

RESEARCH ARTICLE

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

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ABSTRACT

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

KEY WORDS: Chemical communication, Waterborne signal, Deposited cue, Semiochemicals, Sex discrimination

INTRODUCTION

Semiochemical communication is widespread and used by animals across multiple contexts, from finding food to attracting mates (Wyatt, 2014). Behavioural responses to conspecific chemical cues, and specifically sex discrimination, have been investigated in a wide range of taxa, both aquatic and terrestrial (Dunham and Oh, 1992; Bouchard, 2001; Cooper and Pérez-Mellado, 2002; Park et al., 2004; Hutter et al., 2011; Stamps and Shaw, 2019; Kudo et al., 2022). Importantly, sexually mature animals that fail to discriminate between the sexes and/or species incur a reproductive cost (Burdfield-Steel and Shuker, 2011; Lerch and Servedio, 2022). To find and/or attract a mate, animals can use two main types of chemical signals: (i) diffusing signals and/or (ii) trail deposited on the substrate. For instance, male copepods can find females using pheromone plumes that receptive females produce as trails or clouds (Bagøien and Kiørboe, 2005; Kiørboe et al., 2005). Similarly, both male and female apple snails can follow opposite-sex trails, and, in

addition, males are attracted to waterborne sex pheromones of females (Takeichi et al., 2007).


Most studies examining the role of pheromones in sexual selection have focused on diffusing chemical signals used in long-range mate attraction and mate recognition (Johansson and Jones, 2007). Furthermore, there is a strong taxonomic bias towards insects and mammals (Brennan and Keverne, 2004; De Pasqual et al., 2021), and a focus on organisms with obvious male bias sexual dimorphism sensory organs and searching behaviour (Naka, 2018). However, such knowledge is non-existent or very limited for several animal groups, particularly micrometazoans such as the phylum Tardigrada. Tardigrades are a microscopic animal phylum categorized in the superclade Panarthropoda (Yoshida et al., 2017; Jørgensen et al., 2018) yet are largely neglected in behavioural ecology and evolutionary research. Even though individuals must be surrounded by water to be active, they colonised terrestrial environments, such as mosses and lichens, thanks to their cryptobiotic adaptations (Møbjerg and Neves, 2021). They have relatively simple sensory organs: some species present light-sensitive eyes, but all have body segments containing cells with characteristics of mechanoreceptors and chemoreceptors (Møbjerg et al., 2018). Sexual dimorphism is mainly limited to a female bias in body size, with very few reported examples of secondary sexual characters (Gąsiorek et al., 2019, 2021) and parental care (Pilato et al., 2006). Of the ca. 1500 tardigrade species described so far (Degma et al., 2023), mating behaviour has been described for only 10 species (Sugiura and Matsumoto, 2021b), with only one showing secondary sexual dimorphism. Moreover, detailed records of both pre-mating and mating phases have been described for only four species, of which none show secondary sexual characters (Bingemer et al., 2016; Sugiura et al., 2019; Bartel and Hohberg, 2020; Sugiura and Matsumoto, 2021a). Together, these studies suggest a crucial role of semiochemicals in intraspecific sexual contexts (i.e. mate attraction and sperm transfer) through diffusing cues. In addition, a recent study showed that tardigrades could detect deposited chemical cues (trail) in interspecific (i.e. predator–prey) contexts (Meyer et al., 2020). In that study, prey avoided areas previously occupied by predators while the latter preferred the areas occupied by prey.

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

Using the gonochoristic tardigrade *Macrobiotus polonicus* (Pilato et al., 2003), we tested the role of both waterborne diffusing signals and deposited cue trails (in water-free

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environments) in sex discrimination and mate searching behaviours of both females and males. As our study system shows limited sexual dimorphism in sensory morphology and ecology, we hypothesised that (i) both females and males would preferentially associate with the opposite sex, and that (ii) both sexes could detect and follow an opposite-sex deposited-trail cue.

MATERIALS AND METHODS

Culture

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

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

All individuals used for this study were virgins. Virgin individuals were obtained by isolating eggs and then rearing hatchlings individually in 3 cm diameter plastic Petri dishes, kept in the same conditions as the main cultures. We determined the sexual maturity of individuals by observing motile sperm and mature oocytes in the male and female gonad, respectively. Each trial was conducted with individuals from the same strain (experiment 1: $n=23$ trials with AT.002 and $n=10$ with IT S218; experiment 2: $n=16$ trials with AT.002 and $n=11$ with IT S218).

