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Response of *Pteromalus cerealellae* to conspecific odor: Evidence for female- and male-produced pheromones?

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ABSTRACT

Pteromalus cerealellae (Ashmead) (Hymenoptera: Pteromalidae) is an ectoparasitoid of several pests of stored products. We investigated the role of semiochemicals in mediating intraspecific communication in this parasitoid using electroantennogram (EAG) and behavioral bioassays. Responses of virgin male and female *P. cerealellae* were tested to airborne volatiles from live male and female conspecifics and to whole body extracts of both sexes. Females showed significantly greater EAG responses than males to whole body extracts of both sexes. Results from Y-tube olfactometer bioassays demonstrated significant attraction of both sexes to live conspecifics of the same (intrasexual attraction) or opposite sex (cross attraction). Females also showed significant attraction to whole body extracts of females, but not to the extract of males. These findings provide preliminary evidence for possible existence of a female-produced sex pheromone and close range cuticular chemicals by both sexes of *P. cerealellae*, which may play a role in courtship and species recognition. Further studies are needed to characterize these chemicals and determine their roles in the behavioral ecology of *P. cerealellae*.

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1. Introduction

Pteromalus cerealellae (Ashmead) (Hymenoptera: Pteromalidae) is an ectoparasitoid of several insect pests of stored products including *Sitotroga cerealella* Olivier, *Callosobruchus maculatus* (Fab.), *Lasioderma serricorne* (Fab.), *Prostephanus truncatus* (Horn), and *Sitophilus* spp. (Ashmead, 1902; Brower, 1991; Mbata et al., 2005; Onagbola et al., 2007). Females lay eggs in host larvae, which typically are concealed within seeds. The ongoing interest in the potential utilization of *P. cerealellae* for biological control of stored product insects (Brower, 1991; Mbata et al., 2005; Onagbola et al., 2007) is hindered by lack of information on several aspects of its biology and life history strategy, including the cues it uses in mate finding.

Many studies have reported the involvement of sexual attractants in sexual communication of parasitoids from several families (Matthews, 1975; Weseloh, 1976; Vinson, 1978; Simser and Coppel, 1980; Leal et al., 1997; Pompanon et al., 1997; Steiner et al., 2006). The majority of the available studies have implicated female-produced sex pheromones as cues used in mate finding by parasitoids (Weseloh, 1976; Vinson, 1978; Yoshida, 1978; Decker et al., 1993; Quicke, 1997). Female-produced sex pheromones typically serve to attract males from long range to enable mate finding

(Vinson, 1972; Eller et al., 1984; Swedenborg and Jones, 1992; Godfray, 1994; McNeil and Brodeur, 1995; Quicke, 1997; Jewett and Carpenter, 1999; Cheng et al., 2004). Female-derived pheromones have also been demonstrated to mediate close range courtship behavior in several species of parasitoids including members of the family Pteromalidae (King et al., 1969; Yoshida, 1978; Ruther et al., 2000). Males of numerous species of parasitoids including *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) (van den Assem, 1986; Ruther et al., 2007) and *Laelius utilis* Cockerell (Hymenoptera: Bethyridae) (Howard, 1992) have also been implicated to release chemicals which may influence the behaviors of conspecific females (van den Assem et al., 1980; Isidoro and Bin, 1995; Isidoro et al., 1996; Ruther et al., 2007). The plausible roles of cuticular hydrocarbons of parasitic wasps as pheromones have also been reported by several authors (Howard, 1993, 2001; Singer, 1998; Steiner et al., 2006). Cuticular hydrocarbon components of *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) (Syvertsen et al., 1995), *N. vitripennis* (Steiner et al., 2006), *Roptrocercus xylophagorum* (Ratzeburg) (Hymenoptera: Pteromalidae) (Sullivan, 2002), and *Lariophagus distinguendus* (Först) (Hymenoptera: Pteromalidae) (Steiner et al., 2005) were reported to function in attracting parasitoids to their mates.

