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1 **Behavioral asymmetries in the mealybug parasitoid *Anagyrus* sp. near**
2 ***pseudococci*: do lateralized antennal tapping predicts male mating success?**

3

4 Donato Romano ¹, Giovanni Benelli ^{2*}, Cesare Stefanini ^{1,3}, Nicolas Desneux ⁴,
5 Ricardo Ramirez-Romero ⁵, Angelo Canale ², Andrea Lucchi ²

6

7 ¹ The BioRobotics Institute, Sant'Anna School of Advanced Studies, Pontedera, Italy

8 ² Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy

9 ³ Department of Biomedical Engineering, Khalifa University, Abu Dhabi, UAE

10 ⁴ INRA (French National Institute for Agricultural Research), Univ. Nice Sophia
11 Antipolis, CNRS, UMR 1355-7254, Institut Sophia Agrobiotech, 06903 Sophia
12 Antipolis, France

13 ⁵ Department of Agronomy, CUCBA, University of Guadalajara, Zapopan, Jalisco,
14 Mexico

15

16 * Corresponding author. Tel.: +390502216141. Fax: +390502216087. E-mail
17 address: benelli.giovanni@gmail.com (G. Benelli)

18

19

20 **Abstract**

21

22 Lateralization is a fundamental principle of the brain organization widespread
23 among vertebrates but rather unknown in invertebrates. Evidences of lateralized
24 courtship and mating behavioural traits in parasitic wasps are extremely rare. Here,
25 courtship and mating sequences, and the presence of mating lateralization in *Anagyrus*
26 sp. near *pseudococci*, one of the most effective biological control agents of mealybugs,
27 were investigated. Courtship and mating behaviour in *A. sp. near pseudococci* consisted
28 in the male chasing of the female, pre-copula, copula and post-copula phases. Males
29 mating success was not related to the duration of chasing and pre-copula. High-speed
30 videos showed population-level lateralization in *A. sp. near pseudococci* during
31 courtship. Most the wasps used the right antenna to start antennal tapping and this led to
32 a higher mating success, although lateralization had no impact on the frequency of the
33 antennal tapping. Both females and males displayed this behavior. Higher mating
34 success was detected when females displayed antennal tapping during sexual
35 interaction, though male tapping is performed with a higher frequency. From an intra-
36 sexual point of view, higher antennal tapping frequencies characterized successful
37 individuals. To the best of our knowledge, this report on behavioural asymmetries of
38 mating traits in *A. sp. near pseudococci* represents a quite rare evidence of lateralized
39 behavior in parasitic wasps of economic importance. Our findings add basic knowledge
40 on the behavioural ecology of this biocontrol agent with potential implications on the
41 optimization of mass-rearing procedures aimed at using this parasitoid in Integrated Pest
42 Management.

43

44 **Keywords:** *Anagyrus* sp. near *pseudococci*; biological control; courtship;

45 Encyrtidae; high-speed video analysis

46

47 **Key message**

48

49 • Evidences of asymmetric mating traits in parasitic wasps are limited

50 • We studied mating laterality in *Anagyrus* sp. near *pseudococci*, a biocontrol
51 agent of mealy bugs

52 • High-speed videos showed population-level lateralization in during courtship

53 • Lateralization had no impact on the frequency of the antennal tapping

54 • Parasitoids used the right antenna to start antennal tapping, this led to higher
55 mating success

