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1 First evidence of sex-specific responses to chemical cues in tardigrade
2 mate searching behaviour

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9
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11 Keywords: chemical communication, waterborne signal, deposited cue, semiochemical, sex
12 discrimination

13
14 SUMMARY STATEMENT

15 We found evidence of pre-mating Darwinian sex roles despite limited sexual dimorphism. Our work
16 highlights the value of studying both sexes and non-model systems in chemical communication
17 research.

18
19 ABSTRACT

20 Chemical cues are widely used in intra- and interspecific communication, either as substances deposited
21 in the substrate or as molecules diffused in water or air. Tardigrades are an emerging microscopic study
22 system in which chemical communication and its role in reproduction are poorly known. Here,
23 we assess sex differences in the detection of (a) short-range diffusing signals and (b) deposited cue
24 trails during the mate-searching behaviour of freely moving virgin male and female *Macrobiotus*
25 *polonicus*. We tracked individual behaviour (a) in simultaneous double-choice chambers, where live
26 conspecifics of each sex were presented in water and (b) of freely moving pairs on agar without
27 water. We found that males, but not females, preferentially associated with opposite-sex individuals in
28 trials conducted in water. In contrast, neither sex detected nor followed cues deposited on agar. In
29 conclusion, our study suggests that mate discrimination and approach are male-specific traits and are
30 limited to waterborne chemical cues. These results support the existence of Darwinian sex roles in pre-
31 mating behaviour in an animal group with virtually non-existing sex differences in morphology or
32 ecology.

34 INTRODUCTION

35

36 Semiochemical communication is widespread and used by animals across multiple contexts, from
37 finding food to attracting mates (Wyatt, 2014). Behavioural responses to conspecific chemical cues,
38 and specifically sex discrimination, have been investigated in a wide range of taxa, both aquatic and
39 terrestrial (Dunham and Oh, 1992; Bouchard, 2001; Cooper Jr. and Pèrez-Mellado, 2002; Park *et al.*,
40 2004; Hutter, Zala and Penn, 2011; Stamps and Shaw, 2019; Kudo, Fujii and Ishikawa, 2022).
41 Importantly, sexually mature animals that fail to discriminate between the sexes and/or species incur a
42 reproductive cost (Burdfield-Steel and Shuker, 2011; Lerch and Servedio, 2022). To find and/or attract
43 a mate, animals can use two main types of chemical signals: (i) diffusing signals and/or (ii) trail
44 deposited on the substrate. For instance, male copepods can find females using pheromone plumes that
45 receptive females produce as trails or clouds (Bagøien and Kiørboe, 2005; Kiørboe, Bagøien and
46 Thygesen, 2005). Similarly, both male and female apple snails can follow opposite-sex trails, and, in
47 additions, males are attracted to waterborne sex pheromones of females (Takeichi, Hirai and Yusa,
48 2007).

49 Most studies examining the role of pheromones in sexual selection have focused on diffusing chemical
50 signals used in long-range mate attraction and mate recognition (Johansson and Jones, 2007).
51 Furthermore, there is a strong taxonomic bias towards insects and mammals (Brennan and Keverne,
52 2004; De Pasqual *et al.*, 2021), and on organisms with obvious male bias sexual dimorphism sensory
53 organs and searching behaviour (Naka, 2018). However, such knowledge is non-existent or very limited
54 for several animal groups, particularly micrometazoans such as the phylum Tardigrada. Tardigrades are
55 a microscopic animal phylum categorized in the superclade Panarthropoda (Yoshida *et al.*, 2017;
56 Jørgensen, Kristensen and Møbjerg, 2018) yet largely neglected in behavioural ecology and
57 evolutionary research. Even though individuals must be surrounded by water to be active, they
58 colonised terrestrial environments, such as mosses and lichens, thanks to their cryptobiotic adaptations
59 (Møbjerg and Neves, 2021). They have relatively simple sensory organs: some species present light-
60 sensitive eyes, but all have body segments containing cells with characteristics of mechanoreceptors
61 and chemoreceptors (Møbjerg *et al.*, 2018). Sexual dimorphism is mainly limited to a female bias in
62 body size, with very few reported examples of secondary sexual characters (Gąsiorek *et al.*, 2019;
63 Gąsiorek, Kristensen and Kristensen, 2021) and parental care (Pilato *et al.*, 2006). Of the ca. 1500
64 tardigrade species described so far (Degma and Guidetti, 2023), mating behaviour has been described
65 for only ten species (Sugiura and Matsumoto, 2021b), with only one showing secondary sexual
66 dimorphism. Moreover, detailed records of both pre-mating and mating phases are described for only
67 four species, of which none show secondary sexual characters (Bingemer, Hohberg and Schill, 2016;
68 Sugiura *et al.*, 2019; Bartel and Hohberg, 2020; Sugiura and Matsumoto, 2021a). Together, these studies
69 suggest a crucial role of semiochemicals in intra-specific sexual contexts (i.e., mate attraction and sperm
70 transfer) through diffusing cues. In addition, a recent study showed that tardigrades could detect

