STUDIES ON THE BIOLOGY AND HOST LOCATION BEHAVIOR OF *PTEROMALUS CEREALELLAE* (ASHMEAD) (HYMENOPTERA: PTEROMALIDAE), A PARASITOID OF *CALLOSOBRUCHUS MACULATUS* (F.) (COLEOPTERA: CHRYSOMELIDAE)

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A Dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctor of Philosophy

Auburn, Alabama
May 10, 2008

STUDIES ON THE BIOLOGY AND HOST LOCATION BEHAVIOR OF *PTEROMALUS CEREALELLAE* (ASHMEAD) (HYMENOPTERA: PTEROMALIDAE), A PARASITOID OF *CALLOSOBRUCHUS MACULATUS* (F.) (COLEOPTERA: CHRYSOMELIDAE)

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DISSERTATION ABSTRACT

STUDIES ON THE BIOLOGY AND HOST LOCATION BEHAVIOR OF *PTEROMALUS CEREALELLAE* (ASHMEAD) (HYMENOPTERA: PTEROMALIDAE), A PARASITOID OF *CALLOSOBRUCHUS MACULATUS* (F.) (COLEOPTERA: CHRYSOMELIDAE)

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(Doctor of Philosophy, May 10, 2008)
(M.Tech., Federal University of Technology, Akure, Nigeria, 2003)
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272 Typed pages

Directed by Henry Y. Fadamiro

Pteromalus cerealellae is an ectoparasitoid of several pests of stored products. Information on several aspects of its biology and life history strategy is grossly lacking. My dissertation focuses on some aspects of the biology, behavior and the cues used by *P. cerealellae* to locate its host, *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae). In Chapter II, I investigated the influence of environmental factors on longevity and reproductive performance of *P. cerealellae*. Male and female wasps benefited greatly from sugar feeding with significant increases in lifespan and progeny

production. The suitability of freeze-killed larvae of *C. maculatus* as hosts for rearing *P. cerealellae* was investigated in Chapter III. Live and freeze-killed *C. maculatus* larvae were equally suitable as hosts for *P. cerealellae* with no significant difference in progeny production. In Chapter IV, I characterized the pre-imaginal stages of *P. cerealellae* for the first time using morphological structures revealed by microscopic techniques and recorded four larval instars for *P. cerealellae*. The external morphology of antennal sensilla of *P. cerealellae* was examined in Chapter V using scanning electron microscopy. Three morphologically different olfactory sensilla types were recorded on the antennae of both sexes with major sexual differences in the abundance of the multiporous sensilla trichodea type III and the multiporous placoid sensilla. In Chapter VI, I documented the mating behavior of *P. cerealellae*. Females exhibit "calling behavior" to initiate courtship. Males play the active role, exhibiting most of the observed pre-mounting behaviors. The role of semiochemicals in mediating intraspecific communication in *P. cerealellae* was investigated in Chapter VII using electroantennogram (EAG), behavioral (olfactometer) and analytical (gas chromatography) techniques. Results suggest the existence of male- and female-produced semiochemicals, possibly of cuticular origin, in this species. In Chapter VIII, the role of host-related semiochemicals in the host location of *P. cerealellae* was investigated using EAG, behavioral and analytical techniques. Results suggest the use of multiple olfactory stimuli in host finding by female *P. cerealellae* with preference for stimuli from host habitats (cowpea seed odor).

VITA

Ebenezer Oloyede Onagbola, son to Chief Emmanuel (Late) and Mrs. Maria Onagbola, was born on January 2, 1973 in Lagos Island, Lagos State, Nigeria. He graduated from The Federal University of Technology, Akure, Ondo State, Nigeria with a Bachelor of Technology (B. Tech.) degree in Biology (Food Storage Technology Option) in the year 2000 and Masters of Technology (M. Tech.) degree in Food Storage Technology in the year 2003. He started graduate program in Entomology in the Department of Entomology and Plant Pathology, Auburn University, Auburn, Alabama, USA, in 2004.

ACKNOWLEDGEMENTS

My research and development as a man and scientist benefited greatly from constant guidance, support and patience of my adviser and brother, Dr. Henry Yemisi Fadamiro. His enthusiasm and generosity with time has contributed immensely to the success of this work. I am also indebted to the other committee members: Dr. Micky D. Eubanks, Kathy Flanders, Dr. Arthur G. Appel and the dissertation outside reader, Dr. Jack W. Feminella for their genuine interest, time and valuable advice. I would like to acknowledge Dr. Wayne Clark and Dr. Henry Fadamiro for the teaching experience I gained from working with them. My profound gratitude go to Professors O. O. Odeyemi (Mrs), C. O. Adedire and Dr. M. O. Ashamo for having considered and recommended me to my advisor. Elly Maxwell, Shelia Boyt, Drs Chen Li, Vincent Umeh, and Hongyi Wei, Akinyode Morakinyo, Yinfang Xiao, Clement Akotsen-Mensah, Ramohan Rao Balusu, Timothy Nafziger and Prithwiraj Das whom I worked with at one time or the other in Fadamiro's lab, made my time productive and fun-filled. I am deeply grateful to my parents and siblings for their love and supports in giving me the opportunity to be educated. Special thanks to Ms. Erica Deon Phelps for her love and encouragement. I am deeply grateful to my pastors for their unceasing prayers and I am particularly grateful to God for making my dream a reality. My sincere thanks also go to the Department of Entomology and Plant Pathology for financial assistance in the form of a Graduate Research/Teaching Assistantship.