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57

58 **Introduction**

59

60 Lateralization (i.e. the different specialization of the right and left sides of the
61 nervous system reflected in left-right behavioural asymmetries) is a fundamental
62 principle of the brain organization widespread among vertebrates (Rogers et al. 2013;
63 Vallortigara et al. 2011; Vallortigara and Rogers 2005; Vallortigara and Versace
64 2017). Recent evidences support the hypothesis that lateralization can increase neural
65 capacity, enabling the brain to perform simultaneous processing (Vallortigara 2000;
66 Vallortigara and Rogers 2005). Later, it has been highlighted that also invertebrates,
67 endowed with simpler nervous systems, showed lateralized traits (Ades and Ramires
68 2002; Backwell et al. 2007; Benelli et al. 2015a, b, c; Rigosi et al. 2015; Rogers and
69 Vallortigara 2008-2015; Rogers et al. 2013-2016; Romano et al. 2015; Romano et al.
70 2016a; Versace and Vallortigara 2015). However, behavioral asymmetries in insects
71 are still scarcely investigated (Frasnelli et al. 2012). Behavioral asymmetries of
72 courtship and mating behaviour represent a fascinating issue. Recently, lateralized
73 displays in the courtship and mating behaviour have been reported for tephritid flies
74 (Diptera: Tephritidae), (Benelli et al. 2015c), stored product beetles such as the
75 confused flour beetle (Coleoptera: Tenebrionidae), the khapra beetle (Coleoptera:
76 Dermestidae), and the rice weevil (Coleoptera: Curculionidae), (Benelli et al.
77 2017a,b; Romano et al. 2016a), earwigs (Dermaptera: Labiduridae), (Kamimura
78 2006), and the parasitoid *Leptomastidea abnormis* (Girault) (Hymenoptera:
79 Encyrtidae) (Romano et al. 2016b).

80 Basic knowledge about the presence of behavioural asymmetries in parasitic
81 wasps is extremely scarce. To the best of our knowledge, this topic was investigated

82 for the first time in parasitic wasps by Romano et al. (2016b). These authors reported
83 that the encyrtid *L. abnormis* showed a population-level lateralization of male
84 courtship display, with right-biased male antennal tapping (i.e. a key step during
85 courtship that allow the acquisition of information about mate quality) on the
86 female's head. However, a deeper understanding of laterality of mating traits in
87 parasitoids may lead to the optimization of mass rearing monitoring processes,
88 helping to explain potential mating failures (Giunti et al. 2016).

89 *Anagyrus pseudococci* (Girault) is a koinobiont endoparasitoid commonly
90 used worldwide as a biological control agent against mealybugs (*Planococcus* spp.
91 and *Pseudococcus* spp.) (Daane et al. 2012; Fortuna et al. 2015; Heidari and Jahan
92 2010). Triapitsyn et al. (2007) demonstrated the existence of two morphotypes in the
93 population of *A. pseudococci* released in biological control projects carried out in
94 California for the management of *Planococcus ficus* (Signoret). The two
95 morphotypes differed only for the color of the first antennal funicle segment of the
96 female, partially black (basal half) and white (distal half) in *Anagyrus pseudococci*
97 (Girault), and entirely black in the other morphotype, which was named *Anagyrus* sp.
98 near *pseudococci* (Girault). *Anagyrus pseudococci* is known only from Sicily (Italy),
99 Argentina and Cyprus, *A. sp. near pseudococci* occurs in the Mediterranean
100 Countries (Sicily included), in the Palaearctic Asia, in Brasil and USA. This study
101 deals with the latter species, which is the one mass-reared and commercialized by
102 BioPlanet (Cesena, Italy).

103 While several studies have been conducted on host-parasitoid interactions of
104 *A. sp. near pseudococci* (Franco et al. 2008; Güleç et al. 2007; Heidari and Jahan
105 2010; Suma et al. 2012), no information is available about its courtship behavior.

106 Notably, *A. sp. near pseudococci* females rely on their antennae, endowed with
107 sophisticated sensilla, performing antennal tapping during host location and selection
108 (Bugila et al. 2014; Fortuna et al. 2015). Since a left-biased lateralized antennal
109 tapping has been recently reported at population-level in *L. abnormis*, a close-related
110 encyrtid species (Romano et al. 2016b), we hypothesized a key role of lateralized of
111 antennal tapping during courtship and mating behavior of *A. sp. near pseudococci*.
112 Therefore, in this research, the courtship and mating behavior of *A. pseudococci* was
113 investigated under laboratory conditions, producing an ethogram. Furthermore,
114 antennal tapping frequencies, their laterality, and the following success in mating
115 approaches were characterized based on the analysis of high-speed video recordings.

