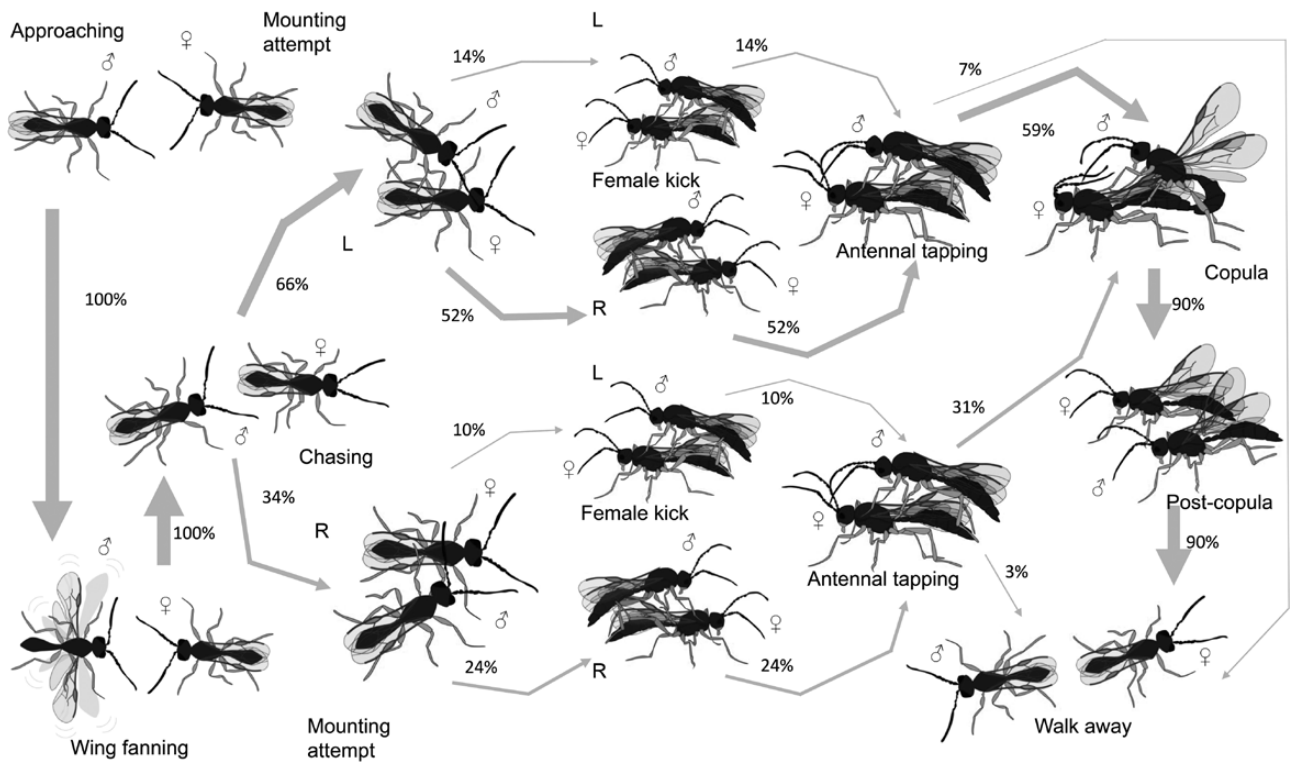


Figure 2

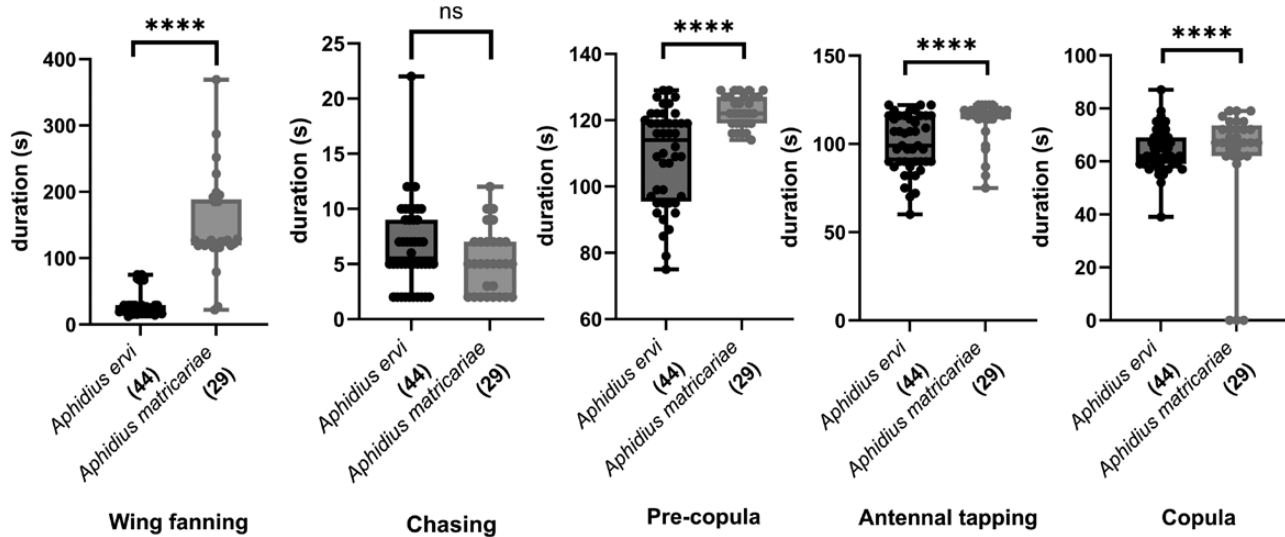