In a preliminary study of mating behavior of *P. cerealellae*, we obtained results which implicate possible involvement of male- and female-produced semiochemicals in mediating courtship in this species (Onagbola and Fadamiro, unpublished data). First,

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courtship is initiated when the female begins to exhibit “calling behavior”, which consequently results in alert-related (including antennation and wing fanning) behavioral responses in conspecific males. Our observations also suggest possible involvement of male contact semiochemicals in inducing sexual receptivity in females. Based on these results, this study was designed to investigate the roles of semiochemicals in mediating mate location in this species and to further determine the existence of male- and female-produced semiochemicals in mate finding by *P. cerealellae*. Specifically, we conducted electroantennogram (EAG) and behavioral responses of both sexes to airborne volatiles from live male and female conspecifics and to whole body extracts of both sexes.

2. Materials and methods

2.1. Insects

Pteromalus cerealellae was reared in our laboratory on the larvae of the cowpea bruchid, *C. maculatus*. The host insect was reared on cowpea seeds, *Vigna unguiculata* Walp. (California Black Eyed variety) in 1-l wide-mouthed Mason glass jars. A fresh culture was started every five days by placing ~25 pairs of 3-day-old mated *C. maculatus* in a glass jar containing ~100 g of cowpea seeds held at 30 ± 1 °C, L:D 12:12 and $70 \pm 5\%$ r.h. (Mbata et al., 2005; Onagbola et al., 2007). The beetles were allowed to lay eggs on the seeds for 24-h after which they were removed with an aspirator. The infested seeds were incubated at the conditions specified above until the larvae had reached the fourth instar stage, which were then provided to *P. cerealellae* for parasitization. The parasitoid was maintained by transferring about 30 adult pairs into a glass jar containing *C. maculatus*-infested cowpea seeds at a stage when most of the bruchid larvae were at the fourth larval instar (this occurred at ~15 days after infestation of cowpea seeds under our rearing conditions). The jars were held at the environmental conditions stated above for *C. maculatus*. Adult *P. cerealellae* were removed from the jars after five days of oviposition. Parasitized host larvae were incubated in a growth chamber at the above environmental conditions until the adult parasitoids started to emerge (~11 days after parasitization at the above conditions). Pupae of the parasitoids (still within parasitized hosts) were immediately transferred into 100 mm × 15 mm disposable Petri dishes to collect adult parasitoids immediately upon emergence.

2.2. Solvent extraction of adult *P. cerealellae*

Extraction of chemical stimuli from male and female *P. cerealellae* was conducted using a method similar to that described by Steiner et al. (2006). Three hundred virgin (6–12 h-old) adult *P. cerealellae* of each sex were anaesthetized by chilling in a Percival freezer (Fisher Scientific) at -20 °C for ~5 min and then soaked in 3 ml laboratory grade hexane in a 4 ml glass vial for up to 4 h. The extraction process was repeated ten times for a total of 3000 adults per sex. The extracts were stockpiled in organic glass vials and concentrated under Nitrogen (N_2) to obtain a concentration of ~1 insect equivalent per 0.2 μ l (5 insects per μ l of extract). Each extract was made in three replicates, dehydrated in ~1 g of anhydrous Sodium Sulfate for ~12 h and stored in a freezer at -20 °C until used. The hexane extracts were kept in the freezer until they were used for experiments.

2.3. Electroantennogram recordings

The EAG techniques used in this study were similar to those previously described by Chen and Fadamiro (2007a,b). Glass