116

117 **Materials and methods**

118

119 Insect rearing and general observation

120

121 Commercially mass-reared specimens of *A. sp. near pseudococci* were
122 provided before adult emergence by BioPlanet (Cesena, Italy). Immediately after
123 emergence, parasitoids were sexed, singly stored in clean glass vials, and fed with a
124 tiny drop of water and honey 1:1 (v:v). Virgin sexually mature males and females
125 (age 2 days old), were used in all observations. All experiments were conducted
126 during June 2016 in laboratory conditions described by Romano et al. (2016b). All
127 experiments were carried out in a Petri dish arena (50 mm diam. ×10 mm high) from
128 10:00–18:00 h. The arena was surrounded by a white wall of filter paper (Whatman

129 no. 1, height 30 cm), to reduce the effect of external cues that could affect the *A. sp.*
130 near *pseudococci* behavior (Benelli and Canale 2012).

131

132 Courtship and mating

133

134 To investigate courtship and mating behavior of *A. sp.* near *pseudococci*, a virgin
135 male and five virgin females were gently transferred into a testing arena using a clean
136 glass vial (diam.: 10 mm; length: 50 mm). Male behavior was focally observed for 45
137 min, or until the end of mating. For each replica we observed the duration of the
138 following phases: (i) chasing (i.e., time spent by the male enriching the female); (ii) pre-
139 copula (i.e., time spent by the male mounting the female, until genital contact); (iii)
140 copula (i.e., from the male's insertion of the aedeagus into the female genital chamber
141 until genital disengagement); (iv) post-copula (i.e., time spent by the male on the female
142 thorax or motionless on the substrate close to the female after genital disengagement)
143 and (v) the duration of the whole courtship and mating sequence. Successful and
144 unsuccessful mating attempts were noted. A total of 47 insect pairs were tested. Males
145 and females that did not engaged any courtship approach or did not move for more than
146 30 min were discarded. 30 mating pairs were considered for statistical analysis.

147

148 Antennal tapping video characterization

149

150 Preliminary observations revealed that antennal tapping during courtship
151 behavior can be performed by *A. sp.* near *pseudococci* males and females. We video-
152 recorded the antennal tapping behaviour performed by males or females during

153 courtship behavior. Only a single antennal tapping sequence was analyzed for each
154 wasp (Benelli et al. 2012), to avoid pseudo-replications. The video recording began
155 once a male mounted a female and the antennal tapping started. The mean pulse
156 frequency (Hz), (i.e. the inverse of the average duration of the tapping during
157 antennation, recorded throughout the frame-by-frame analysis at a rate of 1000 frames
158 per second [fps] of video recordings), and the relationship between frequency and
159 mating success were analyzed. Furthermore, we evaluated the presence of population-
160 level behavioral asymmetries in *A. sp. near pseudococci* by observing which antenna
161 was used to palpate the partner first and if behavioral asymmetries had any effect on
162 male mating success. Sex differences in antennal tapping frequency and lateralization
163 were also noted.

164 Eighty-nine pairs of insects were tested. Females constrained in confined spaces
165 were discarded; for laterality observations, we considered only females that are
166 approached by males when they were free in the middle of the arena (Romano et al.
167 2016b). We analyzed 50 mating pairs performing antennal tapping during the courtship
168 behavior.

169 The high-speed video recordings were made using a HotShot 512 SC high-speed
170 video camera (NAC Image Technology Inc., Simi Valley, CA, USA). Sequential
171 images from each antennal tapping were captured at a rate of 1,000 fps with an exposure
172 time of 1 ms and a video duration of 8.20 s (Romano et al. 2016b). The area where
173 insects were expected to perform antennal tapping was lit with four LED illuminators
174 (RODER SRL, Oglanico, TO, Italy) that emit light (420 lm each) at $\lambda = 628$ nm
175 (Briscoe and Chittka 2001).