Experiment 1: waterborne (diffusing) cues

We used 33 focal individuals (16 females and 17 males) to test whether tardigrades could differentiate the sexes using diffusing signals. We created a choice chamber arena (Fig. 1A) consisting of a 4-well slide (Ibidi, Gräfelfing, Germany) and two fishing lure rings (4 mm inner diameter). The arena surface was coated with 250 μ l of 1.5% agar (BD BACTO™ Agar) with the placement of the lure rings following a printed scheme in which their edges were at an 8 mm distance from each other, and each was 4 mm from the centre of the arena. When the agar was solidified, we added 1 ml of mineral water to flood the arena so chemical cues could diffuse within the chambers. This setup allowed us to provide the focal individual only with diffused chemical cues from the signallers as they could neither see nor touch them. The signallers were able to move freely within the lure area. Some of them were used twice for two different focal individuals (one of each sex), with at least 12 h of interval between the two trials (i.e. the next day). Different arenas were used for every trial conducted on the same day, and all arenas were thoroughly cleaned at the end of each day.

We placed the signallers inside the lure rings in darkness (swapping female and male sides between trials) and let them habituate for 30 min. Afterwards, we added the focal individual in the centre of the arena and filmed its behaviour for 30 min.

A stereomicroscope with a diffused bottom light linked to a camera (and the S-EYE software) was used. Position tracking was done automatically (see 'Video analysis', below). The preferential association was assessed as latency to cross, and time spent within the critical area of the opposite sex signaller.

Experiment 2: trail-deposited cues

Individuals used in the first experiment were randomly grouped in mixed-sex pairs (both focals and signallers). We used 27 couples to test whether tardigrades could follow a trail-deposited cue. The individuals were placed in the same type of Ibidi chamber slides as in experiment 1, but without water and the fishing lure rings. An ultra-thin continuous water film coated agar and the tardigrades, which allowed them to move freely in the arena but limited any chemical cue to the tardigrades' trails. We used the same microscope and camera setup as in experiment 1. After starting the recording, we placed the first individual and waited (ca. 5 min) for it to move. Then, we added the second individual and continued recording for 30 min. The order of individuals (male versus female) was alternated between trials. Position data for both individuals were obtained automatically (see 'Video analysis', below). In addition, we documented in detail the number and type of all observed interactions (i.e. bumps and following behaviour).

Video analysis

To obtain the position of the focal individuals during each trial during both experiments, we analysed the videos in R v.4.2.0 with the function 'trackR' from the package 'trackR' (<https://swarm-lab.github.io/trackR/>). The tracking was checked, and tracking position errors fixed with the function 'trackFixer' of the same package.

To determine whether the focal individuals were inside a critical area (dashed lines, Fig. 1A) for the waterborne-diffused cues, we applied a distance-to-centre function to the output (xy coordinates) given by trackR. For the trail-deposited cue experiment, we calculated the distance between individuals' paths every 3 s. Only those that differed by less than one body length distance from each other were considered to overlap (i.e. perpendicular overlap was excluded). We then assessed the proportion of time the paths of both animals overlapped.

Statistical analyses

All statistical analyses were conducted in R v.4.2.0 (<http://www.R-project.org/>). We ran generalised linear models using the 'brm' function from the 'brms' package v.2.17.5 (Bürkner, 2017).

In the diffusing signal experiment, for the latency to cross the first critical area, the predictor was the sex of the focal individual, whereas, for the proportion of time spent inside a critical area, the predictor was included as four types of dyad interactions (focal–signallers): female–female, female–male, male–female and male–male. The ID of the focal individual was included as a random variable. The focals that did not cross any critical area were removed from the analysis in both models. To test whether the path of one sex overlapped more often with the path of the other, we used sex as a predictor of the proportion of time an individual was on top of the other one's trace. We included trial ID as a fixed effect for the trail data as the couples could interact. To assess whether the absence of water affected motility, we compared the distance travelled in both experiments, where the focal individual's sex was the predictor. We used default priors for each response distribution type: binomial (logit link) distribution for the proportion of time spent inside each critical area and the proportion of time an individual was on top of