capillary (1.1 mm I.D.) filled with 0.1 M KCl solution was used as electrodes. The reference electrode was connected to an isolated head of adult female *P. cerealellae* while the recording electrode was connected to the cut tip of the antenna (flagellum). Chlorinated silver–silver chloride junctions were used to maintain electrical contact between the electrodes and input of preamplifier. The analog signal was detected through a probe (INR-II, Syntech®, the Netherlands), captured and processed with a data acquisition controller (IDAC-4, Syntech®), and later analyzed with EAG 2000 (Syntech®) software on a computer. A ten-microliter aliquot of each solution was applied to a piece of 7 × 40 mm filter paper strip (Whatman® no. 1). After allowing for solvent evaporation, the impregnated filter paper strip was inserted into a glass Pasteur pipette (~14 cm in length, Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.) constituting an odor cartridge. The control stimulus was a similar pipette containing a filter paper strip impregnated with a 10 μ l aliquot of hexane. The tip of the pipette was placed about 3 mm into a small hole in the wall of a metal tube (13 cm long, 8 mm diameter) which was oriented toward the antennal preparation (≈ 0.5 cm away from the preparation). In this way, the stimuli were provided as 0.2 s puffs of air into a continuous humidified air stream at 1000 ml/min generated by an air stimulus controller (CS-55, Syntech®, the Netherlands). At least 2 min were allowed between successive stimulations for antennal recovery.

The EAG response of antennae of virgin (6–12 h-old) *P. cerealellae* of both sexes was tested with the following three treatments: whole body extracts of conspecific males, whole body extracts of conspecific females, and hexane control. A test series consisting of the above treatments was randomly applied to an antennal preparation starting with the hexane control. Recordings were obtained from at least 12 antennal preparations for each sex. Absolute EAG data were first subjected to standard least square modeling to determine the effects of treatments and sex and sex × treatments interaction on EAG response ($P < 0.05$). EAG responses of male or female parasitoids to the different treatments were then compared with one-way ANOVA followed by Tukey's HSD test ($P < 0.05$; JMPIN Version 5.1, SAS Institute Inc., 2003). Student's *t*-test analysis was used to investigate sexual differences in EAG responses to each treatment ($P < 0.05$).

2.4. Behavioral (Y-tube olfactometer) bioassays

A Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) was used to test the attraction of virgin (6–12 h-old) adult male and female *P. cerealellae* to airborne volatiles from live male and female conspecifics and to whole body extracts of both sexes. The olfactometer system used in this study has been previously described by Chen and Fadamiro (2007a). The system consists of a central tube (13.5 cm long, 24 mm diam.) and two lateral arms (5.75 cm long, 24 mm diam.) which are separately connected to an extending glass tube (14.5 cm long, 19 mm diam.). There is a sieve inlaid in the extending glass tube 5.25 cm away from the connection to prevent escape of insects and to serve as an end point of each lateral arm. Humidified and purified air was passed from an air pump into each of the extending arms of the olfactometer at a rate of 200 ml/min. To minimize visual distraction for the parasitoids, the Y-tube olfactometer was placed inside a white paper box, which was open on the top (for illumination) and on the front side (for observation). Illumination was provided by a vertically hanging office lamp (20 W, 250 Lux) above (~50 cm high) the olfactometer tube.

The first experiment was conducted to investigate responses of virgin male and female *P. cerealellae* to airborne volatiles from live male and female conspecifics. Humidified and purified air from the

air pump was passed over three hundred male or female parasitoids which were placed in a 20 cm long, 24 mm diam. volatile collection chamber (VCC, Analytical Research Systems, Gainesville, FL) into an extending arm of the olfactometer. Clean laboratory air was pumped at the same rate (200 ml/min) through a similar VCC into the other arm of the Y-tube olfactometer. Male or female parasitoids were individually released at the base of the central arm of the Y-tube and observed for maximum of 5 min. A parasitoid that did not make a choice within this period was removed, discarded, and not included in the analyses. Parasitoids that walked to the end of one of the arms and remained there for at least 10 s were recorded as having made a choice between the odor stimulus (treatment) and the control (humidified and purified laboratory air). After three individual parasitoids had been tested, a fresh odor stimulus was used and the olfactometer arms were reversed (180°). After each subset of 6 parasitoids had been tested, the olfactometer apparatus was rinsed with soap water and acetone, and then air-dried. Parasitoids were used only once and at least twenty-four males or females were tested per choice test. All bioassays were conducted at 25 ± 1 °C and $60 \pm 5\%$ r.h.