176

177 Data analysis

178

179 Data concerning courtship duration, mating duration and mating success were
180 analyzed with JMP 7 (SAS, 1999). Data normality was checked using Shapiro–Wilk
181 test ($P < 0.05$). The variance between values was analyzed with Fisher’s F-test ($P < 0.05$).
182 Differences in pre-copula duration, copula duration, and whole duration of the mating
183 sequence were analyzed using a general linear model with a normal error structure and
184 two fixed factors (i.e. laterality and mating outcome) ($P < 0.05$) (Benelli et al. 2017b).
185 Differences in male and female antennal tapping frequency were analyzed using a
186 general linear model with a normal error structure and three fixed factors (i.e. laterality,
187 sex and mating outcome) ($P < 0.05$).

188 Differences in male mating success were analyzed using a generalized linear
189 model with a binomial error structure and one fixed factor (laterality): $y = X\beta + \varepsilon$ where
190 y is the vector of the observations (i.e. the male success or failure), X is the incidence
191 matrix, β is the vector of fixed effects (i.e. laterality) and ε is the vector of the random
192 residual effects. A probability level of $P < 0.05$ was used to assess the significance of
193 differences among values.

194 Laterality differences between the numbers of parasitoids using left or right
195 antennae during courtship approaches were analyzed using a χ^2 test with Yates
196 correction ($P < 0.05$) (Sokal and Rohlf 1981).

197 Concerning the high-speed video recordings of parasitoid courtship and mating,
198 to check inter-rater reliability among laterality data, two blind observers re-analyzed a
199 subset of the data [i.e. 39 high-speed videos (video ID numbers: 1-9, 11-19, 21, 22, 24-
200 26, 28-30, 32, 35, 37-39, 41-44, 46-49), Bisazza et al. 2001]. Inter-rater reliability was

201 calculated (Cohen 1960; Gwet 2014; Romano et al. 2016b). The concordance index was
202 0.95 and Cohen's kappa was 0.874.

203

204 **Results**

205

206 Courtship and mating behavior

207

208 Courtship and mating sequence of *A. sp. near pseudococci* is quantified in the
209 ethogram depicted in **Fig. 1**. After the detection of a female, the male started chasing
210 her, then attempt to mount on the thorax of the female, which constantly walked, and
211 the pre-copula phase started. Receptive females bend dorsally their abdomen allowing
212 the insertion of the aedeagus into their genital chamber. At the end of the copula, genital
213 disengagement occurred and the male remounted for a short period the female or stayed
214 still on the substrate close to her (**Fig. 1**). Results showed that no significant differences
215 in the duration of chasing ($F_{1,29}=0.006$; $P=0.941$), pre-copula ($F_{1,29}=0.027$; $P=0.872$),
216 and the whole courtship and mating sequence ($F_{1,29}=2.900$; $P=0.100$) were detected
217 between successful and unsuccessful mating approaches (**Fig. 2**).

218

219 High-speed video characterization of lateralized antennal tapping

220

221 In *A. sp. near pseudococci*, the antennal tapping was displayed both by females
222 (60% of the observed wasps) and males (40%) (**Fig. 1b**). Mating success was higher
223 when females displayed antennal tapping during sexual interactions ($\chi^2=4.818$; $df.=1$;

224 $P=0.029$), In addition, males displaying antennal tapping performed it with higher
225 frequencies, compared to females ($F_{1,49}=7.2689$; $P=0.010$) (**Fig. 3**).

226 The preferential use of the right antenna to start antennal tapping led to higher
227 mating success, compared to left-biased interactions ($\chi^2=7.589$; $d.f.=1$; $P=0.006$) (**Fig.**
228 **3**). However, the lateralized use of antennae showed no effect on antennal frequency
229 ($F_{1,49}=0.004$; $P=0.953$). As a general trend, higher frequencies of antennal tapping were
230 observed in successful mating pairs ($F_{1,49}=4,726$; $P=0.035$). Males performing antennal
231 tapping with higher frequency were more successful in mating over males performing it
232 with lower frequency and the same was true for females (**Fig. 3**).