Style manual or journal used: Biological Control

Computer software used: Microsoft® Office XP, JMPIN® Version 5.1, SAS Institute Inc., 2003 and SAS for Windows, 2003.

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Figure 2 Responses of mated female *P. cerealellae* to various host-related stimuli treatments in a Y-tube olfactometer. Figure shows responses of mated female parasitoids when given a choice between hexane (control) and different host-related stimuli: extracts of uninfested cowpea seeds, bruchid (*C. maculatus*)-infested cowpea seeds, larval frass, bruchid larvae, adult female bruchid, and adult male bruchid. Grey

bars indicate the percentage responses to the control; white bars indicate the percentage responses to the tested stimuli. N = 40 individuals per choice test. Asterisks (*) indicate significant differences within a choice test ($P < 0.05$, χ^2) 244
Figure 3 Gas chromatographs of the various extracts tested in electroantennogram and behavioral tests: uninfested cowpea seeds, bruchid (*C. maculatus*)-infested cowpea seeds, larval frass, bruchid larvae, adult female bruchid, and adult male bruchid. Numbers on the peaks represent the various recognizable peaks (compounds) as analyzed by the GC. Peaks from different extracts having the same number were identified as being the same based on retention times (RT) 245

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Cowpea Seeds

Cowpea, *Vigna unguiculata* (L.) Walp. is an important crop in many parts of the semi-humid tropics where it provides more than half of the plant protein in human diet (Rachie, 1985; Mbata et al., 2000). It is widely cultivated and eaten in tropical Africa (Ohiagu, 1985). Nigeria is the world's leading producer of cowpea, accounting for 2,317,000 metric tones (2004 data), which is approximately 59 % of the total world production (FAOSTAT, 2005). Cowpea is commonly called black eye peas in the United States of America (USA). California is responsible for roughly 80% of black eye peas production in the USA (NASS, 2003; Johnson and Valero, 2004). A major constraint to cowpea production worldwide is the infestation by a number of insect pests at different stages of the crop growth, resulting in poor yield in the unprotected crop (Soyelu and Akingbohungbe, 2007).

The cowpea bruchid, Callosobruchus maculatus

The major most important post harvest pest of cowpea is the cowpea bruchid, C*allosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae) (Jackai and Daoust, 1986; Johnson and Valero, 2004). *Callosobruchus maculatus* is a cosmopolitan pest of cowpea of West African origin, from where it has spread throughout the tropical and subtropical

world as a pest of seed legumes (Southgate, 1978). Females of *C. maculatus* lay eggs on surfaces of various seed legumes, including cowpeas (*V. unguiculata*) and mung beans (*V. radiata* (L.) Wilczek) (Southgate, 1979). Larval and pupal developmental times may range from 9 d to 8 mo. and 3 to 53 d, respectively, depending on environmental conditions (Dick and Credland, 1984). Newly emerged adults may spend one to several quiescent days inside the seed before eating its way out. Adults range in size from 2 - 5 mm and have patterns of reddish-brown, black, white and tan depending on sex and polymorphic dispersal stage (Larson and Fisher, 1938; Utida, 1972). Infestation of cowpeas by *C. maculatus* usually starts from the field when pods are maturing just before the yellowing stage and continues in storage (Huignard et al., 1985; Hagstrum, 1985; Ouedraogo et al., 1996). Bruchid infestation often reduces the market value of cowpea seeds and sometimes renders the seeds unacceptable for human consumption (IITA, 1989; Mbata et al., 2000). Cowpea bruchids multiply rapidly in storage (Ouedrago et al., 1996; Sanon et al., 1998) and untreated populations can cause complete loss of stored cowpea within 6 months (Caswell, 1961; Jackai and Daoust, 1986). For instance, estimated annual losses to cowpea in Nigeria due to cowpea bruchids are in the range of 30-40% of stored cowpea crop in storage, translating to > $30 million (US Dollars) (Jackai and Daoust, 1986; Youdeowei, 1989).