Using the same protocols described above, a second experiment was conducted to test the behavioral response of virgin male and female *P. cerealellae* to whole body extracts of conspecific males and females. Whole body extract of male or female parasitoids was delivered as a 10- μ l sample placed on No. 1 filter paper strips (7 × 40 mm, Whatman® no. 1) resulting in 50 adult male or female equivalent per loading. After allowing for solvent evaporation (~15 s), the filter paper strip was inserted into one arm of the Y-tube olfactometer. A similar filter paper strip containing a 10- μ l aliquot of hexane (laboratory grade) was inserted into the second arm (solvent control). Male or female parasitoids were individually released at the base of the central arm of the Y-tube and observed for maximum of 5 min. At least twenty-four individuals of each sex were tested per choice test.

For each experiment, data obtained on the percentage responses of adult male or female parasitoids to each stimulus versus control were separately subjected to chi-square (χ^2) analyses (Parker, 1979)

to test for significant deviation (at $P < 0.05$) from an expected 1:1 (stimulus:control) response.

3. Results

3.1. Electroantennogram (EAG) responses

The EAG amplitude in response to odors of conspecifics was in the range of 0.02–0.60 for males and 0.30–1.05 for females. Standard least square modeling revealed significant effects of stimuli ($F = 37.44$; $df = 2$; $P < 0.0001$), sex ($F = 46.45$; $df = 1$; $P < 0.0001$), and sex × stimuli interaction ($F = 8.48$; $df = 2$; $P = 0.001$) on EAG responses of *P. cerealellae*. Based on the recorded significant treatment (stimuli) effect, EAG response was separately compared for males and females. One-way ANOVA revealed significant effect of treatment on EAG response of males ($F = 4.71$; $df = 2, 33$; $P = 0.016$) and females ($F = 47.20$; $df = 2, 48$; $P < 0.0001$). Whole body solvent extracts of both sexes elicited significant EAG responses in both male and female *P. cerealellae*, compared to hexane control (Fig. 1). Student's *t*-test revealed significant sexual differences in EAG response to whole body extract of males ($df = 1, 27$; $t = 4.32$; $P = 0.0002$), whole body extract of females ($df = 1, 27$; $t = 4.93$; $P < 0.0001$), and hexane control ($df = 1, 27$; $t = 3.56$; $P = 0.001$), with females showing greater EAG response than males in all cases (Fig. 1).

3.2. Olfactometer response of *P. cerealellae* to airborne volatiles from live conspecifics

Male and female *P. cerealellae* were significantly attracted to airborne volatiles from live male and female conspecifics (Fig. 2). Male *P. cerealellae* showed significant attraction to airborne volatiles from live males ($\chi^2 = 7.26$; $df = 1, 23$; $P = 0.007$; 74%) and live females ($\chi^2 = 16.13$; $df = 1, 23$; $P < 0.0001$; 87%). Similarly, female parasitoids also showed significant attraction to airborne volatiles from live males ($\chi^2 = 4.80$; $df = 1, 23$; $P = 0.029$; 70%) and live females ($\chi^2 = 13.44$; $df = 1, 23$; $P = 0.0002$; 81%).