233

234 **Discussion**

235

236 Recently, asymmetries of mating traits have been found in several insect species
237 (Benelli et al. 2015c, 2017a; Romano et al. 2016a, b), suggesting that laterality would
238 have crucial relevance in the acceptance and coordination of two conspecifics during
239 courtship and mating behavior. It has been argued that lateralization at population level
240 has evolved as a characteristic feature of social species, while solitary species display
241 more frequently asymmetries at individual level (Ghirlanda and Vallortigara 2004;
242 Vallortigara and Rogers 2005; Vallortigara 2006; Ghirlanda et al. 2009; Rogers and
243 Vallortigara 2008-2015; Rogers et al. 2013a). However, a number of recent studies on
244 invertebrates, reported population level lateralization in different solitary species,
245 proposing that behavioral asymmetries in solitary animals could be related to frequent
246 and prolonged social interactions occurring during their life cycle, such as courtship and
247 mating and/or agonistic approaches (Ades and Ramires 2002; Backwell et al. 2007;

248 Frasenelli et al. 2012; Benelli et al. 2015a, b, c; Benelli et al. 2017a; Romano et al.
249 2015; Romano et al. 2016a, b). Focusing on insect courtship and mating behavior,
250 recent research reported evidences of lateralized mating traits in earwigs (Kamimura
251 2006), olive fruit flies (Benelli et al. 2015c), rice weevils and confused flour beetles
252 (Benelli et al. 2017a; Romano et al. 2016a), and even parasitoid species (Romano et al.
253 2016b).

254 In this study, we investigated the poorly known courtship and mating behavior
255 of the parasitic wasp *A. sp. near pseudococci*, a biological control agent of mealybugs,
256 showing a lateral bias in the sexual interactions. Our observations allowed describing
257 the mating sequences of this species that included the chasing of the female by the male
258 and a pre-copula phase, where the male mount the female courting her until copula
259 occurs. In addition, a post-copula phase was observed, where the male remounted for
260 few seconds the female and/or the mating pairs stay still and close each other.
261 According to our data, mating success was not related with the duration of chasing, pre-
262 copula and the whole duration of the courtship and mating sequences. Furthermore,
263 lateralization of the antennal tapping performed during the pre-copula phase in *A. sp.*
264 near *pseudococci* was evaluated, revealing that both females and males of this parasitoid
265 exhibited a tendency in using the right antenna over the left one, when started antennal
266 tapping session. In agreement with our results, a right-biased antennal tapping was
267 observed also during the courtship of *L. abnormis* (Romano et al. 2016b), another
268 encyrtid species occupying an ecological niche closely-related to that of *A. sp. near*
269 *pseudococci*, even if with lower temperature requirements (Tingle and Copland 1989).
270 However, while in *L. abnormis* only the males perform antennal tapping on the potential
271 mate (Romano et al. 2016b), in *A. sp. near pseudococci*, antennal tapping was displayed

272 by both sexes. In addition, the males of *A. sp. near pseudococci* carried out antennal
273 tapping with a higher level of pulse frequency over females, and the mating success was
274 higher in mating pairs where females bring up the antennae to palpate those of males,
275 which were held forward and still during the mount. We hypothesize that males
276 produced aphrodisiac secretions on antennal glands, which induced antennal tapping in
277 females. Indeed, it has been reported that *A. sp. near pseudococci* presents sexual
278 dimorphism of antennae, since male's antennae are provided with sophisticated
279 glandular structures that are absent in the females (Fortuna et al. 2015). Therefore, the
280 production of alluring substances could act as a selective mechanism to persuade
281 females on the male quality (see also Benelli and Romano 2017; Romano et al. 2016a).

282 Interestingly, from an intra-sexual point of view, individuals performing
283 antennal tapping with higher frequency of pulses, outperformed individuals with lower
284 values of the frequency of antennal tapping in terms of mating success. This indicates
285 the important role that tactile stimuli play, aside olfactory cues, in better allocating or
286 harvesting contact pheromones. Finally, *A. sp. near pseudococci* used preferentially the
287 right antenna to start antennal tapping behavior. In addition, right biased individuals
288 were more successful in mating. This phenomenon may be due to the prolonged mating
289 interaction occurring also in other insect species including another encyrtid (Benelli et
290 al. 2015c, 2017a; Romano et al. 2016a, b) as well as may be due to a higher number of
291 sensory structures and/or glandular areas on the right antenna (Anfora et al. 2010;
292 Romano et al. 2016b).