Control of pests of stored cowpea

Several traditional measures for protecting harvested cowpea are in use in subsistence agriculture, but their efficacy is often unverified (van Alebeek, 1996). In domestic situations, subsistent farmers use fresh and deodorized oils (Ajayi et al., 1987;

Ketoh et al., 2002), plant powders such as pulverized *Piper* spp. (Ivbijaro and Agbaje, 1986), sand or wood ash (Chinwada and Giga, 1997) and other plant extracts to preserve cowpea from insect damage (Ketoh et al., 2002). These treatments are only effective in domestic situations. Conventional farmers typically use chemical insecticides including methyl bromide and phosphine gas to protect their stored grains (Mbata, 2004a), but these insecticides are known to have various setbacks (Bond, 1984; Leesch et al., 1995). For instance, the use of methyl bromide has been scheduled to end worldwide by 2020 under the terms of Montreal Protocol (UNEP, 1998). The use of other pesticides for protection of stored products is also facing restriction due to evolution of pests' resistance to pesticides (Hagstrum et al., 1999; Phillips et al., 2000) and potential non-target effects of conventional pesticides (Ketoh et al., 2002).

The inherent problems associated with chemical insecticides, such as development of resistance by insects, environmental pollution, contamination of stored grains and insecticidal poisoning have prompted scientists to seek alternative methods of pest control (Fields, 1992; Mbata et al., 2000). Johnson and Valero (2004) and Fields and White (2001) suggested the use of temperature treatments for protection of stored-products. However, some pests of stored products including *Rhizopertha dominica* (Mbata and Phillips, 2001) and *Tribolium castaneum* (Qaisrani and Beckett, 2003) have been reported to show tolerance to such temperature treatment.

Isman (2000) suggested the use of essential oils and Schmale et al., (2001) suggested complementing the use of essential oils from aromatic plants with the use of natural enemies. Ketoh et al. (2002), however, observed that plant extracts added to stored cowpea reduced the population of the parasitoid, *Dinarmus basalis* (Rondani)

(Hymenoptera: Pteromalidae). Biorational alternatives such as pheromone-based technology has also been successfully used to manage several stored products insects including the Indian meal moth, *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae) (Brady et al., 1971; Zhu et al., 1999), almond moth, *Ephestia* (*Cadra*) *cautella* (Walker) (Kuwahara et al., 1971; Allison and Cardé, 2006) and Mediterranean flour moth, *Anagasta* (*Ephestia*) *kuehniella* Zeller (Traynier and Wright, 1972; 1973). However, little is known about the identity of the pheromones of *C. maculatus* (Phillips et al., 1996; Nojima et al., 2007), making pheromone-based technology an unviable management tactic for this pest at present. Complementing biological control with non-toxic means of control including planting of resistant crop varieties in integrated pest management programs was suggested by Dorn (1998). Dorn (1998) emphasized that this approach will be of particular significance for on-farm storage in preserving the density and diversity of natural enemies. For example, in Africa, eggs of *C. maculatus* were observed to be parasitized in the field and in storage by a minute egg parasitoid, *Uscana lariophaga* (Hymenoptera: Trichogrammatidae) (Lammers and van Huis, 1990; van Huis et al., 1991; Sagnia, 1994).

Biological control of pests of stored products through the use of parasitoids

Parasitoids play a major role in sustainable agriculture through their ability to regulate populations of herbivorous insect pests (Godfray, 1994; Wäckers, 2004). Many parasitoid species are associated with insect pests of stored products (Brower et al., 1996) and they represent potential biological agents for such pests (Donnelly and Phillips, 2001). Parasitoids which have been investigated as biological control agents of *C.*

maculatus include *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) (Williams and Floyd, 1971; Brower et al., 1996), *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) (Jacob and Evans, 2004), *D. basalis* (Ouedrago et al., 1996; Sanon et al., 1998), *Eupelmus vuiletti* (Crw) (Hymenoptera: Eupelmidae) (Cortesero et al., 1993), *Heterospilus prosopidis* (Viereck) (Kistler, 1985), *Lariophagus distinguendis* (Först) (Hymenoptera: Pteromalidae) (Bellows, 1985), *Uscana lariophaga* Stephan (Hymenoptera: Pteromalidae) (van Huis et al.,1991; Sagnia, 1994), and *Pteromalus cerealellae* (Ashmead) (Hymenoptera: Pteromalidae) (Brower, 1991; Mbata et al., 2004b).

