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Definitive evidence for cuticular pheromones in a cricket

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Abstract. The Orthoptera include many species established as important model systems in the study of animal behaviour, particularly in relation to communication and mating systems. Although most interest has focused on auditory communication, increasing circumstantial evidence suggests that there may be a widespread additional communication channel in the form of cuticular contact pheromones. Using the field cricket, *Gryllus bimaculatus*, we conducted a behavioural assay which demonstrated that males can distinguish the sex of conspecifics using such a channel. Male response to females (courtship song) was completely abolished by using an organic solvent to remove cuticular hydrocarbons and associated compounds from a stimulus female. It was subsequently restored by painting the washed female with the dissolved extract. This technique controls for the possibility, inherent in previous tests, that the lack of response to washed body parts might be due to the washing process itself. The composition of the cuticles of males and females was analysed using gas chromatography. This revealed that the two sexes differ markedly in the quantities of the majority of the compounds found in the cuticular extract that had previously been shown to be used in mate recognition. This suggests that mate recognition is likely to be due to the relative concentrations of several cuticular compounds, rather than a single 'sex pheromone'. It supports previous assertions of the existence of contact pheromones in the Orthoptera, suggesting that they may be widespread in this group.

The field cricket *Gryllus bimaculatus* has been extensively used as a model system for neuroethological studies (e.g. Horseman & Huber 1994; Libersat et al. 1994; Sakai et al. 1995; for a review see Huber et al. 1989), and in investigations of male–male competition and female choice (e.g. Simmons 1988, 1991; Simmons & Zuk 1992; Adamo & Hoy 1994, 1995). Although much of this work has focused on the role of male song, there is some circumstantial evidence for the existence of an additional communication channel in the form of cuticular pheromones. van Hörmann-Heck (1957) showed that males require contact to differentiate between the sexes, and more recently, Adamo & Hoy (1994) showed that males will court an anaesthetized female, but not a female coated in plastic. Work on related species has provided stronger evidence. In *Teleogryllus commodus* (Rence & Loher 1977), *Acheta domesticus* and *Teleogryllus oceanicus* (Hardy & Shaw 1983), stroking a male's antenna with the antenna of a conspecific will elicit aggressive behaviour if the antenna is from a male, and courtship song if it is from a female. In addition, both these studies showed that washing the stimulus antenna in an organic solvent abolishes this response, although there remains the possibility of effects of the solvent other than in removing cuticular compounds. In this paper we describe a behavioural assay which controls for side-effects of using a solvent and present results of gas chromatographic analysis of the compounds responsible for sex recognition.

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BEHAVIOURAL EVIDENCE

Methods

Crickets were bulk reared at 29°C with an 18:6 h day:night cycle in two steel gauze insectaries measuring 40 × 40 × 60 cm provided with egg boxes as a substrate. They were fed rodent pellets and water ad libitum. We collected eggs from the culture by providing damp cotton wool for laying, and reared them to the penultimate instar in separate plastic boxes measuring 30 × 16 × 12 cm. During the last nymphal instar we transferred males and females to individual plastic pots, 9 cm in diameter, again provided with food and water. To ensure that they were sexually mature, all test males had been adult for at least 10 days and were allowed to mate at least a day before being used in the trials. All matings were preceded by courtship singing by the male, typically as soon as he contacted the female, and in all cases within 90 min of being placed in the same pot as a female.

To control for differences in behaviour between the sexes previously implicated in sex recognition (see Rence & Loher 1977), we conducted a series of experiments in which we presented a male cricket with a dead conspecific. In each trial, we placed a male or female, killed by freezing, into a plastic pot, 9 cm in diameter, and left it for half an hour to warm up to ambient temperature (25°C). We added a single male and observed him for 90 min or until he produced courtship song for at least 5 s. The small size of the enclosure meant that all males contacted the stimulus individual within seconds of being added. We conducted 48 trials without re-using a test male or dead individual. To test for spontaneous singing, we observed half of the males for 90 min in the absence of a dead conspecific before the trials and half afterwards.

