



Experimental evidence for a female sex pheromone in *Arrenurus manubriator* (Acari: Hydrachnida; Arrenuridae)

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Abstract. We present experimental evidence for a water-borne female-produced sex pheromone in aquatic parasitengonine mites. Water that has contained adult female *Arrenurus manubriator* Marshall will elicit arrestant behaviour in conspecific adult males, and if the cue is sufficiently strong, the males will assume a readiness posture (with 4th pair of legs held over the back, bent anteromedially at the genotibial joint) that is typically a precursor to coupling. Water that has not been exposed to female mites does not induce any behavioural response from male mites. Female-conditioned water that has been passed through a C-18 column does not elicit any response from male *A. manubriator*, while the rehydrated residue from the column does induce arrestant behaviour and may result in the readiness posture. The results from the C-18 extraction indicate that the pheromone is nonpolar in nature.

Introduction

Pheromone communication is presumably widespread among the Acari. Based on the review by Sonenshine (1985) and surveying the literature since then, there are many references for certain distantly-related groups such as ticks, spider mites, and several families of astigmatid mites but very few studies of chemical communication in other mite taxa. Several authors have inferred from behavioural observations that parasitengonine mites probably use sex pheromones. Males of various terrestrial and aquatic genera are reported to only produce spermatophores, or to increase production of spermatophores, following contact with a female or when placed in water that has recently housed females (Proctor 1991, 1992a; Witte 1984, 1991; Wohltmann 1996). Males of some *Neumania* spp. (Unionicolidae) rapidly fan their legs while remaining stationary over recently-deposited spermatophores, which is interpreted to be an adaptation for dispersing their pheromones (Proctor 1991, 1992b). Female mites have been observed to change direction and swim towards spermatophores when passing within 12–20 mm (Witte 1984, 1991).

In most cases, inferences about sex pheromone use among aquatic Parasitengona have been based upon casual observations and not controlled experiments. One exception is Baker (1996), who housed male *Arrenurus acutus* Marshall in

Table 1. Reaction of individual male *Arrenurus manubriator* when exposed to distilled water and female-conditioned water. In trials 1–3 the distilled water was presented first, in trials 4–6 the female-conditioned water was presented first.

Trial	Number out of 10 males responding to distilled water	Number out of 10 males responding to female-conditioned water	Sign Test
1	0	8	P<0.05
2	0	8	P<0.05
3	0	10	P<0.05
4	0	10	P<0.05
5	0	8	P<0.05
6	1	10	P<0.05

one of 4 chambers, with each chamber separated from the females by a water space. He recorded that females stayed closest to the chamber that contained males, with a declining trend as the separation increased to a limit of 3 cm of water. Proctor (1992a) also provided quantitative evidence that spermatophore deposition by male *Limnesia undulata* Müller (Limnesiidae) increased when females were present.

While maintaining laboratory colonies of *Arrenurus manubriator*, separated by gender, we noticed that males would stop swimming and orient towards a pipette used previously in the females' container. An arrestant response to sex pheromones has been previously reported in phytoseiid and tetranychid mites (Sonenshine 1985). Many males were in a readiness posture (legs IV closely pressed to the dorsum, bent anteromedially at the genuotibial joint) which is a preliminary step in mating behaviour of many *Arrenurus* spp. (Böttger 1962; Lundblad 1929; Proctor and Smith 1994; Proctor and Wilkinson 2001). These observations prompted our hypothesis that males were responding to some water-borne substance that was secreted by females. The purpose of our study was to demonstrate the presence of a female-emitted chemical cue that can be extracted from female-conditioned water and elicits mating behaviour in males.

Material and methods

Adult *A. manubriator* used in this study were from a laboratory colony established from inseminated females collected from Lake Opinicon (located approximately 50 km north-east of Kingston, Ontario, Canada). Larvae of this species forego a parasitic association and do not feed (Smith 1998), hence this species is relatively easy to culture. Deutonymphs and adults were fed a surplus of living *Cypris pubera* Müller ostracods from laboratory colonies. Within two days after transformation to adults, mites were sorted by gender into two separate containers and maintained in

Table 2. Reaction of individual male *Arrenurus manubriator* when exposed to female-conditioned water passed through a C-18 column and exposed to untreated female-conditioned water. In trials 1 and 2 the column-passed water was presented first, in trials 3 and 4 the untreated female-conditioned water was presented first.