Host location by insect parasitoids

Parasitoid host location has received significant attention due to their importance in the control of pest insects (Cortesero et al., 1993; Steidle and Schöller, 1997; Steidle et al., 2003; Mbata et al., 2004b). The process of locating and parasitizing a host is a sequential one. A parasitoid must first find an appropriate host habitat where its host may be located (Sokolowski and Turlings, 1987). It then begins to search for the host (Vinson, 1976; 1998). Finally, the parasitoid evaluates the suitability of the host before acceptance (Vinson, 1975; Quicke, 1997). Host location and host acceptance in parasitic wasps is an active field of research, much of which is centered around identification of stimuli and characterization of behavioral responses (Godfray, 1994; Quicke, 1997). Insect parasitoids actively move through their environment to explore resources for feeding, nesting, mates, refugia, oviposition, and even new or different habitats. The success of this searching depends largely on the insect's search strategy relative to resource

availability and distribution, the insect's efficiency in locating the resources, and its ability to adjust to environmental changes by adaptation (Bell, 1991). Natural selection favors adaptations in morphology, physiology and behavior that are the best suited compromises or trade-offs with respect to the various demands of an organism's environment (Begon et al., 1990).

Insect parasitoids experience intense selection pressure to locate hosts for oviposition, and employ a complex assemblage of cues leading them towards the host (Godfray, 1994). The host insect, in turn, tries to avoid detection or parasitization pressure and so, uses a broad range of cryptic strategies or minimization of odor emission (Vet et al., 1991). The developing stages of endophytic hosts (which feed and pupate within seeds, wood, stems, or flowerheads) are physically protected within plant tissues and particularly inconspicuous (Hawkins and Lawton, 1987). To locate concealed hosts, visual or chemical cues derived from the host habitat (e.g., plants or grains) as well as chemical or physical (including mechanical [vibrational]) cues derived from the host may be used by searching parasitoids (Vet et al., 1991; Vet, 2001).

Host location by the use of chemical cues

The efficiency of parasitoid foraging is enhanced through the use of cues that are associated with their hosts. Parasitoids have evolved to use volatile cues that are reliable and consistent in indicating the presence of suitable hosts (Vet and Dicke, 1992). Parasitoids use direct and indirect cues to locate their hosts (Vet et al., 1991; Vet and Dicke, 1992; Morgan and Hare, 1998). Cues derived directly from the stage of host used by parasitoids tend to be innate and are the most valuable because they reduce the time

spent by parasitoids in search of suitable habitats or host stages (Vet et al., 1991). Cues directly from the host tend to be non-volatile contact kairomones (e.g., Hare et al., 1993; Hare, 1996), although some parasitoids also exploit volatile pheromones of their hosts as kairomones (Haris and Todd, 1980; Hardie et al., 1994; Feener et al., 1996; Mbata et al., 2004b).

Many parasitoids rely on less reliable, but more detectable, cues, which usually originate from the sources other than the attacked hosts and are directly associated with host presence (Vet et al., 1991; Vet and Dicke, 1992). Vet and Dicke (1992) divided indirect cues available for foraging parasitoids into 3 categories: (i) cues from hosts of the same species, but a different stage from that parasitized (infochemical detour cues, such as host pheromones); (ii) cues produced by plants damaged by herbivores (herbivore-induced synomones); and (iii) cues produced by healthy and damaged plants (plant volatiles). Volatile chemicals emanating from infested plants or from hosts' by-products such as frass or mandibular gland secretions can be important long-distance and contact cues for parasitoids of concealed hosts (Vet and Dicke 1992; Potting et al. 1995; Dutton et al., 2000; Vet, 2001), as they may guide parasitoids during approach into the microhabitats of their hosts (Turlings et al., 1998). When parasitoids arrive at the host microhabitat, they may switch to another set of cues (Vinson, 1998).

Host location by the use of physical cues

Parasitoids may also use mechanical cues including vibrations due to host activities (Shade et al., 1990; Vet and Alphen, 1985; Hailemichael et al., 1994; Wäckers et al., 1998), visual cues (Smith et al., 1993; Wäckers and Lewis 1999), and tactile cues

including antennal searching (Vet and Alphen, 1985) and ovipositor probing (Vet and Alphen, 1985) during host searching. For parasitoid species which specialize on the developing stages of endophytic hosts, the task of host location is even more challenging. Mechanical cues are more likely to be used by such parasitoids since (i) larval stage is the active feeding stage (Shade et al., 1990; Meyhöfer et al., 1994), and (ii) host odor produced from feces (or frass) or even from the host itself, if any, might not be released through the concealing plant tissue (Godfray, 1994). Vibrations emanating from hosts which are feeding or moving have been reported to induce behavioral responses in a number of parasitoid species (Lawrence, 1981; Vet and Alphen, 1985; Meyhöfer and Casas, 1999).

Parasitoids also use visual cues from a distance during host finding, as do honeybees when searching for flowers (e.g. Giurfa and Lehrer, 2001; Ne'eman and Kevan, 2001) or as close-range cues to guide foraging females to plant parts that are structurally damaged or discolored due to herbivory (Hawkins, 1988; Sugimoto et al., 1988; Smith et al., 1993) or to plant structures that the parasitoids have associated with the host (Wardle and Borden, 1990). Visual cues can lead parasitoids into the vicinity where hosts may be found and retain them within such contaminated areas. Parasitoids of concealed hosts may rely on infrared radiation from the host's body (Richerson and Borden, 1972) and, consequently, on plant- or host-derived physical cues.