Figure 3

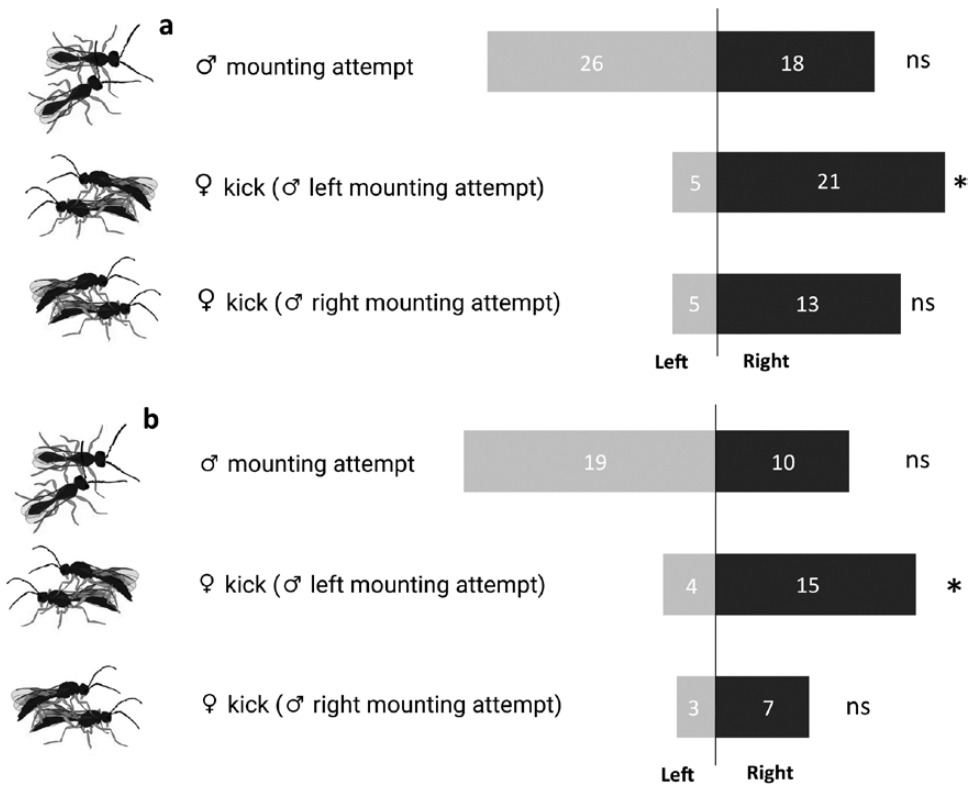


Figure 4