71 deposited chemical cues (trail) in inter-specific (i.e., predator-prey) contexts (Meyer *et al.*, 2020). In
72 this study, prey avoided areas previously occupied by predators while the latter preferred the areas
73 occupied by prey.

74

75 Chemical signals can serve as honest or deceptive indicators of quality, age, or other reproductive
76 factors and may be used to compete for mates (Johansson and Jones, 2007). Typically, the female
77 transmits signals, and the male responds, leading to a potential blind spot in research regarding female
78 response to pheromones (Hare and Simmons, 2019). To address this, it is vital to study both sexes'
79 reactions to chemical cues and their impact on mate-searching behaviour, which will also contribute to
80 a broader understanding of the evolution of these signals.

81

82 Using the gonochoristic tardigrade *Macrobiotus polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003
83 we tested the role of both waterborne diffusing signals and deposited cue trails (in water-free
84 environments) in sex discrimination and mate searching behaviours of both females and males. Since
85 our study system shows limited sexual dimorphism in sensory morphology and ecology, we
86 hypothesised that (i) both females and males would preferentially associate with the opposite sex, and
87 that (ii) both sexes could detect and follow an opposite-sex deposited-trail cue.

88

89 METHODS

90

91 *Culture*

92 We used two strains of a laboratory culture of the moss-living eutardigrade *Macrobiotus polonicus*
93 (strain AT.002 (Stec *et al.*, 2021) and IT S218). The second strain has been obtained from a moss sample
94 collected in Anzola Emilia, Bologna, Italy (44°34'08.2"N, 11°10'44.1"E) in December 2019. Females
95 of this species lay eggs freely in the environment (Pilato *et al.*, 2003) and have a sperm storage organ
96 (Vecchi *et al.*, 2022). The mating behaviour of *M. polonicus* has not been described fully; however, we
97 have observed sperm-release similar to what Sugiura *et al.* (2019) observed in closely related species.

98

99 Tardigrades were kept in 5 cm-diameter plastic Petri dishes with a scraped bottom (to aid motility) filled
100 with mineral water and fed *ad libitum* with algae (*Chlorococcum hypnosporum* and *Chlorella* sp.;
101 Sciento UK) and rotifers (*Lecane* sp.) or nematodes (*Panagrellus pycnus*) according to what was
102 available in culture, inside a climate chamber at 16 °C, 2:22 LD cycle. Half of the medium was partially
103 changed weekly. Thanks to the transparent cuticle, the sex and reproductive state of *M. polonicus* can
104 be determined non-invasively using light microscopy (400× magnification).

105

106 All individuals used for this study were virgins. Virgin individuals were obtained by isolating eggs and
107 then rearing hatchlings individually in 3 cm-diameter plastic Petri dishes, kept in the same conditions

108 as the main cultures. We determined the sexual maturity of individuals by observing motile sperm and
109 mature oocytes in the male and female gonad, respectively. Each trial was conducted with individuals
110 from the same strain (Experiment 1: n= 23 trials with AT.002 and n = 10 with IT S218; Experiment 2:
111 n = 16 trials with AT.002 and n = 11 with IT S218).

