



# Olfactory Mating Signals in the Migratory Locust *Locusta migratoria*

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Received: 27 March 2023 / Revised: 22 September 2023 / Accepted: 2 October 2023  
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## Abstract

Swarming locusts cause huge plagues across the world threatening food production. Before swarms form, locust populations exhibit a dramatic phase change from a solitary to a gregarious phase. The cause of this phase change is a complicated interplay of conspecific and environmental cues and is, especially for one of the major pests, the migratory locust *Locusta migratoria*, still not well understood. Here we study the behavior of both solitary and gregarious *L. migratoria* towards the headspace odors of conspecifics. As we do not find a general attraction of gregarious animals to the headspace of gregarious conspecifics, swarm formation does not seem to be mainly governed by olfactory aggregation cues. When testing for potential mating signals, we observe that the headspace of virgin gregarious females is highly attractive only towards virgin males of the same phase, while mated gregarious males and solitary males, regardless of their mating state, do not become attracted. Interestingly, this phase-specific attraction goes along with the finding, that mating behavior in experiments with inter-phasic pairings is extremely rare. Our data suggest that odor emissions in *L. migratoria* play a significant role in a mating context.

**Keywords** Migratory locusts · Chemical ecology · Mating · Aggregation · Behavior

## Introduction

The devastating effect of swarms of gregarious locusts has been reported since biblical times. While solitary and gregarious locusts had been considered to be different species, in 1921, Uvarov discovered the fascinating phenomenon of phase polyphenism (Pflüger and Bräunig 2021; Simpson et al. 2011). As we now know from several locust species (Topaz et al. 2012), *Locusta migratoria* (Linnaeus 1758; Order: Orthoptera, Family: Acrididae) exists in solitary and gregarious phases. These phases differ in morphological, anatomical, and behavioral features (Greenwood and Chapman 1984; Latchininsky 2019; Wang et al. 2014; Wei et al. 2017), with e.g., the solitary phase being more camouflaged, while the gregarious phase is conspicuously colored. The shift between both phases is a complex

phenomenon, that to some extent is still considered a puzzle. In desert locusts *Schistocerca gregaria* (Forsskål 1775; Order: Orthoptera, Family: Acrididae), factors such as environmental cues and sensory cues from conspecifics, including visual, tactile, and olfactory cues, seem to be involved (Nakano et al. 2022; Roessingh et al. 1998; Simpson et al. 2011). The role of olfaction in the locusts' phase polyphenism has been explored over the past few decades (Guo et al. 2020; Wei et al. 2017). It is well understood that odor profiles are dynamic within the life stages and between the phases (Wei et al. 2017). Most of the existing literature, however, focuses on *S. gregaria* and suggests that headspace odors from gregarious animals are attractive to gregarious and repulsive to solitary conspecifics (Roessingh et al. 1993, 1998; Rogers et al. 2003). However, as *S. gregaria* and *L. migratoria* even differ in their responses to the pivotal body odor phenylacetoneitrile (PAN) that is present in both species (Pener and Simpson 2009; Torto et al. 1996; Wei et al. 2019), the general function of headspace odors in *L. migratoria* so far remains elusive. Moreover, phase shift dynamics also differ directionally between the phases in *S. gregaria* (Simpson et al. 1999). However, there is a lack of understanding about *L. migratoria* that prompts further research to understand the behavioral response of *L. migratoria* to conspecific smells.

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57 Here, we provide headspace odor collections of differ- 84  
 58 ent developmental stages of gregarious *L. migratoria* to 85  
 59 individual animals in a binary choice arena. Some studies 86  
 60 have so far focused on the behavioral responses towards 87  
 61 animal-released odors mainly in *S. gregaria* (Obeng-Ofori 88  
 62 et al. 1993; Torto et al. 1996). We, therefore, performed a 89  
 63 comprehensive set of experiments to test, whether stage-,  
 64 phase-, and/or sex-specific odor blends provoke attraction  
 65 or repulsion to the different stages, phases, and sexes in *L.*  
 66 *migratoria*. At the same time, interphasic mating occurs in  
 67 *S. gregaria* (Golov et al. 2018a) but this phenomenon has  
 68 not been studied in *L. migratoria*. We, therefore, extend our  
 69 study to mating assays between the different phases of *L.*  
 70 *migratoria*. In conclusion, we aim to increase our under-  
 71 standing of *L. migratoria* with regard to the behavioral  
 72 impact of its body odors.