In general, there can be variation in the cues used by parasitoids in host location depending on the habitat of the hosts, the stage of host being parasitized, and the phase of the host-finding process. The host-searching insects evaluate the informational content and the reliability of the different cues and use them interactively in a hierarchical order

(Turlings et al., 1993; Godfray, 1994; Vet et al., 1995; Völkl, 2000). In order to reveal the mechanisms underlying host location in a multisensory context, experimental studies become necessary to (i) investigate which cue(s) are involved in the host location mechanisms of *P. cerealellae*, and (ii) determine the relative importance of semiochemicals from first (cowpea seeds) and second (bruchid-specific odors) trophic levels in host location by this species.

Study organism

The study parasitoid, *Pteromalus cerealellae* (Ashmead) is a member of the Hymenopteran family Pteromalidae (Chalcidoidea), which includes > 3500 species inhabiting different habitats. Pteromalids are excellent study organisms because they exhibit life histories which are widespread among Hymenopteran parasitoids and, thus, research findings on them might be transferable to other parasitoid taxa. For example, courtship behavior and mating systems are better understood in this family than in many other parasitoid taxa (van den Assem, 1986). Most members of the pteromalid family are easy to work with in that they have short generation time, high offspring production, and some of the hosts which can be used to rear them are commercially available.

Pteromalus cerealellae is a solitary, generalist larval and prepupal ectoparasitoid of several concealed pests of stored products (Brower, 1991; Wen and Brower, 1994). Described in 1902 as *Catolaccus cerealellae* Ashmead (Ashmead, 1902) and placed in the genus *Habrocytus* Thomson, where it remained until Boucek (1977) lumped this genus under *Pteromalus* Swederus because no clear diagnostic character enables separation of the 2 groups. Native to West Africa (Southgate, 1978), it has been reported

from many parts of the world, including USA (Wen and Brower, 1994). It was reported to be monophagous on *Sitotroga cerealella* Olivier (Lepidoptera: Gelechidae) (Krombein et al., 1979; Boucek, 1988), but earlier report by Flanders (1930) indicated its potential to attack other pests of stored grains. Wen and Brower (1994) reported the potential host range of this parasitoid to include *Lasioderma sericorne* (Fab.) *Rhyzopertha dominica* (Fab.), *Callosobruchus maculatus* (Fab.), *Sitophilus zeamais* (Motch.). *Pteromalus cerealellae* is markedly polyphagous (Brower, 1991), so it is considered a potential candidate for biological control of pests of stored products (Brower, 1991; Mbata et al., 2004b; Onagbola et al., 2007). Brower (1991) reported the cowpea bruchid, *C. maculatus* to be its most susceptible stored-product beetle host. Females of *P. cerealellae* lay eggs in host larvae, which typically are concealed within grain seeds. Generally, information on the biology and life history strategy of this species is scant. For example, little is known about the developmental biology of *P. cerealellae* (Noble, 1932; Fulton, 1933). Most of the available publications on *P. cerealellae* focus on its potential utilization for biological control of pests of stored products (Smith et al., 1995; Wen and Brower, 1994; Mbata et al., 2004b; Onagbola et al., 2007). Only one published work is available on host location behavior of *P. cerealellae* (Mbata et al., 2004b); moreso, information on sexual communiation by *P. cerealellae* is completely lacking.

Dissertation outline, goals and objectives

My dissertation is made up of 3 themes with the basic goal of studying some aspects of the developmental biology and host location behavior of *P. cerealellae*. Theme 1 (Chapters II and III) focuses on the developmental biology and examines the influence

of some environmental factors on lifespan and progeny production by females of this species. Theme 2 (Chapters IV and V) is on morphological studies of *P. cerealellae* and the focus of theme 3 (Chapters VI, VII and VIII) is on mate and host location behaviors.

In Chapter II, the influence of diet (sugar feeding), host provision, and mating on the longevity, fecundity, and progeny sex ratio of *P. cerealellae* was investigated. Sugar feeding (25% sucrose solution) increased male lifespan by a factor of 3 to 4 relative to sugar-starved (provided water only) or completely starved (provided no water and no sugar solution) males, irrespective of mating status or host provision. Sugar feeding also increased longevity of females which were not provided hosts, but had no effect on longevity of females which were provided hosts. Females had a significantly greater longevity than males: mean (± SE) longevity of sugar-fed unmated females provisioned with no hosts (36.3 ± 1.2 d) was significantly greater than mean longevity of sugar-fed unmated males provisioned with no hosts (29.7 ± 0.9 d). A negative effect of mating on longevity was recorded only in the absence of hosts but not when hosts were provided. In general, sugar feeding resulted in only a modest increase in progeny production by female *P. cerealellae*: cumulative lifetime progeny (mean ± SE) of sugar-fed females (64.2 ± 9.2) was not significantly greater than that of sugar-starved (44.5 ± 6.8), or completely starved (54.5 ± 9.3) females. However, progeny of sugar-fed females was female-biased (53% females) compared to male-biased progeny recorded for completely starved females (37% females).