112

113 *Experiment 1: Waterborne (diffusing) cues*

114 We used 33 focal individuals (16 females and 17 males) to test whether tardigrades could differentiate
115 the sexes using diffusing signals. We created a choice chamber arena (Fig. 1A) consisting of an Ibidi 4-
116 well slide (Ibidi, Germany) and two fishing lure rings (4 mm inner diameter). The arena surface was
117 coated with 250 μ l of 1.5% agar (BD BACTO™ Agar, USA) with the placement of the lure rings
118 following a printed scheme. We placed the lure rings with their edges at an 8 mm distance from each
119 other, each 4 mm from the centre of the arena (distances same as in Bartel and Hohberg, 2020). When
120 the agar was solidified, we added 1 ml of mineral water to flood the arena so chemical cues could diffuse
121 within the chambers. This setup allowed us to provide the focal individual only with diffused chemical
122 cues from the signallers since they could not see nor touch them. The signallers were able to move
123 freely within the lure area. Some of them were used twice for two different focal individuals (one of
124 each sex), with at least 12 h of interval between the two trials (i.e., the next day). Different arenas were
125 used for every trial conducted on the same day, and all arenas were thoroughly cleaned at the end of
126 each day.

127

128 We placed the signallers inside the lure rings in darkness (swapping female and male sides between
129 trials) and let them habituate for 30 min. Afterwards, we added the focal individual in the centre of the
130 arena and filmed its behaviour for 30 min. A stereomicroscope with a diffused bottom light linked to a
131 camera (and the S-EYE software) was used. Position tracking was done automatically (see video
132 analyses section). The preferential association was assessed as latency to cross, and time spent within
133 the critical area of the opposite sex signaller.

134

135 *Experiment 2: Trail-deposited cues*

136 Individuals used in the first experiment were randomly grouped in mixed-sex pairs (both focals and
137 signallers). We used 27 couples to test whether tardigrades could follow a trail-deposited cue. The
138 individuals were placed in the same type of Ibidi chamber slides as in Experiment 1, but without water
139 and the fishing lure rings. An ultra-thin continuous water film coat agar and the tardigrades which
140 allowed them to move freely in the arena but limited any chemical cue to the tardigrades' trails. We
141 used the same microscope and camera setup as in the previous experiment. After starting the recording,
142 we placed the first individual and waited (ca. five min) for it to move. Then, we added the second
143 individual and continued recording for 30 min. The order of individuals (male vs. female) was alternated
144 between trials. Position data for both individuals were obtained automatically (see video analyses

145 section). In addition, we documented in detail the number and type of all observed interactions (i.e.,
146 bumps and following behaviour).

147

148 *Video analyses*

149 To obtain the position of the focal individuals during each trial during both experiments, we analysed
150 the videos in R v.4.2.0 with the function "trackR" from the package "trackR" (Garnier, 2022). The
151 tracking was checked, and tracking position errors fixed with the function "trackFixer" of the same
152 package.

153 To determine whether the focal individuals were inside a critical area (dashed lines, Fig. 1A) for the
154 waterborne-diffused cues, we applied a distance-to-centre function to the output (xy coordinates) given
155 by trackR. For the trail-deposited cue experiment, we calculated the distance between individuals' paths
156 every three seconds. Only those that differed by less than one body length distance from each other
157 were considered to overlap (e.g., perpendicular overlap is excluded). We then assess the proportion of
158 time the paths of both animals overlapped.

159

160 *Statistical analyses*

161 All statistical analyses were conducted in R v.4.2.0 (R Core Team, 2022). We ran generalised linear
162 models using the "brm" function from the "brms" package v.2.17.5 (Bürkner, 2017).