Trial	Number out of 10 males responding to column-passed female-conditioned water	Number out of 10 males responding to female-conditioned water	Sign Test
1	0	8	P<0.05
2	0	8	P<0.05
3	1	10	P<0.05
4	1	4	n.s.

Table 3. Reaction of individual male *Arrenurus manubriator* when exposed to female-conditioned water passed through a C-18 column and exposed to the reconstituted residue from the C-18 column. In trials 1 and 2 the column-passed water was presented first, in trials 3 and 4 the C-18 residue was presented first.

Trial	Number out of 10 males responding to column-passed female-conditioned water	Number out of 10 males responding to the residue from the C-18 column	Sign Test
1	0	9	P<0.05
2	0	9	P<0.05
3	1	9	P<0.05
4	0	10	P<0.05

isolation at room temperature. Adults used in the experiments were a mixture of ages varying from 3–8 m.

Glass-distilled water was used for all experiments. Female-conditioned water was produced by storing 250 rinsed female *A. manubriator* in a new glass beaker containing 250 ml of water for 24 hr at room temperature (approximately 21 °C). Females were not fed during this period, to exclude any kairomones that may be emitted by food organisms. Distilled water used as a control in the first experiment was also stored in a new glass beaker for 24 hr at room temperature. Control and female-conditioned water were used in experiments immediately after the 24 hr period.

Experiments 2 and 3 involved extraction using a 5 ml Bond Elut C-18 cartridge. The column was pre-wetted by passing 5 ml of 100% methanol through it, then it was rinsed with 5 ml of distilled water. 150 ml of female-conditioned water was passed through the column, followed by 3 ml of distilled water, and the water remaining after extraction was used as one treatment (column-passed). Chemicals remaining in the column were eluted with 1 ml of 100% methanol, this fraction was evaporated under vacuum and chilling using a SpeedVac, and then dissolved in 150

ml of distilled water. This reconstituted portion was used as another treatment (residue from the C-18 column).

Males were tested individually in plastic petri dishes (55 mm diameter, 15 mm high) filled with water. One ml. of water to be tested was introduced to the centre of the petri dish using a 1 ml plastic syringe with a 20-gauge needle, injected at a rate of approximately 1 ml/5 s. The male was observed during and immediately after the introduction of water, and arrestant behaviour with or without the readiness posture was considered a positive reaction whereas continued swimming was considered a negative reaction. Each male was tested twice: first with one treatment and then, after a 5 min. delay, with the other treatment. Ten males were used in each trial of each experiment, and the order of presentation (control vs. experimental treatment) was alternated between each trial.

In experiment 1 we were testing whether males responded to some substance in female-conditioned water or simply to the introduction of distilled water into the container. Three replicates of each order of presentation were completed, a total of 6 trials involving 60 male *A. manubriator*. In experiment 2 we tested whether column-passed female-conditioned water would elicit a response from male *A. manubriator*, with female-conditioned water that had not been passed through the column serving as a positive control. Two trials in each order of presentation were conducted (4 trials in total, 40 male mites). Finally, in experiment 3 we tested whether the residue from the C-18 column would elicit a response from males, using the column-passed female-conditioned water as a negative control. Again, we ran two trials in each order of presentation (4 trials in total, 40 male mites). A sign-test of association was used for analysis of results with $P < 0.05$ as the critical value for acceptance.

Results

When males responded to a potential pheromonal cue they would typically stop swimming and either sit or crawl slowly towards the tip of the syringe needle; in about 25% of the cases, males also displayed the readiness posture. Several males attempted to crawl into the tip of the needle. Males also often exhibited a very rapid short stroke fanning of legs 4, almost appearing to be trembling. If arrested males were disturbed by water currents or contact they would escalate their response to either leg fanning or the readiness posture, and would also frequently turn their posterior towards the stimulus. The few males that did not respond to any treatments were often those that were repeatedly caught on the surface tension.

