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Cuticular lipids and odors induce sex-specific behaviors in the male cricket *Gryllus bimaculatus*

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Abstract

Male crickets display sex-specific (e.g., mating and agonistic) behaviors towards conspecific individuals. One of the key signals for these behaviors is the chemical substance on the cricket body surface. In the present study, we analyzed female and male cuticular substances in behavioral assays. Antennal contact stimulation using female forewings elicited a mating behavior in males, while that using male forewings elicited an agonistic behavior in males. Thin-layer-chromatographic and other techniques analysis showed that saturated cuticular lipids were present in both female and male cuticles and that unsaturated lipids were present only in the male cuticle. Filter papers soaked with saturated or unsaturated cuticular lipids were applied to antennae of male crickets. Males showed mating behavior in response to stimulation with saturated lipids from both females and males but showed avoidance behavior in response to stimulation with male unsaturated lipids. Because cuticular lipids did not induce agonistic behavior in males, we collected odors from male crickets and found that these odors induced agonistic behavior in males. Therefore, we concluded that the key signals for mating, avoidance and agonistic behaviors of male crickets are comprised of at least three different components, saturated and unsaturated cuticular lipids and male odors, respectively.

Keywords: Cricket; *Gryllus bimaculatus*; Pheromone; Insect; Agonistic behavior; Mating behavior; Chemoreception

1. Introduction

During social interactions with conspecific individuals, insects display a wide range of species- and sex-specific behaviors that are elicited by auditory, visual and chemical signals. Cuticular substances are one of the signals that are used for intra-species communication in insects. Individuals detect cuticular substances of conspecific individuals using specific receptors and then respond to them by exhibiting species- and sex-specific behaviors (Fukui and Takahashi, 1983; Takahashi and Fukui, 1983; Nishida et al., 1974, 1975, 1976).

In cricket species, mating and agonistic behaviors have been reported (Alexander, 1961; Loher and Dambach, 1989; Adamo and Hoy, 1995; Sakai et al., 1991; Iwasaki et al., 2006). It is certain that auditory signals of male crickets play important roles in communication, functioning as a calling song that attracts potential mating partners, a courtship song that induces a female to copulation and an aggressive song used by dominant males to inhibit aggression in subordinate individuals (Alexander, 1961; Phillips and Konishi, 1973; Loher and Dambach, 1989). In addition to auditory signals, antennal contact with conspecific individuals is necessary for the actual initiation of mating or agonistic behavior in male crickets (Nagao and Shimozawa, 1987; Hofmann and Schildberger, 2001; Murakami and Itoh, 2003). A few reports show that crickets produce sex-specific odors and that these odors affect cricket behavior (Sexton and Hess, 1968; Otte and Cade, 1976). However, little is known about the details of these contact chemical signals that relate to sex-recognition and that trigger sex- and species-specific behaviors in crickets (Sexton and Hess, 1968; Otte and Cade, 1976; Rence and Loher, 1977; Balakrishnan and Pollack, 1997; Tregenza and Wedell, 1997).

In the present study, we analyzed female and male cuticular substances of *Gryllus bimaculatus* using thin-layer-, column- and gas-liquid-chromatographies, and we assayed the effects of these substances on mating and agonistic behaviors of male crickets.

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2. Materials and methods

2.1. Cricket maintenance and experimental set-up

Male crickets, *G. bimaculatus*, were maintained in a crowded colony under a 14-h light-10-h dark cycle at 28±2 °C. The crickets were given insect food pellets, carrots and water *ad libitum*. Five days prior to experiments, each of the sexually mature adult males (1–2 weeks after imaginal moult) with all appendages intact was isolated in a 100-ml glass beaker to hinder communications with conspecific individuals.

Behavioral assays using male crickets were performed in a circular glass container (12 cm in diameter and 6 cm in height) of which bottom was covered with filter paper. Males were placed into the container and their behavior was observed. Each individual was used once for the behavioral experiment. The filter paper was renewed after each experiment.