163 In the diffusing signal experiment: for the latency to cross the first critical area, the predictor was the
164 sex of the focal individual, whereas, for the proportion of time spent inside a critical area, the predictor
165 was included as four types of dyad interactions (focal-signallers): Female-Female, Female-Male, Male-
166 Female and Male-Male. The ID of the focal individual was included as a random variable. The focals
167 that did not cross any critical area were removed from the analysis in both models. To test if the path of
168 one sex overlapped more often with the path of the other, we used the sex as a predictor of the proportion
169 of time an individual was on top of the other one's trace. We included the trial ID as a fixed effect for
170 the trail data since the couples could interact. To assess if the absence of water affected motility, we
171 compared the distance travelled in both experiments, where the focal individual's sex was the predictor.
172 We used default priors for each response distribution type: Binomial (logit link) distribution for the
173 proportion of time spent inside each critical area and the proportion of time an individual was on top of
174 another one's path, Gaussian for the latency to cross the first critical area, and the distance travelled.
175 The results are presented as back-transformed Bayesian posterior means and 95% high-density interval
176 (HDI) using the "mean_hdi" function from the "tidybayes" package v.3.0.2 (Kay, 2022), computed from
177 $n = 4$ well converging and uncorrelated chains with $n = 4000$ saved iterations each.

178

179 RESULTS

180

181 *Experiment 1: Waterborne (diffusing) cues*

182 In total, ten individuals (five females and five males) did not cross any critical area, and their latency to
183 choose was recorded as 30 min (i.e., the maximum duration of the trials); (Fig. 1B). The first choice in
184 both sexes was biased towards female signallers except for one focal individual of each sex (Fig. 1B).
185 The latency to first cross a critical area was not significantly associated with the sex of the focal
186 individual (n = 23, Bayesian mean estimate [95% HDI]: females = 7.54 [3.91, 10.99] min; males = 3.54
187 [0.20, 6.77] min) (Fig. 1B).

188
189 Females spent a very short but similar time next to either sex, whereas males showed a strong attraction
190 to female signallers (female n = 12, mean of the proportion of time spent next to female [95% HDI] =
191 0.107 [0.034, 0.22]; next to male = 0.08 [0.023, 0.164]; males n = 12, mean of the proportion of time
192 spent next to females [95% HDI] = 0.540 [0.290, 0.778]; next to males = 0.008 [0.002, 0.018]); (Fig.
193 1C).

194

195 *Experiment 2: Trail-deposited cues*

196 The proportion of time an individual spent on top of the other one's path was low (i.e. less than 1% of
197 the experiment duration), and there were no differences between the sexes in this trait (n = 27, mean of
198 the proportion of time a female is on top of a male's path [95% HDI] = 0.009 [0.007, 0.011]; a male is
199 on top of a female's path = 0.006 [0.005, 0.008]); (Fig. 2A).

200

201 Although we did not observe trail-following for either sex, we discovered other behaviours. First,
202 individuals were randomly encountering each other, which we describe as a contact. We noted which
203 sex was initiating the contact (i.e., the individual colliding into the other). Second, individuals followed
204 each other after these collisions (see Movie 1 and Fig. 2B). Half of the couples (n = 15 of 27) collided
205 at least once. In total, 24 contacts were recorded, with 1.6 contacts on average per couple (from 1 to 4
206 contacts in one trial). Half of the contacts led to males moving together in tandem with females while
207 keeping physical contact. This behaviour was more often observed when the male had initiated the
208 contact (Fig. 2B). Females never expressed this behaviour, and when they moved away from the male,
209 they stopped and then started moving in another direction.

210

211 Lastly, we found no sex nor experiment-specific differences in activity level as measured by cumulative
212 distance travelled (Fig. S1).

213

214 DISCUSSION

215

216 *Signal type: waterborne (diffusing) vs deposited trail cues*

217 Our results confirm previous work (Bartel and Hohberg, 2020) that diffusing waterborne signals are
218 used in tardigrade intraspecific chemical communication in a reproductive context. However, we show,

219 for the first time, sex-differences in this context (see next section). We found no evidence that
220 tardigrades can detect sex-specific cues deposited on the agar surface. The latter contrasts with what
221 was found in a predator-prey deposited cue detection context (Meyer *et al.*, 2020). However, that study
222 differs from ours not only in context but also substantially in methodology: they used much larger taxa,
223 with multiple individuals as signallers and focals, longer times to deposit cues on a substrate, and non-
224 simultaneous response. Therefore, we cannot separate two potential explanations for our results: a lack
225 of sex-specific signal or an inability to detect cues from a single signaller. Our observation of the
226 interactions between individuals suggests that, in the absence of water, the detection of conspecifics is
227 compromised. Only in half of the trials did the individuals interact, and of those, only one-third did so
228 more than once (Fig. 2B). In addition, we did not observe any instances of trail-matching behaviour by
229 either sex as assessed by the very rare (1%) spatial overlap of trails (Fig. 2A). Therefore, physical
230 encounters appear to be random in the absence of water.