In Chapter III, the suitability of frozen host larvae for rearing *P. cerealellae* was investigated. The reproductive potential (number and sex ratio of progeny) of female *P. cerealellae* was compared on live (fresh) *C. maculatus* larvae (concealed within cowpea

seeds) versus frozen larvae (obtained by freezing infested cowpea seeds at -20˚C for 48 h) which were subsequently thawed and held at ambient conditions (~25 ± 1ºC, 50 ± 5% r.h.) for 4, 24, 48, 72, 96, and 120 h before exposure to female parasitoids. No significant differences were recorded in the numbers and sex ratios of the progeny produced by female *P. cerealellae* on live larvae compared to frozen host larvae that were thawed and held at ambient conditions for up to 96 h, suggesting that live and frozen larvae of *C. maculatus* are equally suitable for rearing *P. cerealellae.* However, the data showed that progeny production on frozen hosts gradually declined with thawing duration and was significantly reduced at the thawing duration of 120 h. When live and frozen host larvae were simultaneously presented to female *P. cerealellae* at different exposure periods, relatively greater progeny production was recorded on live hosts than on frozen hosts at 12, 24 and 48 h of exposure.

In chapter IV, I documented the development of *P. cerealellae* within fourth-instar larvae of its concealed host, *C. maculatus* infesting cowpea seeds and characterized the preimaginal life stages of the parasitoid for the first time using morphological structures revealed by microscopic techniques including scanning electron microscopy (SEM). *Pteromalus cerealellae* produces hymenopteriform eggs and larvae. Eggs hatch into 13-segmented first-instar larvae with peripneustic condition of spiracles. Larvae have simple, tusk-like mandibles whereas mandibles of pupae and adults are of the conventional toothed types. Using statistical analyses of the sizes of the larval mandibles and head capsules in conjunction with reliable characters including the number of exuviae on the body of parasitoid larvae, cuticular folding, and excretion of the meconium, we recorded 4 larval instars for *P. cerealellae.* The data showed significant

positive correlations between larval mandible lengths and widths of larval head capsules, as well as between mandible lengths and larval instars, suggesting that mandible length is a good predictor of the number of instars in *P. cerealellae*. Developmental time from egg to adult emergence was ~12 d for females and ~11 d for males at 30 ± 1˚C, 70 ± 5% r.h., and L12: D12 photoperiod.

In order to provide requisite information to support my research on the mechanisms used by *P. cerealellae* for mate (ChapterVII) and host location (Chapter VIII), I examined in Chapter V the external morphology of the antennal sensilla of this parasitoid using SEM. Antennae of male and female *P. cerealellae* are geniculate in shape, ~1300 µm in length, and consist of 15 antennomeres. Eight morphological sensilla types were recorded in both sexes, including 4 types of highly abundant and widely distributed sensilla trichodea (types I, II and IV are aporous, while type III is multiporous), basiconic capitate peg sensilla, coeloconic sensilla, chaetica sensilla, and the most conspicuous plate-like placoid sensilla. Detailed examination of sensilla morphological features including pore presence, and numbers suggest that the multiporous type-III sensilla trichodea and the multiporous placoid sensilla may play a role in olfaction, whereas the uniporous chaetica sensilla may function as contact chemoreceptors. The types I and II sensilla trichodea are presumably mechanosensory, while type-IV sensilla trichodea may function as proprioceptors. The basiconic capitate peg sensilla and coeloconic sensilla probably function in thermo-hygro reception. Shape, structure, and size of antennae of males and female were basically similar, but major differences were recorded between sexes in the distribution of some sensilla types. The type-II sensilla trichodea and the multiporous placoid sensilla are relatively more

abundant in females, whereas males have greater number of the multiporous type-III sensilla trichodea than females.