To investigate whether males were reacting to cuticular pheromones or some other cue, such as body shape or texture, we conducted a further set of trials. We presented males with a dead female and observed them as above. On the following day, we presented each male with a dead female that had had its surface cuticular hydrocarbons removed. We achieved this by immersing the dead female in hexane, a volatile organic solvent, for 5 min. The day after a male had been tested with each washed female, we partially restored the female's surface hydrocarbons by painting the body with the hexane extract. To return the entire solution required multiple coats; we used a small paint brush and left the hexane to evaporate between applications (a few seconds). As a further control for the sequence of trials, we washed the painted females in hexane a second time, to remove the re-applied extract, and re-tested each male. After each hexane treatment, we left dead females for at least 4 h at 20°C, to ensure that all the hexane had evaporated, and subsequently stored them at −20°C.

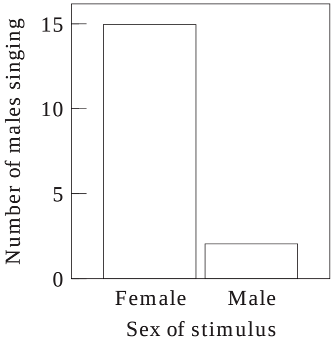

Figure 1. Number of males responding to contact with a dead conspecific with courtship song. $N=24$ for each sex of stimulus individual.

Results

When presented with a dead female, males were much more likely to sing courtship song than in the presence of a dead male (Fig. 1; $\chi^2_1=15.4$, $P<0.001$). Males were never observed to sing courtship song in the absence of a conspecific; solitary males did not sing either before or after the trials ($N=48$).

There is strong evidence that males were reacting to a contact pheromone rather than to some other cue (Fig. 2). Removal of cuticular hydrocarbons abolished the male's response. However, the response was restored by painting the hexane extract back onto the washed females (planned comparison of number of males singing before and after extract re-application: $\chi^2_1=12.8$, $P<0.001$). A second washing again removed the response, making it clear that the result was not an artefact of repeated trials. The process of removing and returning cuticular hydrocarbons clearly reduces the number of males responding to

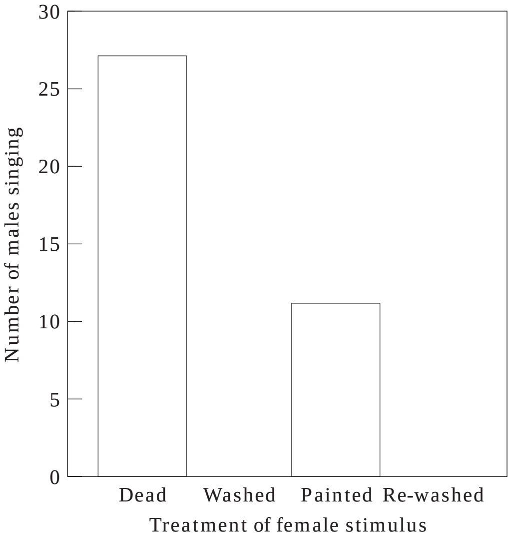

Figure 2. Number of males singing in response to contact with a dead female which was either untreated, washed in hexane, painted with the hexane extract from its cuticle or washed for a second time (having been through the two previous treatments). $N=39$ for each treatment.

the stimulus (planned comparison of number of males singing to unwashed and painted females $\chi^2_1=13.1$, $P<0.001$), although in those that did sing, there was no difference in the latency to singing (untreated mean = 11 min, painted mean = 13 min; $t_{36}=0.38$, NS).

PHYSICAL EVIDENCE

It is clear from our behavioural experiments that males are able to distinguish between the sexes on the basis of cuticular pheromones that can be dissolved in hexane. To provide physical evidence for differences in cuticular composition, we used gas chromatography to analyse the hexane extract that had abolished and restored male courtship song.

Methods

We immersed a mature adult hind leg in 500 µl of hexane containing an internal standard (pentadecane) at a concentration of 10 ppm for 5 min (the same length of time for which females had been washed in the behavioural assay). A 2 µl sample of the extract was injected into a gas chromatograph (Varian 3400) fitted with a 15 m, 0.32 mm bore, 1 µm film, DB-1 capillary column (J&W Scientific, 91 Blue Ravine Road, Folsom, CA 95630-4714, U.S.A.). We optimized separation of extracted components by using a column temperature profile in which the analysis began at a temperature of 75°C rising at 25°C/min to 200°C, holding for 12 min and then rising, again at 25°C/min, to 300°C. Ultra high purity helium at 5 ml/min was used as a carrier gas and components were detected using a flame ionization detector. A study of another species of cricket (Warthen & Uebel 1980) suggested that the majority of hexane soluble compounds are hydrocarbons (more than 84% in *Acheta domesticus*). This is likely also to be the case in *Gryllus* although further analyses are required to identify individual peaks.