2.2. Response behaviors of male crickets

The behavioral responses of male crickets were divided into four major categories: "mating behavior", "avoidance behavior", "agonistic behavior" and "no response" (Table 1). "Mating behavior" was further divided into three subcategories: 1) "approaching" = the male crickets placed their forelegs on the substance and stretched their head forward trying to probe the stimulant with their maxillary palpi but did not bite the stimulant, 2) "courtship" = the males stridulated to produce a courtship song and turned the back towards the substance, 3) "calling" = the males only stridulated to produce a calling song after antennal contact stimulation. "Agonistic behavior" was also divided into four subcategories: 1) "threat posture" = the males raised their body off the ground with all six legs, 2) "antennal fencing" = the males lashed the stimulating substance with their antennae, 3) "aggressive song" = the males stridulated to produce an aggressive song after antennal contact stimulation, 4) "mandible flare" = the males showed antennal fencing with hyperextension of the mandibles.

Table 1
Behavioral responses of male *Gryllus bimaculatus*

Behaviors	Description
Mating behavior	
Courtship	Individual stridulates to produce a courtship song.
Calling	Individual stridulates to produce a calling song.
Approaching	Individual approaches the stimulating substance and probes it with maxillary palpi.
Avoidance behavior	
	Individual avoids antennal contact stimulation and/or kicks against the stimulant.
Agonistic behavior	
Mandible flare	Hyperextension of the mandibles.
Aggressive song	Individual stridulates to produce an aggressive song.
Antennal fencing	Individual rapidly moves its antennae.
Threat posture	Individual raises itself on its forelegs.
No response	
	Individual remains immobile during antennal contact stimulation or only touches the stimulant with its antennae.

2.3. Contact stimulation using isolated forewings

To investigate whether cuticular substances induce mating and agonistic behaviors in male crickets, a stimulant (a cricket forewing or a filter paper with cuticular substances) was held in forceps and was touched to the antennae of a male cricket ("antennal contact stimulation"). To avoid cross contamination of cuticular substances, sexually mature adult female and male individuals (1–2 weeks after imaginal moult) were isolated for at least 24 h prior to collecting their forewings. A 24-h isolation period was deemed necessary because preliminary experiments showed that when isolation period was less than 24 h, males showed both mating and agonistic behaviors to both male and female forewings. After anesthetizing on ice, female and male forewings were dissected within 5 min prior to behavioral tests by cutting the root of the forewings using scissors. Antennal contact stimulation was performed for 3 min; when the antennal contact stimulation to male antennae was applied for more than 3 min, all tested males finally avoided every stimulating substance or stridulated to produce a courtship song with avoidance (preliminary observations). Control experiments were performed using female and male forewings that had been washed three times with *n*-hexane to remove solvent-extractable cuticular substances.

2.4. Chemical analyses of cuticular lipids

To extract whole cuticular lipids, forewings from 30 mature male or female crickets were dissected and immersed in 10 ml *n*-hexane for 10 min. The *n*-hexane extract was then evaporated using a rotary evaporator at 30 °C. The ratio of saturated and unsaturated cuticular lipids was quantified using a thin-layer chromatography/flame ionization detector (Iatroscan) (Katagiri et al., 1985, Kaneko and Katagiri, 2004). The ratio was determined by the peak areas.

Cuticular lipids of both females and males were subjected to gas-chromatography. A capillary column (ULBON HR-1) was run with helium as a carrier gas. The inlet temperature was 300 °C and programmed to increase from 130 °C to 320 °C at 4 °C per minute, using a Shimadzu G-14AH (Ishii et al., 2001). We used *n*-hexacosane ($C_{26:0}$), *n*-heptacosane ($C_{27:0}$), *n*-octacosane ($C_{28:0}$), *n*-nonacosane ($C_{29:0}$), *n*-triacontane ($C_{30:0}$) and *n*-hentriacontane ($C_{31:0}$) as standards of 26-, 27-, 28-, 29-, 30- and 31-carbon chain lengths respectively (GL Sciences). Whole male cuticular lipids separated into saturated and unsaturated lipids (details in the next paragraph) and then were subjected to gas-liquid-chromatography.

2.5. Contact stimulation using saturated and unsaturated cuticular lipids from isolated forewings

The *n*-hexane extracts from female and male forewings were subjected to silver nitrate (10% $AgNO_3$)-immersed silicic acid column chromatography (Unisil, 100/200 mesh) for separation