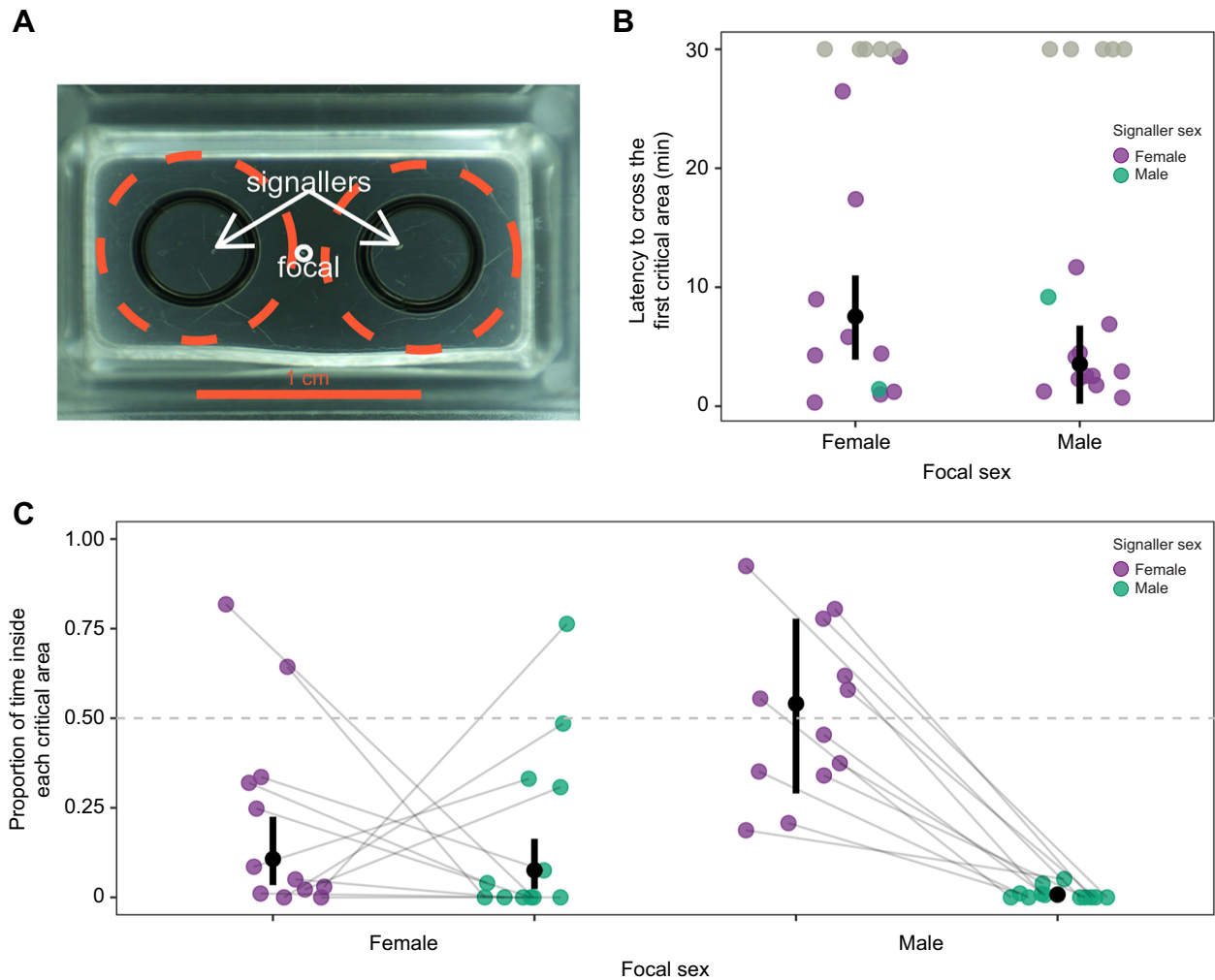


Fig. 1. Experiment 1: waterborne (diffusing) cues. (A) Photograph of the arena (choice chamber) setup from above (total size 21.6×11.4 mm, lure ring chambers 4 mm inner diameter). Dashed lines indicate the critical area. (B) Latency to first cross a side for focal females ($n=16$) and males ($n=17$); five individuals of each sex (grey) did not spend time inside a critical area (non-crosser). (C) Proportion of time spent inside each critical area ($n=11$ females, $n=12$ males, non-crossers are excluded). Black circles represent means of posterior distributions, whereas vertical lines represent the 95% high-density interval (HDI).

another one's path, Gaussian for the latency to cross the first critical area, and the distance travelled. The results are presented as back-transformed Bayesian posterior means and 95% high-density interval (HDI) using the 'mean_hdi' function from the 'tidybayes' package v.3.0.2 (available from Zenodo: <https://zenodo.org/record/7933163>), computed from $n=4$ well-converging and uncorrelated chains with $n=60,000$, 100,000 and 15,000 saved iterations, respectively.