In experiment 1, 54 of 60 male *A. manubriator* exhibited an arrestant response to female-conditioned water, while one individual in one trial responded to both sources of water, and none responded only to distilled water (Table 1). Differences between treatments in all 6 trials were statistically significant. Order of presentation did not appear to have an effect, although the one individual who responded to both stimuli had been first exposed to female-conditioned water.

Generally, column-passed female-conditioned water did not elicit a response from males while female-conditioned water that had not been extracted did result in a reaction (experiment 2; Table 2). In total, 30 of 40 males responded to female-conditioned water, of which only two individuals (trials 3 and 4, Table 2) responded to both stimuli. In both of these cases, the unextracted female-conditioned water had been presented first. Except for trial 4, the differences between treatments were statistically significant. The reconstituted residue eluted from the C-18 column induced arrestant behaviour in 37 of 40 males, while only one male responded to the column-passed female-conditioned water and that individual reacted to both treatments (experiment 3; Table 3). The male in question was in trial 3, in which the residue from the C-18 column had been presented first. Differences between treatments in all 4 trials were statistically significant.

Discussion

The behaviour of male *A. manubriator* was quite consistent in these experiments. Few males failed to respond to any treatment, and the few males that reacted to both had first received the water source that was believed to contain the pheromone. Most likely, the effects of the pheromone had not worn off for the last-mentioned males. Böttger (1962) observed that male *Arrenurus globator* (Müller) would exhibit leg-crooking or present the end of the cauda towards the source when contacted by female conspecifics, other mite species, and even when ostracods and copepods passed nearby. Presumably the pheromonal cue is used for releasing mating behaviour in males and that other stimuli, such as water currents or physical contact, can contribute to their excitation (see also Proctor and Smith "1994").

Two categories of acarine sex pheromones defined by Sonenshine (1985) were arrestant and attractant sex pheromones; *Arrenurus manubriator* in these experiments reacted both ways. While the simplest conclusion is that there is a single pheromone that induces both responses, it is also possible that more than one pheromone is involved. Extraction with the C-18 column infers that the chemical(s) involved would be non-polar in character. We have been unable thus far to extract sufficient quantities to produce measurable peaks with mass spectroscopy.

Our study demonstrates the presence of a female-emitted pheromone. Baker (1996) presented evidence that at least some male *Arrenurus* spp. also produce pheromones. Böttger (1962) questioned whether 3 of the 4 pairs of dorsal glands on the cauda of male *A. globator* were secreting chemicals detected by the females. To test this possibility he coated dead males with paraffin, to seal shut the glandularia, but females would still mount these dead males; either the males were not producing a pheromone or the wax was an ineffective block (Proctor and Smith 1994). The rapid short stroke fanning of leg 4 that we observed in *A. manubriator* and that Lundblad (1929) noted for *A. globator* could coincide with release of pheromones by the male, and serve to disperse pheromones much as has been suggested for the leg fanning behaviour of many *Neumania* spp. (Proctor 1991, 1992b;

Proctor and Wilkinson 2001). Alternatively, leg fanning in *A. manubriator* could be an adaptation for increasing water flow across sensory receptors for better reception of female-emitted pheromones.

Many questions still remain regarding pheromone communication in *A. manubriator* and *Arrenurus* species in general. First, chemical(s) involved need to be identified. It is not clear whether these signals are long-lasting in the environment, nor whether production is continuous or varies with a female's mating status, nutrition, age, temperature, etc. It also remains to be seen whether use of arrestant/attractant sex pheromones is widespread among *Arrenurus* species or is restricted to certain species groups. Species that forego larval parasitism, such as *A. manubriator*, make it feasible to maintain laboratory colonies and to further test hypotheses regarding mating behaviour and pheromone communication in aquatic mites.

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