293 To the best of our knowledge, this report on behavioral asymmetries of mating
294 traits in *A. sp. near pseudococci* represents a quite rare evidence of lateralized behavior
295 in parasitic wasps of economic importance. Our findings add basic knowledge to the

296 behavioral ecology of this biocontrol agent with potential implications on the
297 optimization of mass-rearing procedures aimed to employ this parasitoid in Integrated
298 Pest Management.

299

300 **Author contributions**

301

302 DR and GB designed the research, conducted the experiments and analyzed the
303 data. All authors contributed new reagents and/or analytical tools. All authors wrote and
304 approved the manuscript.

305

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307

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316

317 **Compliance with ethical standards**

318

319 **Conflict of interest**

320

321 The authors declare no conflicts of interest.

322

323 **Ethical standards**

324

325 All applicable international and national guidelines for the care and use of
326 animals were followed. All procedures performed in studies involving animals were in
327 accordance with the ethical standards of the institution or practice at which the studies
328 were conducted.

329

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331

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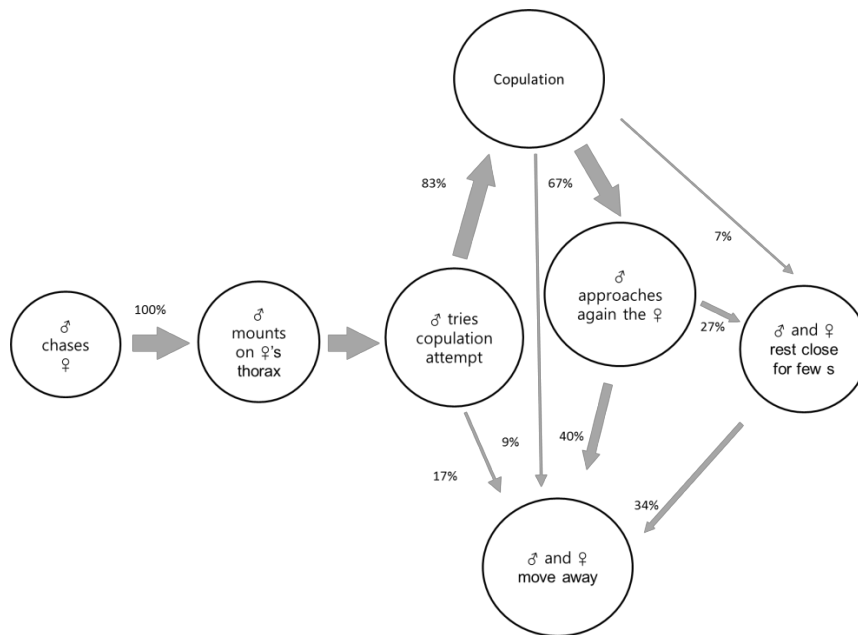
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Figure 1. (a) Ethogram quantifying the courtship and mating behaviour of the encyrtid parasitoid *Anagyrus pseudococci*. **(b)** Presence of lateralized antennal tapping behaviour in males and female wasps during courtship. The thickness of each arrow indicates the proportion of individuals displaying different behavioural phases. Green arrows indicate females showing right-biased antennal tapping, red arrows showed females using first the left antenna. Orange arrows indicate males showing right-biased antennal tapping, brown arrows showed males using first the left antenna.