RESULTS

Experiment 1: waterborne (diffusing) cues

In total, 10 individuals (5 females and 5 males) did not cross any critical area, and their latency to choose was recorded as 30 min (i.e. the maximum duration of the trials; Fig. 1B). The first choice in both sexes was biased towards female signallers except for one focal individual of each sex (Fig. 1B). The latency to first cross a critical area was not significantly associated with the sex of the focal individual ($n=23$, Bayesian mean estimate [95% HDI]: females latency=7.54 min [3.91, 10.99 min]; males latency=3.54 min [0.20, 6.77 min]) (Fig. 1B).

Females spent a very short but similar time next to either sex, whereas males showed a strong attraction to female signallers

(female $n=12$, mean of the proportion of time spent next to female [95% HDI]=0.107 [0.034, 0.22]; next to male=0.08 [0.023, 0.164]; males $n=12$, mean of the proportion of time spent next to females [95% HDI]=0.540 [0.290, 0.778]; next to males=0.008 [0.002, 0.018]) (Fig. 1C).

Experiment 2: trail-deposited cues

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

Although we did not observe trail following for either sex, we discovered other behaviours. First, individuals were randomly encountering each other, which we describe as a contact. We noted which sex was initiating the contact (i.e. the individual colliding into the other). Second, individuals followed each other after these collisions (see Movie 1 and Fig. 2B). Half of the couples ($n=15$ of 27) collided at least once. In total, 24 contacts were recorded, with 1.6 contacts on average per couple (from 1 to 4 contacts in one trial).

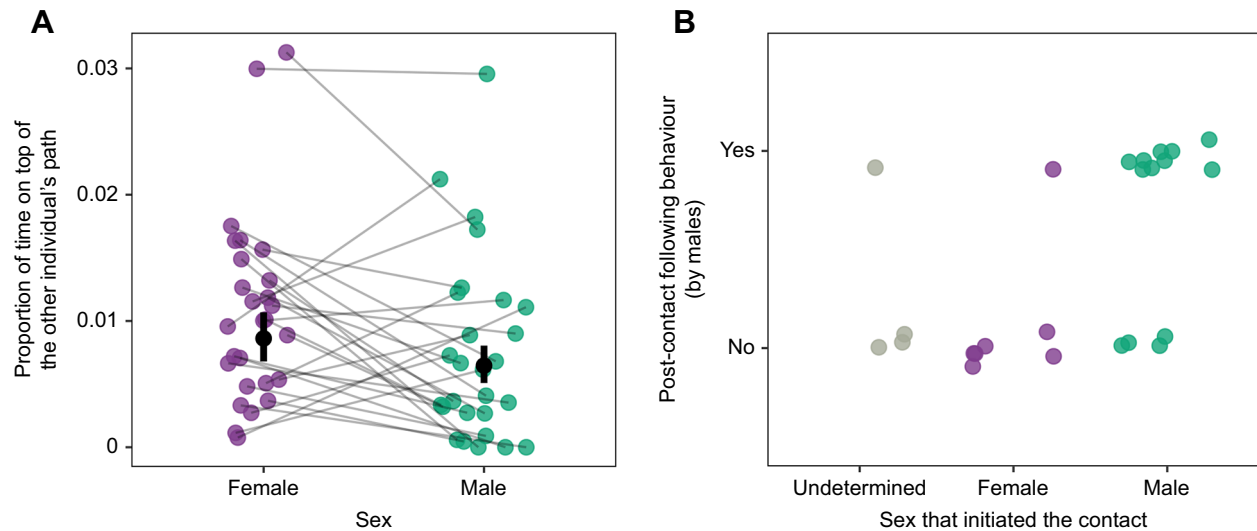


Fig. 2. Experiment 2: trail-deposited cues. (A) Proportion of time when the paths of the two individuals overlapped ($n=27$ trials). Black circles represent means of posterior distributions, whereas vertical lines represent the 95% HDI. Grey lines connect the two individuals used in the same trial. (B) Following behaviour observed according to the individual that initiated a contact during the trail experiment ($n=24$ recorded contacts). Only males showed this behaviour. The colour refers to the sex that initiated the contact.