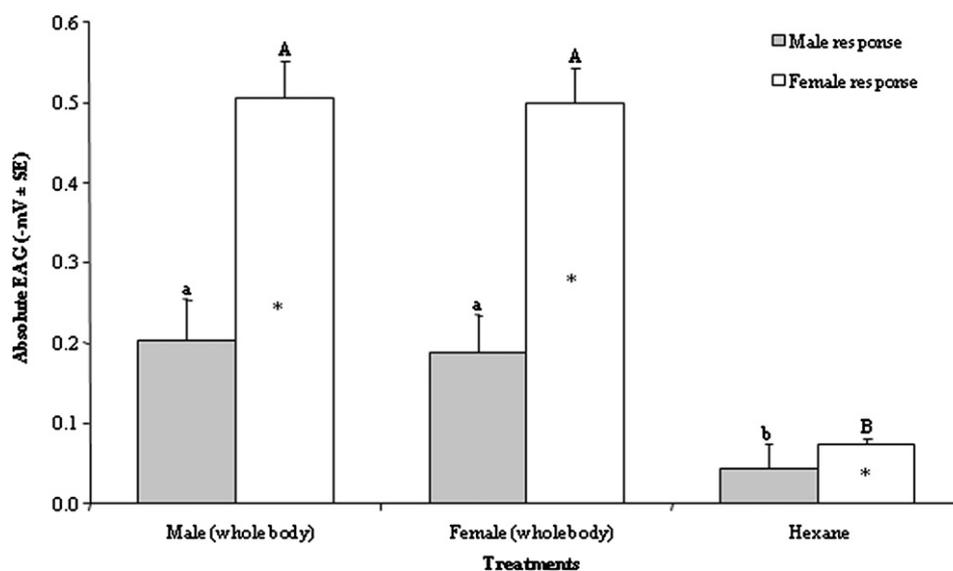


Fig. 1. Electroantennogram (EAG) responses of *P. cerealellae* to whole body (WB) extracts of conspecifics. Figure shows absolute EAG ($-mV \pm SE$) responses of males (gray bars) and females (white bars) to WB extracts of male and female conspecifics and hexane (control). Means for the same sex having letters in common are not significantly different (Tukey's HSD test; $P < 0.05$). Asterisks (*) indicate significant differences between the sexes (Student's *t*-test; $P < 0.05$).

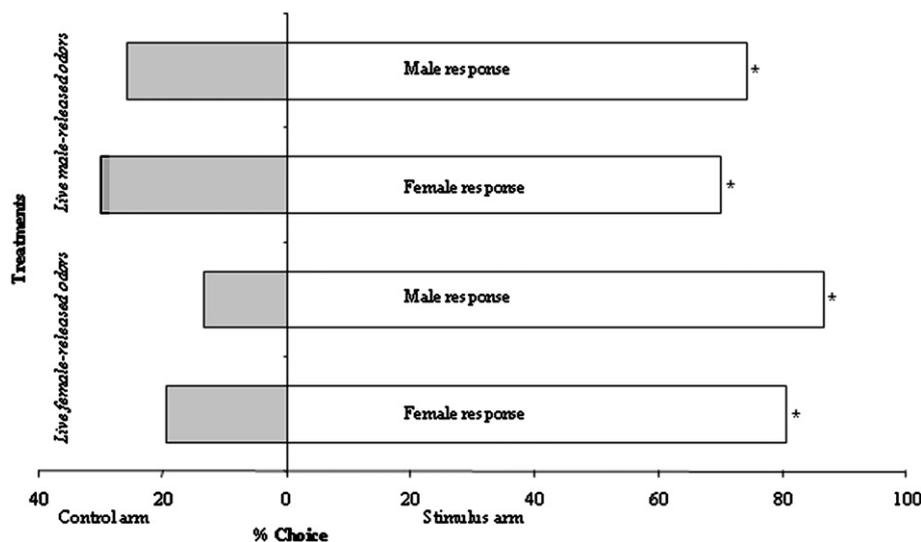


Fig. 2. Responses of male and female *P. cerealellae* in a Y-tube olfactometer when given a choice between humidified and purified laboratory air (control) and airborne volatiles from conspecific males or females. White bars indicate the percentage of male or female wasps responding to the control, while gray bars indicate the percentage responding to the tested stimuli. Asterisks (*) indicate significant differences within a choice test (χ^2 , $P < 0.05$).

3.3. Olfactometer response of *P. cerealellae* to whole body extracts of conspecifics

Whole body extract of female *P. cerealellae* elicited significant attraction in conspecific females ($\chi^2 = 4.17$; $df = 1, 23$; $P = 0.041$; 71%), but not in conspecific males ($\chi^2 = 1.50$; $df = 1, 23$; $P = 0.221$; 63%). In contrast, whole body extract of males did not elicit significant attraction in conspecific males ($\chi^2 = 0.67$; $df = 1, 23$; $P = 0.414$; 58%) or in conspecific females ($\chi^2 = 2.67$; $df = 1, 23$; $P = 0.103$; 67%) (Fig. 3).