## 73 Methods and Materials

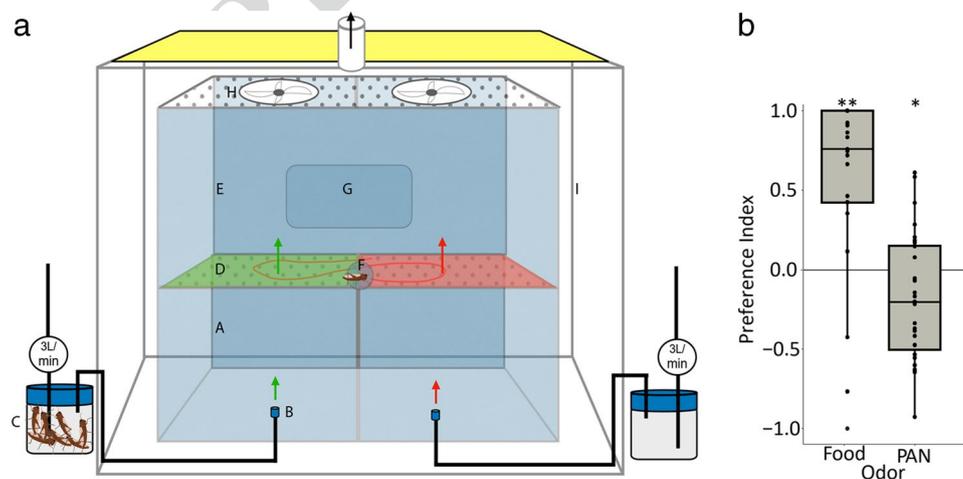
### 74 Animal Breeding

75 We used *L. migratoria* that we bought from a local pet shop.  
 76 The gregarious and solitary animals used for tests were kept  
 77 separated for a minimum of 5 generations. Both phases were  
 78 maintained at the Max Planck Institute for Chemical Ecology  
 79 in climate chambers with a 14:10 h light:dark cycle, at a  
 80 temperature of  $30 \pm 2$  °C, and humidity of  $50 \pm 5\%$ . The gre-  
 81 garious animals were kept with around 300–400 first instar  
 82 animals in a cubic cage (side length, 30 cm  $\times$  30 cm  $\times$  30 cm).  
 83 The numbers of animals were reduced continuously during

aging, to finally reach around 200 adult animals in the same  
 cage. The solitary animals were separated on the day of  
 hatching into individual cylindrical boxes (height, 10.5 cm;  
 diameter 8 cm). Each solitary animal was supplied with  
 a separate ventilation system. Both phases were fed with  
 wheat grass provided by our greenhouse.

### Bioassay Arena

The behavioral setup includes a cuboid base, an arena sur-  
 face consisting of a mesh, and an arena enclosure (Fig. 1a).  
 The base of the behavioral setup consists of two separate  
 polypropylene boxes (A) (16 cm  $\times$  30.5 cm  $\times$  25 cm) with  
 air diffusers opening upwards at the middle of the lower  
 surface (B) of each of the boxes. The diffuser is connected  
 to the odor/control source (C). The base is high enough to  
 evenly distribute the odor at the base before the air enters the  
 arena. The in-house air is controlled by two flowmeters con-  
 nected to the odor and control source via a 6/4 mm Teflon  
 pipe. The air inlet is kept at 3L/min for each side. From the  
 source, the air is introduced to the base of each side of the  
 setup through the diffuser. Two perforated polypropylene  
 plates of size 25 cm  $\times$  30 cm, with perforations of 2 mm  
 diameter and distributed evenly at every 2 mm are placed on  
 each of the two boxes making a rectangular behavioral arena  
 (D). The arena has no division in the middle, allowing the  
 locust to move in all directions within the arena. This design  
 results in an arena divided into two zones, one with odor  
 and one without that the animal can chose depending on the  
 valence of the tested odor. The arena is enclosed by another  
 rectangular polypropylene box (26 cm  $\times$  62 cm  $\times$  39 cm)