231

232 However, the behaviour observed after these physical encounters is insightful: only males followed
233 females and mostly by maintaining physical contact (see Movie 1). In their natural habitat, the encounter
234 rate can be quite variable: in another *Macrobiotus* species, the number of sexually mature females
235 showed a seasonal 7-fold change (Schuster and Greven, 2013). Therefore, males might have evolved
236 this following behaviour to improve their mating opportunities. Furthermore, this behaviour might also
237 be linked to the potential assessment of female receptivity and/or mating status using cues only
238 detectable in close range. For example, it is well established that cuticular hydrocarbons (CHCs), in
239 addition to their contribution to desiccation resistance (Hadley, 1981), have a key role in mate choice
240 in the sister phylum to Tardigrada – Arthropoda (reviewed in Ingleby, 2015). Our current knowledge
241 on tardigrade cuticle is mostly limited to interspecific morphological differences and their potential role
242 in anhydrobiotic ability (reviewed in Czerneková and Vinopal, 2021). Unfortunately, the presence of
243 CHCs in tardigrades (and their potential role in reproduction) remains unknown.

244

245 In many polyandrous species, males locate and establish a permanent association with immature
246 females, i.e., pre-copulatory mate guarding (Parker, 1974). For example, male beetles are known to
247 mount females for several hours during the day to defend the females from intruding males (Arakaki *et*
248 *al.*, 2004). Similarly, pre-copulatory mate guarding could explain our own observation of the males'
249 following behaviour. However, in our experiments, females were ready-to-mate (i.e., eggs are visible
250 in the ovary; see Poprawa, Schlechte-Wehnicz and Hyra, 2015), and not immature, as is usually the case
251 in pre-copulatory mate guarding.

252

253 The biology of our study system can also explain the differences seen in cue type responses. Tardigrades
254 need a thin layer of water to be active, but mating in eutardigrades that lay free eggs requires that sperm
255 be released into the environment, which then swim towards the female cloaca (reviewed in Sugiura and

256 Matsumoto, 2021b). Thus, mating requires water to be present between individuals rather than just
257 covering the cuticle. Therefore, it would not be surprising if pre-mating reproductive behaviours, such
258 as sex discrimination or mate choice, are mediated by waterborne chemical cues and not by following
259 a deposited trail cue. In fact, trail following is a widespread feature of chemical communication in
260 aquatic environments, from crustaceans to fish (reviewed in Kamio, Yambe and Fusetani, 2022).

261

262 *Sex differences in response to waterborne signal*

263 In accordance with previous mating observations (reviewed in Sugiura and Matsumoto, 2021b), our
264 data quantitatively support male-biased mate-searching and mating initiation behaviours. When
265 waterborne cues were available, males and females had a similar latency to approach one of the
266 signallers (Fig. 1B), irrespective of the sex of the signaller (Fig. 1B). However, males spent significantly
267 more time next to female signallers, whereas females showed no sex-based preference (Fig. 1C). These
268 results suggest that tardigrades can discriminate between the sexes, with males preferentially
269 associating with females.