4. Discussion

The results of this study demonstrated the olfactory response of male and female *P. cerealellae* to their conspecifics, as have been reported for several other parasitoid species (Yoshida, 1978; Vinson,

1978; Simser and Coppel, 1980; Espelie et al., 1996; Steiner et al., 2005, 2006). Females showed greater EAG response than males to all tested stimuli. Similar results showing greater EAG response of females compared to conspecific males have been reported for various species of parasitoids including *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Braconidae) (Vaughn et al., 1996), *N. vitripennis* (Beukeboom and van den Assem, 2001), *Apanteles obliquae* Wilkinson (Hymenoptera: Braconidae) (Jyothi et al., 2002), and the decapitating phorid fly, *Pseudacteon tricuspis* Borgmeier (Diptera: Phoridae) (Chen and Fadamiro, 2007b). The recorded sexual differences in EAG response of *P. cerealellae* may be due to differences in antennal morphology. In a previous study of antennal morphology of *P. cerealellae*, we recorded relatively greater abundance of the multiporous (and presumably olfactory) placoid sensilla on female antennae (Onagbola and Fadamiro, 2007). Differences in the sensitivity of male and female wasps to odor

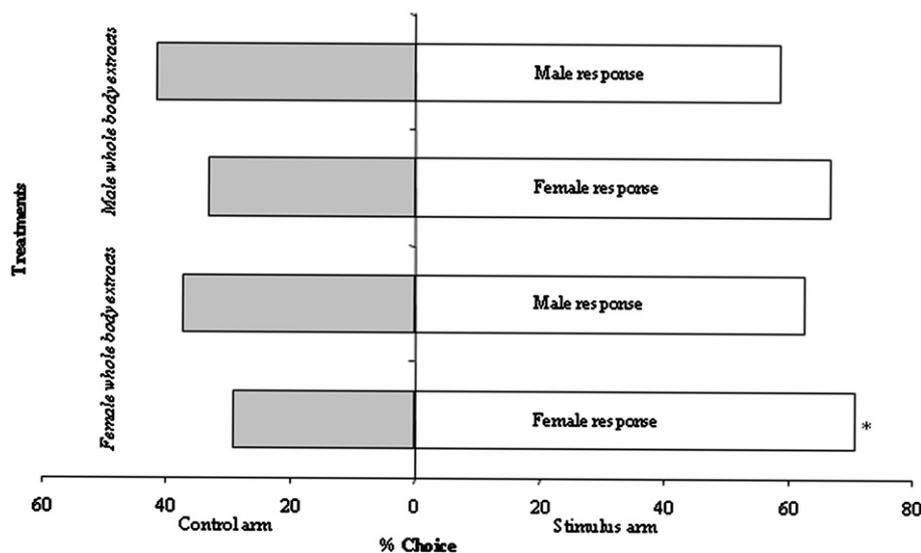


Fig. 3. Responses of male and female *P. cerealellae* in a Y-tube olfactometer when given a choice between filter paper impregnated with hexane (control) and filter paper impregnated with 10- μ l aliquot of whole body hexane extracts of male or female *P. cerealellae*. White bars indicate the percentage of male or female wasps responding to the control, while gray bars indicate the percentage responding to the tested stimuli. Asterisks (*) indicate significant differences within a choice test (χ^2 , $P < 0.05$).

stimuli, as recorded for *P. cerealellae* in the present study, may also arise from sexual differences in the higher-order processing of incoming peripheral olfactory information (Whitman, 1988; Whitman and Eller, 1992; Jyothi et al., 2002).

The results of the behavioral bioassays showed significant attraction of both sexes of *P. cerealellae* to airborne volatiles from live conspecifics of the same (intrasexual attraction) and opposite sex (cross attraction), suggesting the presence of volatile attractants in the effluvia of both sexes, which may function in species recognition (Howard, 1993; Singer, 1998; Beukeboom and van den Assem, 2001; Cheng et al., 2004). Cross attraction of both sexes to their conspecifics has been reported in several parasitoids. For example, females of *C. nigriceps* (Lewis et al., 1971), *Brachymeria intermedia* (Nees) (Hymenoptera: Chalcididae) (Leonard and Ringo, 1978), *B. lasus* (Walker) (Leonard and Ringo, 1978; Simser and Coppel, 1980), and *C. nigriceps* (Vinson, 1978; Weseloh, 1976) were reported to produce volatile chemicals that attract conspecific males and females.