**Fig. 1** Assay for testing attraction and aversion of odors. **a** Experimental setup: A, polypropylene base; B, air diffusers; C, odor source; D, odor (green) and control (red) zones of arena; E, arena enclosure; F, opening to introduce animal; G, window to remove animal; H, axial fan; I, enclosing chamber. **b** Gregarious *L. migratoria* nymphs show significant attraction to food odors ( $n=23$ ,  $p=0.0018$ ,

*Wilcoxon-signed rank test*) and repulsion to PAN ( $n=31$ ,  $p=0.017$ , *Wilcoxon-signed rank test*). Box represents the 50% of the central data/interquartile range (IQR) with median, the whiskers represent the range (= upper quartile+1.5 IQR/ lower quartile-1.5IQR). \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$

113 to limit the locust within the arena (E). A circular inlet of  
 114 5 cm diameter that can be opened and closed from outside  
 115 is situated at the same level as the behavioral arena plane  
 116 to introduce locusts into the arena (F). A rectangular open-  
 117 ing of 29 cm × 25 cm with a closing door is situated above  
 118 the animal inlet to retrieve the animal after each trial, with  
 119 the least disturbance to the airflow (G). The base, arena,  
 120 and enclosing walls are supported with aluminum hinges  
 121 of 1 cm width. A pair of axial fans (H) (connected to the  
 122 same voltage input) are suspended on top of each side 39 cm  
 123 above the arena to ensure a laminar and vertical airflow (ca.  
 124 3 cm/s) in the arena. Between the fans, an HD USB camera  
 125 covering the whole arena is fitted and connected to a com-  
 126 puter outside. The whole setup is placed inside an enclosing  
 127 chamber 92\*72\*65 cm with solid white walls to exclude  
 128 visual distraction from outside of the arena (I). LED light  
 129 is fitted 11 cm above the enclosure to ensure even lighting  
 130 throughout the arena surface. The air from the enclosure is  
 131 ventilated out at a constant rate of approximately 30L/min  
 132 via an exhaust fitted 15 cm above the behavioral setup. The  
 133 setup is maintained at  $31 \pm 1^\circ\text{C}$  and humidity at 50% during  
 134 the trials.

### 135 Bioassay Procedure

136 For each trial, one animal was introduced into the arena  
 137 through the animal inlet, and each animal was tested only  
 138 once. If an animal refused to enter the arena within the first  
 139 5 min, it was removed, and a new trial was started with a  
 140 new animal. The 10-min recording of the animal's behavior  
 141 started only after the animal made an initial movement of  
 142 at least 1 cm or more into the arena. After every 5 trials, the  
 143 arena was wiped with 70% ethanol and ventilated for an hour  
 144 with clean air to remove any odors and any potential trails  
 145 left by the animals.

146 20 unstarved animals in an air-tight box were used as an  
 147 odor source. After every 5 trials, the animals used as the  
 148 odor source were placed back into the breeding cage for an  
 149 hour after which they were again used as the odor source in  
 150 the setup. In all tests, the side of odor within the arena was  
 151 reversed after half of the trials. The nymphs used were all in  
 152 the late fourth instar and not separated by males or females.  
 153 The virgin animals for experiments were taken 6–8 days post  
 154 eclosion and the mated animals were taken a few hours after  
 155 the male debarks the female or the female finishes oviposi-  
 156 tion. The solitary animals were simply marked on the cage  
 157 on the date of eclosion and used 8 days later for the tests.