(a)



(b)

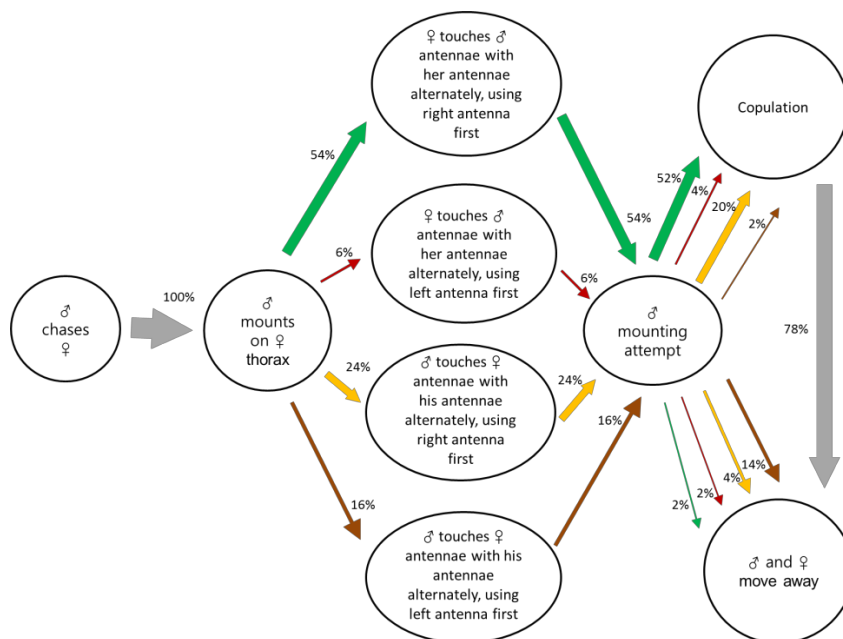


Figure 2. Duration of different courtship and mating phases in successful and unsuccessful approaches of *Anagyrus pseudococci* males towards females. T-bars represent standard errors. Above each column, different letters indicate significant differences (general linear model, $P < 0.05$).

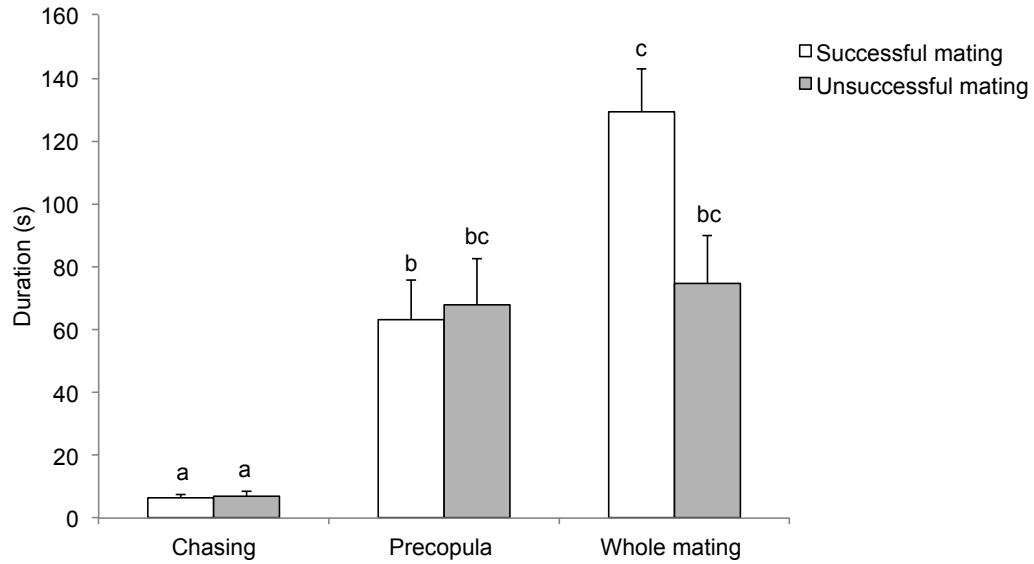
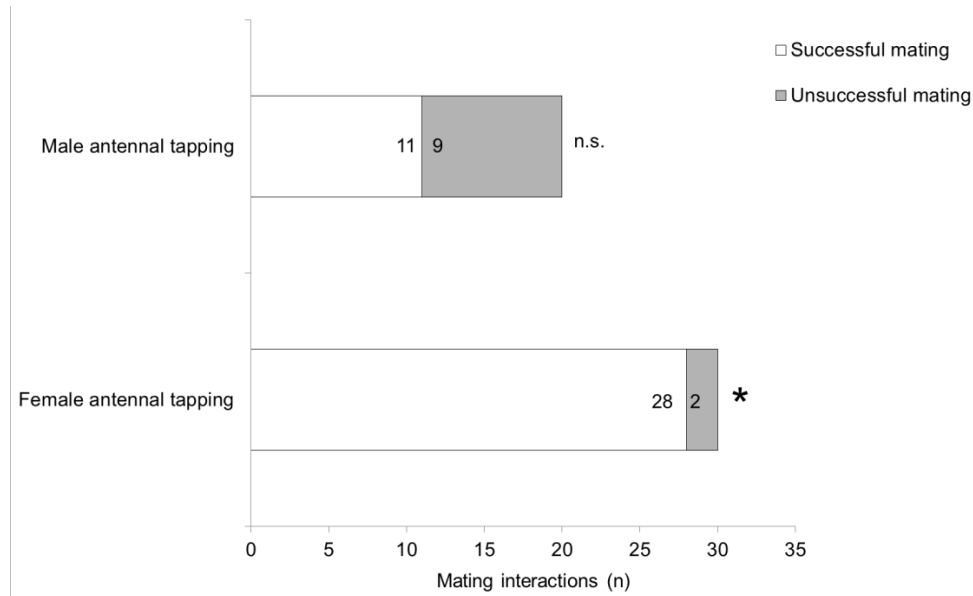