270

271 There are many examples in the literature of males being attracted to opposite-sex chemical cues
272 (Gomez-Diaz and Benton, 2013). In contrast, much less is known about the converse female response
273 (Karlsson Green and Madjidian, 2011; Hare and Simmons, 2019). The latter might be a consequence
274 of the focus on species with sexually dimorphic sensory apparatus (Naka, 2018), which is not the case
275 in tardigrades (Møbjerg *et al.*, 2018). In taxa without obvious sensory sexual dimorphism, both
276 attraction (e.g. Passos *et al.*, 2013) and insensitivity to male chemical signals were observed (e.g.
277 Ratterman, Rosenthal and Jones, 2009). This could be due to a lower female than male sensitivity to
278 pheromones. Invertebrates, including insects and crustaceans, have specialised chemosensory organs,
279 such as antennae, that detect environmental chemicals (Su, Menuz and Carlson, 2009). For example,
280 male moths and beetles have larger and more complex antennae than females, which allow them to
281 detect pheromones from farther away and with greater precision (Jourdan *et al.*, 1995; Symonds,
282 Johnson and Elgar, 2012). These differences in chemical sensory organs may be related to differences
283 in reproductive behaviour and communication between the sexes in invertebrates. Unfortunately, most
284 studies focus on female pheromones and male responsiveness to them (Doall *et al.*, 1998; Marco *et al.*,
285 1998; Winfrey and Fincke, 2017; Stamps and Shaw, 2019; Liu *et al.*, 2022). Thus, the biases towards
286 research on males and conspicuous visual ornaments has left a blind spot regarding female olfactory
287 ornaments (Hare and Simmons, 2019).

288

289 Another non-exclusive explanation could be that females do not gain advantages from responding to
290 chemical cues released by males but are equally attracted to both sexes via aggregating pheromones.
291 These long-range pheromones are emitted by and attractive to both sexes (Wertheim *et al.*, 2005).
292 Individuals may aggregate for the benefit of living in a group, for example by avoiding predation (Raveh

293 *et al.*, 2019), or aiding feeding (Tanaka, Frommen and Kohda, 2018) and reproduction (Roff *et al.*,
294 2017). They are also essential for internally fertilising aquatic animals (Wyatt, 2014), and tardigrades
295 could rely on them to find mates. For example, barnacle cyprids, the mobile larval form of barnacle,
296 release a pheromone that attracts other cyprids to the location. This behaviour is thought to be an
297 adaptation that increases the chances of successful settlement by bringing together many individuals in
298 a small area. This pheromone also plays an essential role in the reproductive process by helping to
299 gather conspecific cyprids in the same location to increase the future chances of fertilisation
300 (Matsumura, Nagano and Fusetani, 1998).

301

302 *Conclusion*

303 This study provides the first step in understanding intersexual communication in tardigrades by
304 comparing distinct types of chemical signals, and the behavioural response of both sexes to them.
305 Tardigrades were able to detect conspecifics using waterborne signals but did not respond to deposited
306 trail cues. Moreover, females and males behaved differently: females showed no sex-based preferences
307 (if any), whereas males discriminated sex through waterborne signals, showing a strong preference to
308 associate with females. Furthermore, in the absence of waterborne cues, males (but not females) can
309 follow opposite-sex individuals, but only while maintaining direct body contact. Our results align with
310 the very limited literature about reproductive behaviour, especially regarding the pre-copulatory stage,
311 in tardigrades. Future work should focus on identifying the chemical compounds used in intersexual
312 communication in this understudied phylum.

313

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316

317 COMPETING INTERESTS

318 We have no competing interests.

319

320 DATA AVAILABILITY

321 The datasets and the annotated R script of the analyses output for this study will be made available on
322 Dryad as respectively, two .csv files and a RMarkdown file (DOI:tbd).

323

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326

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509 FIGURES

510

511 Figure 1: A. Photograph of the arena (choice-chamber) setup from above (total size
512 21.6×11.4 mm, lure rings chambers 4 mm inner diameter). Dashed line: critical area. B. Latency to
513 first cross a side for focal females (n = 16) and males (n = 17); five individuals of each sex (grey) did
514 not spend time inside a critical area (non-crosser). C. Proportion of time spent inside each critical area
515 (female n = 11, male n = 12, non-crossers are excluded). Black dots represent means of posterior
516 distributions, whereas vertical lines represent the 95% high-density intervals.

517

518 Figure 2: A. Proportion of time when paths of both individuals overlapped (n = 27 trials). Black dots
519 represent means of posterior distributions, whereas vertical lines represent 95% high-density intervals.
520 Grey lines connect the two individuals used in the same trial. B. Following behaviour observed
521 according to the individual who initiated a contact during the trail experiment (n = 24 recorded
522 contacts). Only males showed this behaviour. The colour refers to the sex that initiate the contact.