### 158 Mating Experiments

159 To measure the willingness to mate, we used mounting  
 160 behavior by males (virgin animals 6–8 days after the final  
 161 eclosion) as a parameter. The animals were kept together

in the cage and were observed until the first mounting hap-  
 162 pened. The mounted pair was removed and the remaining  
 163 animals were separated into males and females and used for  
 164 experiments within 48 h of separation. Mating tests were  
 165 done in a 10 cm × 10 cm cage built from perforated plates  
 166 on 5 sides and a glass on one side allowing observation.  
 167 Each cage, considered as one data point, consists of four  
 168 males and five females. The proportion of the number of  
 169 males mounting the females was observed at 6 and 12 h.  
 170 This was done inter-phase as a test and intra-phase as control  
 171 experiments.  
 172

### Data Analysis

In the odor choice bioassay procedure, the time (in seconds)  
 174 an individual animal spent in the control or the odor side  
 175 was observed. We considered the exact middle of the setup  
 176 to be the division and the place of the head of the animal  
 177 as the animal's location when the animal spent time in the  
 178 2 cm-wide fringe area of the two zones.  
 179

The preference index was calculated as:  
 $PI = (Time_{inOdor(s)} - Time_{inControl(s)}) / (Totaltime(600s))$ . To test whether the  
 181 preference was significantly attractive or aversive, we used  
 182 the *Wilcoxon-signed rank test*.  
 183

Mounting behavior was analyzed by the *Mann Whitney-U*  
 184 *test*, at 6 h. To compare between multiple groups, at 12 h,  
 185 *Kruskal–Wallis test with Dunn's posthoc test* for multiple  
 186 comparisons was used. In all cases, both for *PI* and mat-  
 187 ing experiments, \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ .  
 188 *Wilcoxon-signed rank test* was performed in R version 4.2.3.  
 189 For multiple group comparison GraphPad InStat was used.  
 190

PAN used for the experiments was ordered in the highest  
 191 purity available from Sigma Aldrich (B19401-250G).  
 192

## Results

### Evaluation of Bioassay

To screen for the behavioral valence of body odors, we used  
 195 a two-zone arena, where the headspace of 20 animals from  
 196 a given stage was infused to one side, while the other side  
 197 was infused with control air (Fig. 1a). The time spent by  
 198 individual animals in either zone was measured to investi-  
 199 gate whether the odor was perceived as attractive (i.e., more  
 200 time spent on the odor side), or repellent (i.e., more time  
 201 spent on the control side).  
 202

To test the assay for functionality, experiments were first  
 203 performed with an attractive food odor (i.e., headspace emit-  
 204 ted by 5 g of shredded wheat grass) and with the known  
 205 repellent PAN (100  $\mu\text{L}$  at 1 mg/mL concentration) diluted  
 206 with mineral oil. PAN is repulsive in this assay to all stages  
 207 and phase of the *L. migratoria* (Chang et al. 2023). When  
 208

209 testing starved fourth instar gregarious nymphs with the food  
210 odor, the animals showed strong attraction to the odor and  
211 spent significantly more time on the side of the arena smell-  
212 ing of wheat (Fig. 1b). Animals of the same cohort tested  
213 with PAN avoided the side with this odor (Fig. 1b), demon-  
214 strating that the assay indeed was suitable for testing both  
215 attraction and aversion.

## 216 Responses of Nymphs in Bioassay

217 Gregarious locusts usually aggregate in conspicuous huge  
218 hopper bands or swarms, whereas locusts of the solitary  
219 phase avoid groups and are rather cryptic. To investigate  
220 whether the aggregation and repulsion in gregarious and  
221 solitary animals, respectively, is governed by olfactory  
222 cues from the gregarious nymphs, the valence of odors  
223 from gregarious nymphs was tested in gregarious and soli-  
224 tary nymphs. Surprisingly, we did not find any significant  
225 response either in gregarious or in solitary nymphs to the  
226 headspace of gregarious nymphs (Fig. 2a). Similarly, no  
227 other tested headspaces from gregarious adults, except for  
228 the headspace of gregarious virgin females, elicited any sig-  
229 nificant attraction in gregarious or solitary nymphs (Supple-  
230 mentary Fig. 1). It thus seems that olfactory cues alone are  
231 not sufficient for the forming of hopper bands in gregarious  
232 *L. migratoria*.