In Chapter VI, I documented the mating behavior of *P. cerealellae* using a JVC digital video camera Model GR-D244U attached to a stereomicroscope (National Microscope, Model DC 3-420, Meiji, Japan). Courtship behavior in *P. cerealellae* consists of a series of behavioral transitions, and is initiated when the female begins to exhibit "calling behavior", which probably results in the release of a sex pheromone for male attraction. Males play the most active role, exhibiting most of the observed pre-mounting behaviors including "alert" (i.e. antennation, swaying of abdomen and wing fanning), "trail", "follow", and "agitate" behaviors. In close proximity to the male, the female exhibits "antennal drumming" behavior, which may suggest involvement of male-produced close-range contact chemicals. During mounting, the male makes antennal contact (touch) with the female's antennae probably to enhance her sexual receptivity. In *copula*, males typically assume posterior dorso-lateral position and are relatively passive. Copulation duration in *P. cerealellae* is short (~12 s), and was neither affected by previous mating experience nor had a significant effect on progeny production and sex ratio. Once copulation has occurred, the male dismounts from the female and does not exhibit post-copulatory behaviors. The female, however, which was relatively passive during copulation begins to exhibit avoidance behavior by running away from the male. Total courtship duration in freshly emerged inexperienced *P. cerealellae* was ~2744 s. Previous mating experience had a significant effect on total courtship duration. Female mating experience significantly increased courtship duration, whereas male mating experience served to reduce courtship duration. Previous mating experience also had a

significant effect on progeny production: the highest number of progeny was produced by inexperienced male × experienced female mating pair, and was significantly male-biased.

The role of semiochemicals in mediating intraspecific communication in *P. cerealellae* was investigated in Chapter VII. 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 electroantennogram (EAG) responses than males to whole body extracts of both sexes, but showed similar EAG responses to male and female whole-body (WB) extracts. Results from Y-tube olfactometer bioassays demonstrated significant attraction of both sexes to live conspecifics of the same (intrasexual attraction) and opposite sex (intersexual attraction). Females also showed significant attraction to WB extracts of conspecific females, but not to extract of conspecific males. However, WB extracts of males and females did not elicit significant attraction in conspecific males. Gas chromatographic (GC) analyses of the chemical profiles of whole body extracts of *P. cerealellae* males and females revealed quantitative and qualitative differences in chemical composition, which may explain the above results. These findings provide preliminary evidence for possible existence of a female-produced sex pheromone and the production of courtship pheromones 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*.

In Chapter VIII, the role of host-related semiochemicals in host location of *P. cerealellae* was investigated using EAG, behavioral (olfactometer), and analytical (GC)

techniques. Responses of mated and unmated female *P. cerealellae* were tested to a variety of host-related chemical stimuli including hexane extracts of uninfested cowpea seeds, (bruchid)-infested cowpea seeds, bruchid larvae (WB), larval frass, adult female bruchids (WB), and adult male bruchids (WB). All of the tested stimuli elicited significant EAG response in unmated and mated female *P. cerealellae,* with mated females exhibiting greater EAG response than unmated females to some treatments. Results from Y-tube olfactometer bioassays demonstrated significant response of mated female *P. cerealellae* to extracts of uninfested cowpea seeds, infested cowpea seeds, adult female bruchids, bruchid larvae, and larval frass, but no significant response was elicited by extract of adult male bruchids. Pair-wise comparisons of the four most attractive stimuli (i.e. uninfested seeds, infested seeds, bruchid larvae, and larval frass) in a four-way olfactometer showed preference of mated female *P. cerealellae* for extract of uninfested cowpea seeds compared to larval frass extract. No significant differences were recorded between other paired treatments. GC analyses of chemical profiles of the various tested stimuli revealed quantitative and qualitative differences in chemical compositions, which may explain the observed greater parasitoid response to cowpea seed extracts.

It is hoped that this dissertation will provide the much-needed information on the morphology and biology of *P. cerealellae*. Findings of this work will also provide new insights regarding the cues mediating sexual communication and host location in this parasitic wasp and advance our knowledge of host-parasitoid interactions. The results may also have practical implications in the utilization of this important parasitoid for biological control of stored-product insects.

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CHAPTER 2: LONGEVITY, FECUNDITY AND PROGENY SEX RATIO OF *PTEROMALUS CEREALELLAE* IN RELATION TO DIET, HOST PROVISION, AND MATING

INTRODUCTION

Parasitoids play a major role in sustainable agriculture through their ability to regulate populations of herbivorous insect pests. Many species of parasitoids are associated with stored-product insects with potential for utilization as biological control agents (Brower et al., 1996). One of such parasitoid species is *Pteromalus cerealellae* (Ashmead) (Hymenoptera: Pteromalidae), an ectoparasitoid of several pests of stored grains (Brower, 1991) including the cowpea bruchid, *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae) (Brower, 1991; Mbata et al., 2005). The ongoing interest in the potential utilization of *P. cerealellae* for biological control of stored product insects (Brower, 1991; Mbata et al., 2005) is, however, hindered by a gross lack of information on several aspects of its biology and life-history strategy. For example, little is known about the effects of food provision and physiological factors such as age and mating on survival and fitness of *P. cerealellae*.