Figure 3. (a) Impact of male and female antennal tapping behaviour on *Anagyrus pseudococci* mating success. The asterisk indicates a significant difference (generalized linear model, $P < 0.05$); n.s. = not significant. **(b)** Influence of left-biased and right-biased antennal tapping on *Anagyrus pseudococci* mating success. Above each column, different letters indicate significant differences (generalized linear model, $P < 0.05$).

(a)



(b)

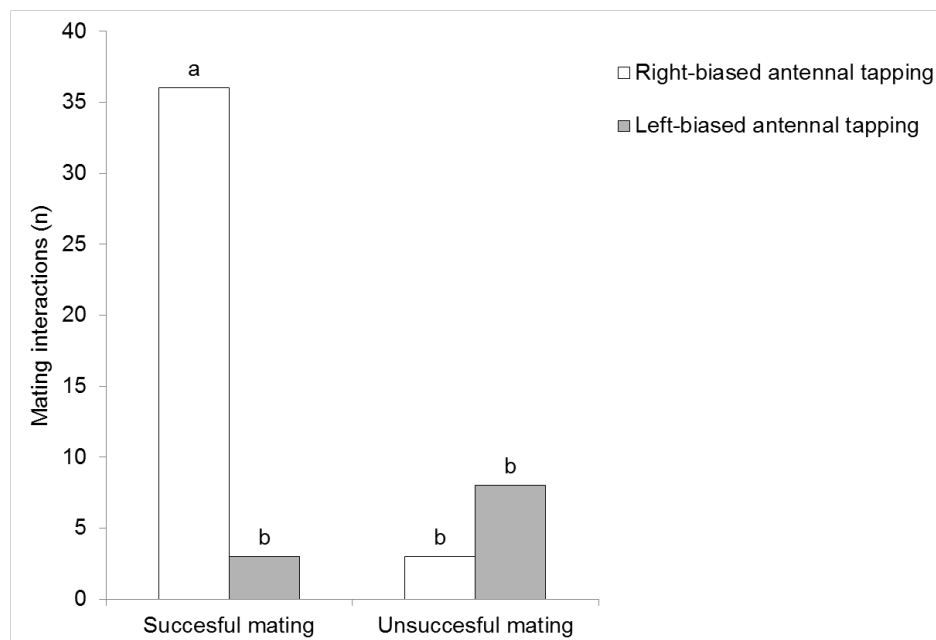


Figure 4. Variations in antennal tapping frequencies of *Anagyrus pseudococci*, in relation to the sex performing antennal tapping, laterality and mating success in both sexes. Above each column, different letters indicate significant differences (general linear model, $P < 0.05$).