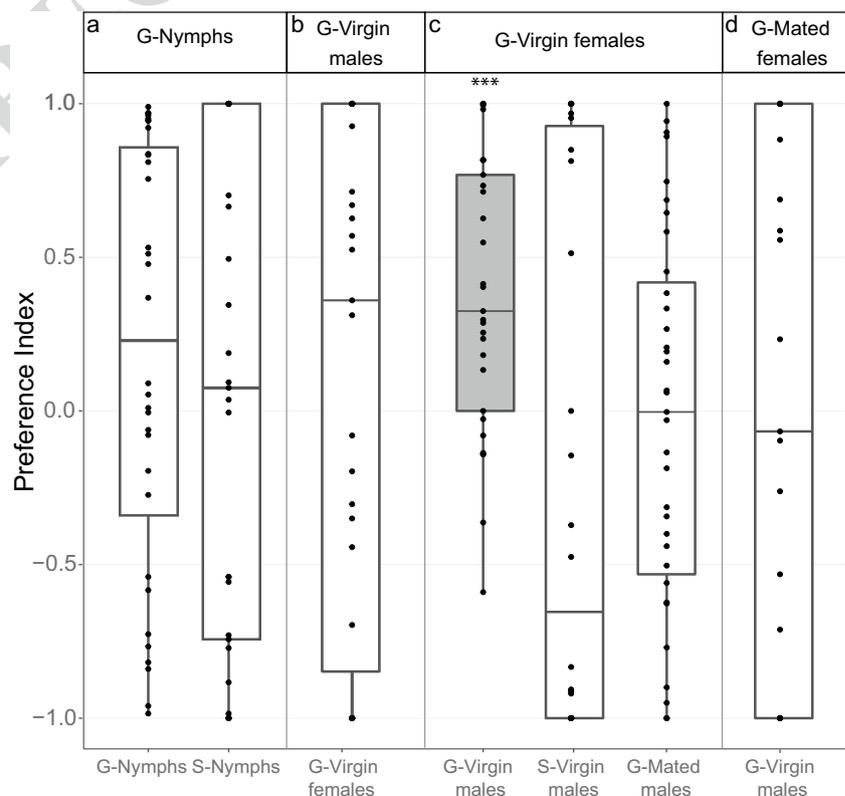
## Responses of Adults in Bioassay

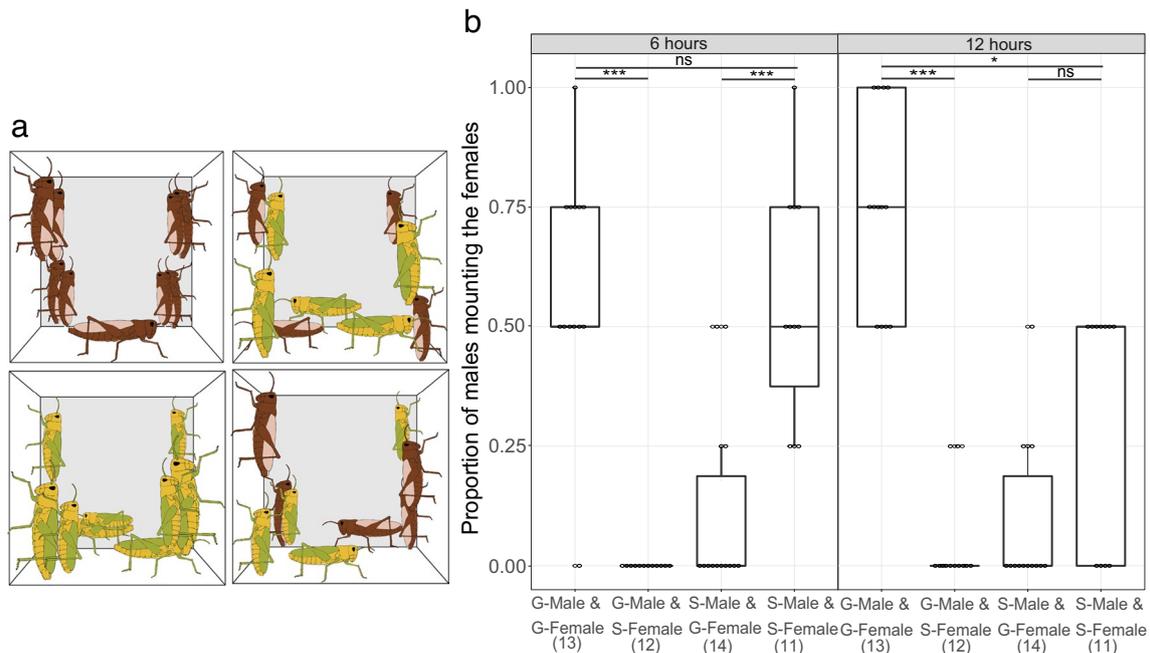
234 We next asked whether attraction towards potential mates  
235 might be governed by olfactory cues. When testing gregari-  
236 ous virgin males and virgin females with the headspace of  
237 the opposite sex, males were significantly attracted to the  
238 female odor, while females did not respond to the male odor  
239 (Fig. 2b, c). Interestingly, when testing for inter-phase attrac-  
240 tion, we found that the odors from gregarious virgin females  
241 did not elicit any response in solitary virgin males (Fig. 2b).  
242 In addition, contrary to the headspace of virgin gregarious  
243 females, the odor emitted by mated gregarious females was  
244 not attractive to virgin gregarious males (Fig. 2d), suggest-  
245 ing that the headspace of mated females is either lacking  
246 attractive compounds or includes repellent compounds that  
247 virgin females are not emitting. At the same time, the attrac-  
248 tion of gregarious males towards the odor of virgin gregari-  
249 ous females was diminished when the males were already  
250 mated (Fig. 2c).

## Mating Experiments

252 Having found that solitary males showed no attraction to  
253 the headspace of virgin gregarious females, we tested if this  
254 results in an inter-phase mating barrier. We conducted mat-  
255 ing experiments, where virgin males were paired with either  
256 virgin females of the same or opposite phase (Fig. 3a). We