Half of the contacts led to males moving together in tandem with females while maintaining physical contact. This behaviour was more often observed when the male had initiated the contact (Fig. 2B). Females never expressed this behaviour, and when they moved away from the male, they stopped and then started moving in another direction.

Lastly, we found no sex- or experiment-specific differences in activity level as measured by cumulative distance travelled (Fig. S1).

DISCUSSION

Signal type: waterborne (diffusing) versus deposited trail cues

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

However, the behaviour observed after these physical encounters is insightful: only males followed females and mostly by maintaining physical contact (see Movie 1). In their natural habitat, the encounter rate can be quite variable: in another

Macrobiotus species, the number of sexually mature females showed a seasonal 7-fold change (Schuster and Greven, 2013). Therefore, males might have evolved this following behaviour to improve their mating opportunities. Furthermore, this behaviour might also be linked to the potential assessment of female receptivity and/or mating status using cues only detectable in close range. For example, it is well established that cuticular hydrocarbons (CHCs), in addition to their contribution to desiccation resistance (Hadley, 1981), have a key role in mate choice in the sister phylum to Tardigrada – Arthropoda (reviewed in Ingleby, 2015). Our current knowledge on tardigrade cuticle is mostly limited to interspecific morphological differences and their potential role in anhydrobiotic ability (reviewed in Czerneková and Vinopal, 2021). Unfortunately, the presence of CHCs in tardigrades (and their potential role in reproduction) remains unknown.

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

The biology of our study system can also explain the differences seen in cue-type responses. Tardigrades need a thin layer of water to be active, but mating in eutardigrades that lay free eggs requires that sperm be released into the environment, which then swim towards the female cloaca (reviewed in Sugiura and Matsumoto, 2021b). Thus, mating requires water to be present between individuals rather than just covering the cuticle. Therefore, it would not be surprising if pre-mating reproductive behaviours, such as sex discrimination or mate choice, are mediated by waterborne chemical cues and not by following a deposited trail cue. In fact, trail following is a widespread feature of chemical communication in aquatic environments, from crustaceans to fish (reviewed in Kamio et al., 2022).

Sex differences in response to waterborne signal

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

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

Another non-exclusive explanation could be that females do not gain advantages from responding to chemical cues released by males but are equally attracted to both sexes via aggregating pheromones. These long-range pheromones are emitted by and attractive to both sexes (Wertheim et al., 2005). Individuals may aggregate for the benefit of living in a group; for example, by avoiding predation (Raveh et al., 2019), or aiding feeding (Tanaka et al., 2018) and reproduction (Roff et al., 2017). Aggregating pheromones are also essential for internally fertilising aquatic animals (Wyatt, 2014), and tardigrades could rely on them to find mates. For example, barnacle cyprids, the mobile larval form of barnacle, release a pheromone that attracts other cyprids to the location. This behaviour is thought to be an adaptation that increases the chances of successful settlement by bringing together many individuals in a small area. This pheromone also plays an essential role in the reproductive process by helping to gather conspecific cyprids in the same location to increase the future chances of fertilisation (Matsumura et al., 1998).

Conclusion

This study provides the first step in understanding intersexual communication in tardigrades by comparing distinct types of chemical signals, and the behavioural response of both sexes to them. Tardigrades were able to detect conspecifics using waterborne signals but did not respond to deposited trail cues. Moreover, females and males behaved differently: females showed no

sex-based preferences (if any), whereas males discriminated sex through waterborne signals, showing a strong preference to associate with females. Furthermore, in the absence of waterborne cues, males (but not females) can follow opposite-sex individuals, but only while maintaining direct body contact. Our results align with the very limited literature about reproductive behaviour, especially regarding the pre-copulatory stage, in tardigrades. Future work should focus on identifying the chemical compounds used in intersexual communication in this understudied phylum.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.C., S.C.; Methodology: J.C., S.C., Ł.M.; Validation: J.C.; Formal analysis: J.C.; Investigation: J.C.; Resources: Ł.M., S.C.; Data curation: J.C.; Writing - original draft: J.C., S.C.; Writing - review & editing: J.C., K.E.K., Ł.M., S.C.; Visualization: J.C.; Supervision: K.E.K., Ł.M., S.C.; Project administration: S.C.; Funding acquisition: S.C.

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Data availability

R code for the statistical analysis and the data related to it from this study are openly available in JYX: <https://doi.org/10.17011/jyx/dataset/88887>.

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