Results

Figure 3 shows typical traces for males (Fig. 3a) and females (Fig. 3b). We were able to distinguish 16 peaks (numbered in Fig. 3), all of which are present in both males and females. It is evident that the sexes differ considerably in the amounts of many of the major constituents of the cuticle, notably in peaks 7, 14 and 15. Univariate analysis of variance of the individual peaks from 10 females and eight males (Table I) revealed that 10 out of the 16 peaks showed significant differences between the sexes at $P=0.05$ or below, even with sequential Bonferroni adjustment (Holm 1979). Males had more detected compounds (mean 1.82 × total detected peak areas of females), but this was mainly due to peaks 7, 14 and 15. If these peaks are ignored, males had a mean of only 0.89 × total detected peak areas of females, so the sexes clearly differ in more than just the overall quantity of detected cuticular compounds.

DISCUSSION

The primary role of the insect cuticle is in providing waterproofing and physical protection. In a wide variety of species, the long chain hydrocarbons that are the major constituents of cuticular wax have also been adapted for use as signals (Howard & Blomquist 1982; Bell & Carde 1984).

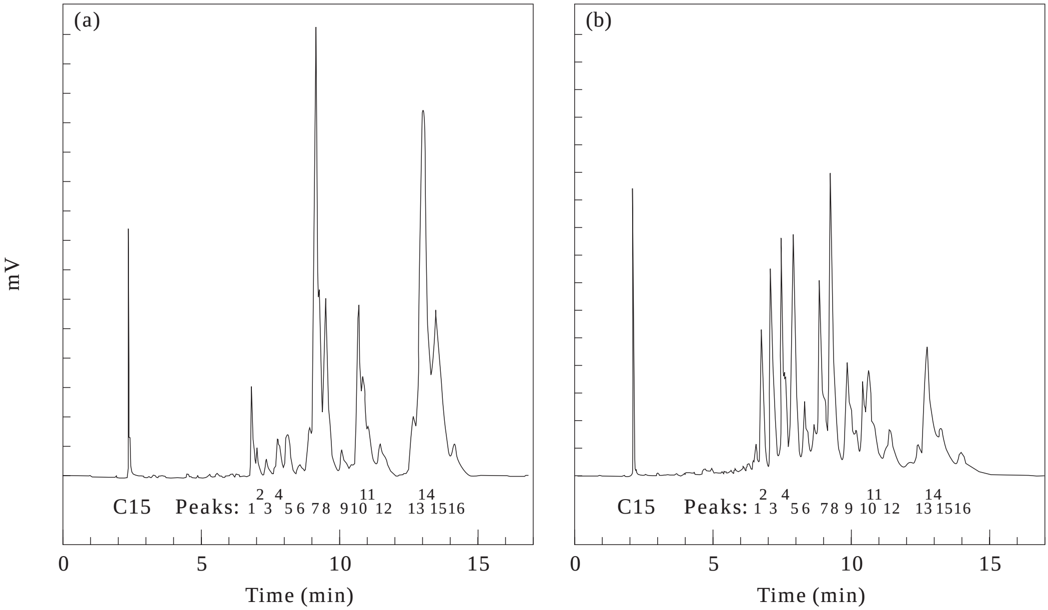

Figure 3. Typical gas chromatograms for (a) males and (b) females. The *Y*-axis gives the output from the flame ionization detector in millivolts. The *X*-axis gives the time since the sample was injected into the column. Longer chain hydrocarbons have longer retention times. The first peak, labelled C15, is a pentadecane internal standard. Other peaks are numbered according to their retention times allowing comparison between males and females.

Table I. Univariate analysis of variance on each hydrocarbon peak comparing 10 females and eight males

Peak	$F_{1,18}$	P	Mean area of male peak relative to female
1	13.6	<0.01*	3.68
2	6.9	<0.05	0.39
3	5.6	<0.05	0.16
4	10.8	<0.01*	0.41
5	20.6	<0.001*	0.24
6	11.6	<0.01*	0.17
7	18.9	<0.001*	3.83
8	0.4	NS	1.17
9	4.7	<0.05	0.37
10	42.2	<0.001*	3.37
11	10.5	<0.01*	1.79
12	0.13	NS	0.88
13	26.5	<0.001*	3.60
14	45.3	<0.001*	5.38
15	58.2	<0.001*	7.60
16	0.38	NS	1.29

*Sequential Bonferroni adjusted significance at $P=0.05$.