**Fig. 2** Attraction of animal headspaces in *L. migratoria*. **a** Preference of gregarious ( $n=32$ ,  $p=0.13$ ) and solitary nymphs ( $n=29$ ,  $p=0.66$ ) tested with headspace odors of gregarious nymphs. **b** Gregarious virgin females ( $n=31$ ,  $p=0.50$ ) tested with odors of gregarious virgin males. **c** Gregarious virgin males ( $n=29$ ,  $p=0.0004$ ), solitary virgin males ( $n=30$ ,  $p=0.25$ ), and gregarious mated males ( $n=35$ ,  $p=0.74$ ) tested with the odor of gregarious virgin females. **d** Gregarious virgin males ( $n=27$ ,  $p=0.95$ ) tested with odors of gregarious mated females. Wilcoxon-signed rank test was used to determine P values. Box represents the 50% of the central data/interquartile range (IQR) with median, the whiskers represent the range (= upper quartile + 1.5 IQR/ lower quartile - 1.5IQR). \*\*\*,  $p < 0.001$





**Fig. 3** Intra- and inter-phase mating behavior in *L. migratoria*. **a** Schematic presentation of the mating experiment for an overview of the crowding of animals in the experiment. Cubic cage (side lengths 10 cm), experimental animals drawn to scale. The gregarious animals are represented in brown while the solitary animals are green and yellow. **b** Proportion of males mounting females in the intra- and inter-

phase combinations of solitary and gregarious animals observed after 6 (left) and 12 (right) hours. *Kruskal–Wallis test with Dunn’s posthoc test* for selected comparisons. Box represents the 50% of the central data/interquartile range (IQR) with median, the whiskers represent the range (= upper quartile + 1.5 IQR/ lower quartile - 1.5 IQR). \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$