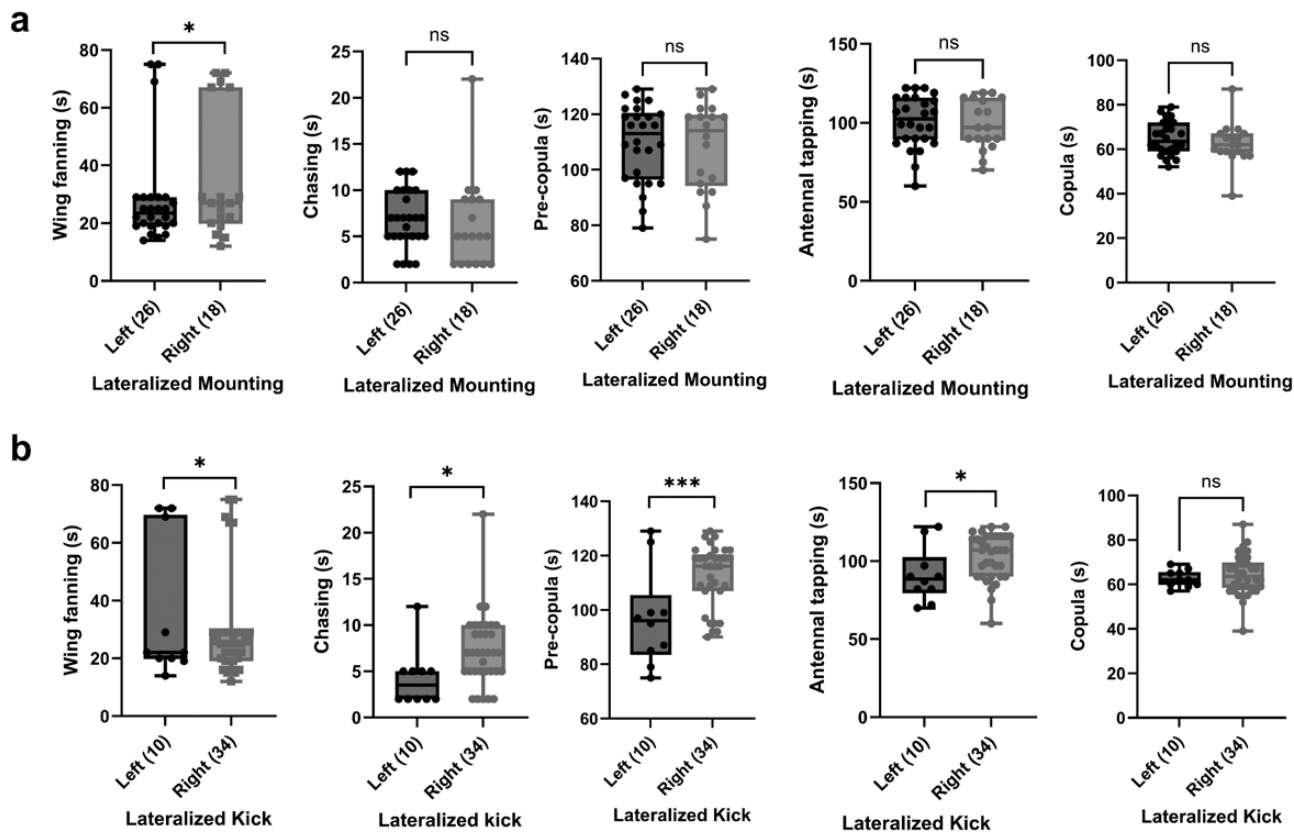
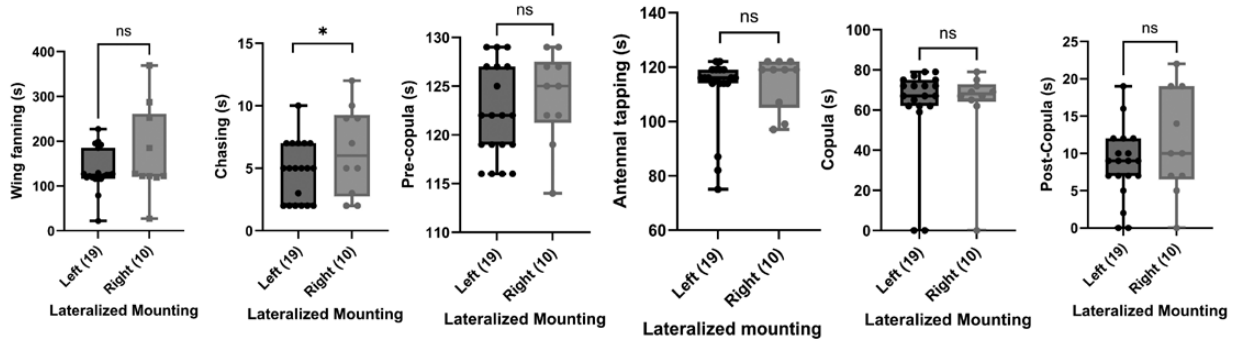