The Orthoptera are notable for their use of acoustic signals and they have been extensively used as model systems for investigations into the evolution of mating systems (Gwynne & Morris 1983). Chemical means of communication have, however, received less attention. Our behavioural assay provides the first definitive evidence for cuticular pheromones in *Gryllus bimaculatus*. To our knowledge, this is also the first study in any Orthopteran to show that behaviours can be switched on by returning cuticular compounds, rather than just switched off by removing them. This controls for the possibility that chemical or physical side-effects of the removal process might be responsible for abolishing the response.

Males invariably sang to live females but only around 70% sang to dead females. This decline in response could either be because cuticular pheromones degrade rapidly after death or, more likely, because the behaviour of live females is important in stimulating the male to sing. In contrast to this study, Adamo & Hoy (1994) found that 12 males presented with a dead female did not sing courtship song. However, when presented with an anaesthetized female, six out of the 12 did sing. Adamo & Hoy did not speculate on why this difference occurs, but it is possible that the killed females had changed in some other way, which might not have been the case in our study. We used our dead females slightly sooner after being removed from the freezer, half an hour as opposed to an hour, but it seems unlikely that such a short period would be sufficient to cause degradation of the substances involved.

When hydrocarbons and associated compounds were removed and then returned to dead females there was a further decrease in the number of males singing. The simplest explanation for this is that some substances would inevitably have been lost as a result of the extraction and re-application procedure, through being left in the tube and on the brush. Alternatively, it might be an indication of variation in pheromone concentration over the body. Reapplied extracts were painted over the entire body which might have led to certain areas having less of a particular component than in the natural state and others having more. Hardy & Shaw (1983) observed the response of males of two species of cricket to four different exposed areas of females. We have re-analysed their data, revealing a significant difference in the frequency of male courtship song according to female body area in *Acheta domesticus* ($\chi^2_3 = 10.54$, $P < 0.05$) but not in *Teleogryllus oceanicus* ($\chi^2_3 = 6.55$, NS). On the other hand, Neems & Butlin (1995) found no differences in hydrocarbon profile in the analysis of different body parts from the grasshopper, *Chorthippus parallelus*. Another possibility is that the additional 12 h that the reapplied extracts had been at room temperature might have meant that they had degraded. Finally, it is possible that in their natural state, cuticular compounds are arranged in a more ordered fashion at a molecular level than when they are painted on, affecting their potency as a stimulus.

Despite the simplicity of this technique, this study provides the best available evidence for cuticular pheromones in an Orthopteran, supporting the conclusions of previous studies, and indicating that communication through the use of cuticular compounds may be common in crickets (van Hörmann-Heck 1957; Rence & Loher 1977; Hardy & Shaw 1983; Adamo & Hoy 1994) and perhaps in other Orthopterans (Ritchie 1990; Neems & Butlin 1995). Use of mass spectrometry and other analytical chemical techniques would allow more detailed characterization of the differences between the sexes. Individual compounds and blends could then be tested using a bioassay similar to the one described in this study.

Our gas chromatographic analysis revealed clear differences between males and females in the concentration of most of the cuticular compounds measured. Males had larger total amounts of detected compounds, but since this could be ascribed to only three peaks it could not be the main reason for the observed differences between the sexes. Also, seven of 16 peaks were larger in females than in males. Males did not appear to show greater total peak area because of a size difference between the sexes; measurement of a large number of individuals from the same population showed no difference between males and females in hind femur length ($t_{2020} = 1.74$, NS), or hind leg mass ($t_{116} = 0.26$, NS). No sex-dependent habitat preference or lifestyle differences have been recorded, so the sex difference is unlikely to be due to different environmental requirements. Males engage in fights with other males, and it is possible that there are some physical benefits of their cuticular composition related to injuries inflicted during fights. However, the finding that both sexes had larger amounts of particular substances than the other indicates that a single explanation such as this cannot explain cuticular composition.

There can be little doubt that some aspect of the sexual dimorphism in cuticular composition revealed in our analysis is responsible for sex recognition. However, unlike some other species, in which a single ‘sex pheromone’ has been isolated, there were no peaks that occurred in only one sex. It seems likely that in *G. bimaculatus* it is the relative concentration of substances in the female cuticle that allows males to recognize potential mates.

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