257 then observed the mounting behavior which is an obliga- 279  
 258 tory and easily observable step in orthopteran mating. 280  
 259 Interestingly, we found that the frequency of mounting was 281  
 260 significantly lower in inter-phase tests. At the end of 6 h, 282  
 261 while 59% of the gregarious males mounted gregarious 283  
 262 females, only 10% of the solitary males mounted the gre- 284  
 263 garious females. At the same time, 54% of the solitary males 285  
 264 mounted the solitary females, while none of the gregarious 286  
 265 males mounted solitary females during the 6 h of observa- 287  
 266 tions (Fig. 3b). While intra-phase combinations resulted in 288  
 267 similar mounting ratios in gregarious and solitary animals 289  
 268 during the first 6 h of the experiments, the mounting behav- 290  
 269 ior of gregarious animals lasted longer, as many solitary 291  
 270 males had already unmounted their solitary females by the 292  
 271 end of 12 h (Fig. 3b). 293

## 272 Discussion

273 The phase state in locusts has been shown to be governed 294  
 274 both by environmental cues and cues from conspecifics 295  
 275 (Nakano et al. 2022). From a sensory point of view, differ- 296  
 276 ent developmental stages of *S. gregaria* aggregate based on 297  
 277 both chemo- and mechanosensory cues (Niassy et al. 1999; 298  
 278 Rogers et al. 2003; Simpson et al. 2001). Contact cues and 299  
 300  
 301  
 302  
 303

short-range odors seem to be pivotal in the gregarious phase, 279  
 while long range auditory cues are more important in the 280  
 solitary phase (Nakano et al. 2022; Pener and Simpson 281  
 2009). The short-range odors emitted and perceived by the 282  
 gregarious animals emanate from food, feces, and the head- 283  
 space of conspecifics. Based on these results we expected, 284  
 that gregarious nymphs of *L. migratoria*, which form hopper 285  
 bands, are attracted by the headspace of gregarious nymphs. 286  
 Surprisingly, we did not find any attraction, and gregarious 287  
 nymphs in most aspects did not differ from solitary ones, 288  
 which were neither attracted nor repulsed by any of the gre- 289  
 garious headspaces. 290

*S. gregaria* have been reported to shift easily from the soli- 291  
 tary to the gregarious phase (Rogers et al. 2003), whereas 292  
 for *L. migratoria*, the solitary phase seems to be the more 293  
 stable state, and a shift to the gregarious phase is difficult 294  
 to induce and needs an extremely high density of animals. 295  
 Corresponding to that the rate of gregarization and solitarisation 296  
 is also different within and between locust species (Topaz 297  
 et al. 2012). *S. gregaria* exhibit a much faster gregarisation 298  
 and a slower solitarisation process (Simpson et al 1999; Wei 299  
 et al. 2017). A higher concentration of odors or even a com- 300  
 bination of multi-modal cues including e.g., mechanosen- 301  
 sory or visual signals might thus potentially be necessary to 302  
 trigger aggregation behavior among *L. migratoria* nymphs. 303

The headspace composition has been shown to be dynamic and dependent on phase and state (Wei et al. 2017). 4-vinylanisole (4 VA) is one of the headspace odors that has been identified as a possible aggregation pheromone in *L. migratoria* (Guo et al. 2020). PAN is another dominant headspace odor in both *L. migratoria* and *S. gregaria*. The role of PAN in *S. gregaria* is still under debate, as some studies suggest a role as an aggregation pheromone (Torto et al. 1994), others supposed repellency (Seidelmann et al. 2005), while yet others even suggest a role in sexual behavior (Seidelmann and Ferenz 2002). In *L. migratoria*, however, PAN is repulsive to animals of all stages and phases (Chang et al. 2023; Wei et al. 2019). The responses of nymphs that we observed in the aggregation tests could thus be expected, considering the odor profile of individual groups. The higher proportion of PAN to 4VA in gregarious males as compared to gregarious females (Wei et al. 2019) could form a background to the neutral response to gregarious male odors, where the attractant 4VA and the repellent PAN balance each other. The strongly attractive response by gregarious nymphs to gregarious females could rely on the higher amount of 4VA, which would dominate over the repellency of PAN. Similar mixture interactions in binary mixtures of opposing valence have also been reported in e.g., *Drosophila* (Mohamed et al. 2019; Thoma et al. 2014).