Figure 5



**a**



**b**

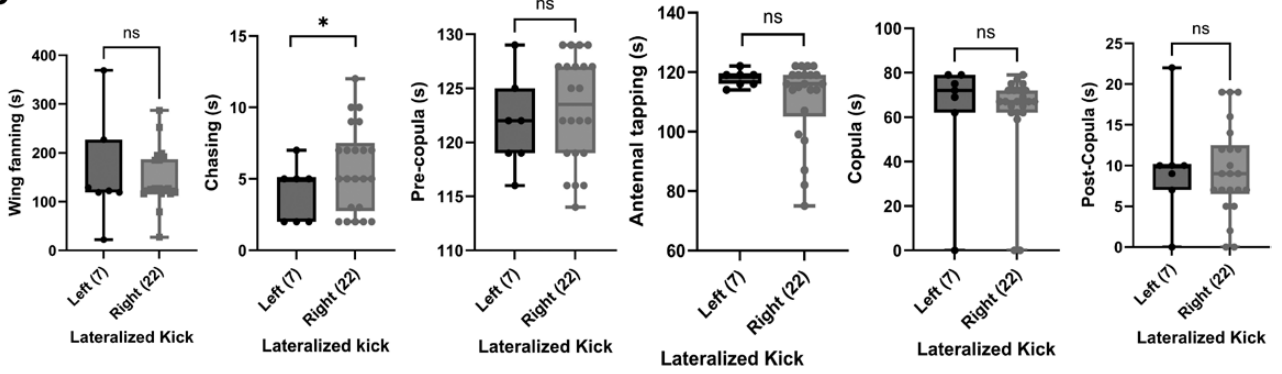
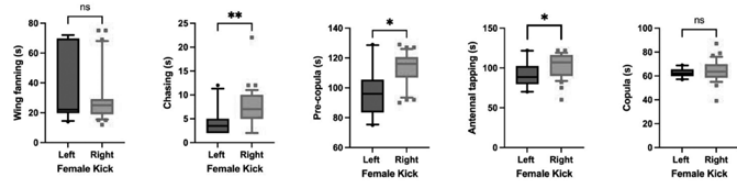
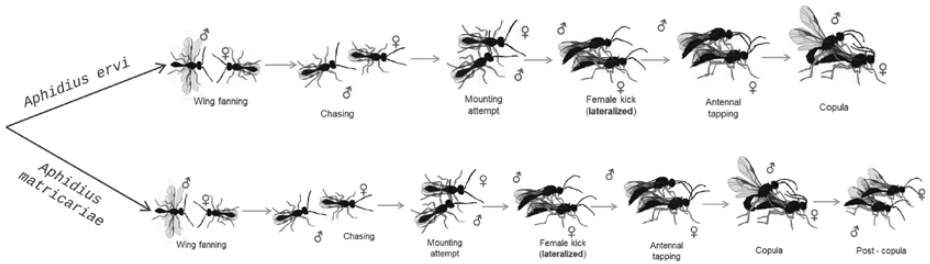


Figure 6

# Courtship and mating behaviors

## Behavioural asymmetries



### Influence of behavioral asymmetries on courtship and mating displays and viceversa

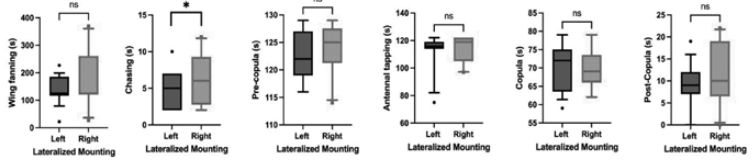
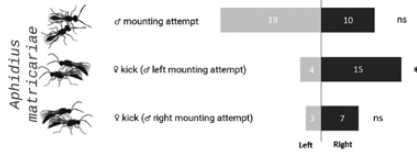


Figure 1