In contrast to the generally attractive airborne volatiles from live males and females, whole body extracts of male and female *P. cerealellae* were not very attractive: only the females showed significant attraction to female extracts. This disparity in the attractiveness of airborne volatiles from live conspecifics versus whole body extracts may suggest the presence of compounds in some of the extracts which are somewhat repellent to the parasitoids, as have been reported in studies involving other insect species (Birch and Haynes, 1982; Hansen, 1984). Whole body extracts of parasitoids typically contain cuticular hydrocarbons (Singer, 1998; Howard, 2001) whose primary function is to protect insects from desiccation (Lockey, 1988). However, cuticular hydrocarbons of insects have also been reported to contain cuticular pheromones (Howard, 2001; Steiner et al., 2006), which have been demonstrated in numerous studies to be involved in orientation and recognition processes of insects (Howard, 1993; Smith and Breed, 1995; Singer, 1998; Howard and Blomquist, 2005; Steiner et al., 2006). The attraction of both sexes of *P. cerealellae* to conspecifics may be due to volatile cuticular pheromones. However, some of the components of the cuticular hydrocarbons may be inhibitory and thus account for the reduced attractiveness of male and female extracts.

The attraction of females to live conspecific males suggests the presence of attractive male volatiles which may function either as a male courtship pheromone or as species recognition chemicals. Howard (1992) and Singer (1998) reported that males of *L. utilis* have unique cuticular hydrocarbons which help conspecific females to recognize them. In an earlier study of mating behavior of *P. cerealellae*, we observed males exhibiting “antennal touch” behavior (Onagbola and Fadamiro, in press), probably to stimulate and induce sexual receptivity in females, as has been reported for other parasitoid species (van den Assem et al., 1980; Isidoro et al., 1996; Ruther et al., 2000; Cheng et al., 2004). These observations suggest possible involvement of male-produced pheromones in the courtship behavior of *P. cerealellae*, as was recently reported for *N. vitripennis* (Ruther et al., 2007).

The attraction of males to conspecific females, as recorded in the present study, may suggest involvement of female-produced volatile attractants in this species, as has been reported for several other parasitoids species (Matthews, 1975; Weseloh, 1976; Vinson, 1978; Yoshida, 1978; Simser and Coppel, 1980; Eller et al., 1984; Leal et al., 1997; Cheng et al., 2004; Steiner et al., 2005). For example, females of *N. vitripennis* (King et al., 1969), *L. distinguendus* (van den Assem, 1970; Steiner et al., 2005), *B. intermedia* (Leonard and Ringo, 1978; Simser and Coppel, 1980), *Cotesia liparidis* (Bouche) (Hymenoptera: Braconidae) (Weseloh, 1976; Vinson, 1978), and *C. nigriceps* (Weseloh, 1976; Vinson, 1978) are known to produce pheromones which enable conspecific males to find them. In our earlier study of

mating behavior of *P. cerealellae*, we observed the display by males of alert-related behaviors (i.e., “antennation” and “wing fanning”) from long range in response to “calling females” (Onagbola and Fadamiro, unpublished data). It was also observed in the same that male *P. cerealellae* usually becomes agitated when it encounters a conspecific female, which may also implicate involvement of female-produced close range contact chemicals (courtship pheromone) in the courtship behavior of this species.

In summary, the results of this study provide preliminary evidence for possible existence of a female-produced sex pheromone and the production of courtship pheromones and close range cuticular compounds by both sexes of *P. cerealellae*, which may play a role in courtship behavior and species recognition. Further studies are needed to characterize and identify the chemicals and determine their roles in the behavioral ecology of *P. cerealellae*.

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