When looking for potential effects of headspace odors on mating behavior, we found a strong attraction of gregarious males to the headspace of virgin gregarious females. As the number of gregarious males is much higher than that of gregarious females in mating swarms and in ovipositing populations (Ellis and Ashall 1957), gregarious males face strong competition for females. The indifferent response of the virgin gregarious females to the headspace of virgin gregarious males is coherent with this proportion, as females do not have to actively respond to males for successful mating. The lack of response of virgin gregarious males to mated females could indicate the presence of a courtship inhibitory factor. Such a factor can be either produced by the female herself to prevent further harassment from males (Engel et al. 2016) or be transferred by the male during mating as an additional passive form of mate guarding (Seidelmann 2006). Male-transferred anti-aphrodisiacs are found in other insects too (Khallaf et al. 2020, vander Meer et al. 1986). In *S. gregaria*, PAN acts as such a courtship inhibition pheromone (Seidelmann and Ferenz 2002). Alternatively, mated females could emit lower amounts of pheromones. The lack of response by mated gregarious males to the odor of virgin gregarious females could be interpreted as a sign of transient abstinence during a recovery phase (Barrozo et al. 2010a) which has been shown in other insects to be combined with a lower sensitivity to female pheromones post mating (Barrozo et al. 2010b).

When testing mating behavior within the phases, we already found differences between the gregarious and the solitary animals. The mounting of solitary males usually lasted for less than 12 h, while that of gregarious males lasted much longer. Mounting of females by males is the longest copulation step and is present in both phases. It has been proposed as an active mate guarding strategy of the male to avoid remating of the female before his sperm has fertilized her eggs (Golov et al. 2018a; Zhu and Tanaka 2002). The duration of the mounting varies depending on sub species, duration of separation of males into cohorts, and phase (Golov et al. 2018a, b; Seidelmann 2006; Tanaka and Zhu 2003). The longer mounting that we observed in the gregarious phase could, hence, be interpreted as an effect of the higher male-male competition faced by gregarious males within the swarm.

In the inter-phase attraction experiments, we found it intriguing that solitary males were not attracted by the headspace of virgin gregarious females. We, therefore, asked whether males and females from different phases would mate at all. In *S. gregaria* gregarious males exhibit frequent mounting attempts when encountering solitary females. Interestingly, we found an opposite trend in *L. migratoria*. Males of a given phase showed only weak interest in females of the other.

In conclusion, we found that odors emitted by nymphs seem to be of less importance in attracting other nymphs into hopper bands. Other sensory cues or odors of a higher concentration, or combinations of these might be the deciding factors. The attraction between the sexes does, however, seem to be relying on female-produced cues as gregarious males were strongly attracted to the odor of virgin gregarious females. Interestingly, solitary males were not attracted to the smell of gregarious females, revealing a certain degree of an inter-phase mating barrier. This postulation was further corroborated by our finding that male mounting behavior in couples of mixed phases is very rare.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10886-023-01456-9>.

**Author's Contributions** All authors were involved in the design of the study; APU performed the experiments; APU and MK analyzed the data; APU and MK wrote the first draft of the manuscript; all authors contributed to the final version of the manuscript.

**Funding** Open Access funding enabled and organized by Projekt DEAL. The study was funded by the Max-Planck Institute.

**Data Availability** Data will be made available on request.

**Declarations**

**Ethical Approval** Not applicable.

**Competing Interests** The authors have no competing interests to declare that are relevant to the content of this article. The authors have no relevant financial or non-financial interests to disclose.

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