A variety of factors may be responsible for the shortened longevity of parasitoids in the field compared with laboratory populations, but poor diet may play a significant role. Several parasitoids, in particular females, are known to utilize host and non-host foods (e.g., sugar sources) with important ramifications on their reproductive success (Jervis et al., 1996). Host feeding (consumption of host tissue) is a common occurrence in female parasitoids and has been observed in over 140 species across 17 families of Hymenoptera (Jervis and Kidd, 1986) and Diptera (Nettles, 1987). In addition to host feeding, several parasitoids have been reported to feed on artificial (e.g., sucrose solution and honey) and naturally occurring (e.g., nectar and homopteran honeydew) sugar sources both in the laboratory and field (Jervis et al., 1993; Heimpel et al., 1997; Olson et al., 2000; Fadamiro and Heimpel, 2001; Lee et al., 2004; Fadamiro and Chen, 2005; Fadamiro et al., 2005). Sugar feeding has been demonstrated to increase survival of adult parasitoids (Jervis et al., 1996; Leatemia et al., 1995; Wäckers, 2001; Fadamiro and Chen, 2005; Fadamiro et al., 2005), and may enhance fecundity, either through a positive effect on the rate of egg maturation or through increased lifespan, or both (Schmale et al., 2001, Heimpel and Jervis, 2005). However, some studies have suggested that host feeding may be a superior source of nutrients for parasitoid survival and fecundity than sugar feeding (Jervis and Kidd, 1986; van Lenteren et al., 1987; Heimpel and Collier, 1996). In contrast, provision of hosts may have a negative impact on parasitoid longevity, possibly by allowing for oviposition which may result in the death of females (Lim, 1986). No previous information is available on the ability of *P. cerealellae* to feed either on the hemolymph or the body tissues of its hosts.

Mating is another factor that can potentially impact insect survival. Mating can increase allocation of resources to reproductive tasks such as oogenesis and egg maturation in females leading to a significant reduction in lifespan (Partridge and Farquhar 1981; Reznick, 1985; Ellers, 1996; Wheeler, 1996; Jacob and Evans, 2000). In fact, several studies have reported a negative impact of mating on longevity of parasitoids (Li et al., 1993; Carpenter, 1995; Jacob and Evans, 2000; Sagarra et al., 2002). In this study, we investigated the effects of diet, host provision, mating, and possible interactions of these factors on the longevity and fecundity of *P. cerealellae*. Knowledge of the influence of these factors on the fitness of *P. cerealellae* should aid in the current efforts aimed at utilizing this parasitoid for biological control of stored-product insects.

MATERIALS AND METHODS

Insects

Callosobruchus maculatus was utilized as host for *P. cerealellae* in this study. The starting culture of *C. maculatus* was initially obtained from Fort Valley State University, Fort Valley, GA, USA (contact: Dr George Mbata), where it has been reared continuously on cowpea seeds (*Vigna unguiculata* Walp.) for several years. *Callosobruchus maculatus* was reared in our laboratory on cowpea seeds (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 ± 1ºC, 70 ± 5% r.h., and L12: D12 (Mbata et al., 2005). The beetles were allowed to lay eggs on the seeds for 24 hours after which they were removed with an aspirator. The infested seeds were incubated at the conditions

specified above for 15 to 18 d (Mbata et al., 2005). Based on the results of a preliminary experiment (unpublished data), fourth-instar larvae of *C. maculatus* were used as parasitoid hosts for *P. cerealellae* in this study.

The original culture of *P. cerealellae* was obtained from Fort Valley State University, Fort Valley, GA, USA where the parasitoid has been reared continuously for several years. *Pteromalus cerealellae* culture was maintained in our laboratory by transferring ~30 adult pairs onto a glass jar containing *C. maculatus*-infested cowpea seeds at a stage when most of the bruchid larvae were at the fourth instar. This was determined in a preliminary experiment to occur ~15 d 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 5 d of oviposition and the attacked *C. maculatus* larvae incubated in a growth chamber at the above environmental conditions until the emergence of adult parasitoids.

Effect of diet, host provision, and mating on longevity

This experiment simultaneously tested the effects and interactions of diet (3 diet treatments), host provision (host provisioned versus no host), and mating (unmated versus mated) on longevity of female and male *P. cerealellae*. The 3 diet treatments evaluated were: i) completely starved (provided no water and no sugar); ii) sugar-starved (provided water only); and iii) sugar-fed. The various combinations of the three factors (diet, host feeding, and mating) resulted in a total of 12 treatment combinations for each sex. We demonstrated in a preliminary experiment that *P. cerealellae* is arrhenotokus (female progeny produced only when parent female has mated). Freshly emerged adults of *P. cerealellae* were placed in groups of 2

individuals either of the same sex (unmated treatments) or of opposite sex (mated treatments) in a 6-cm diameter plastic Petri dish containing either 10 uninfested cowpea seeds (no host treatments) or infested cowpea seeds containing ~80 fourth instars of *C. maculatus* (host provisioned treatments). Petri dishes were then randomly assigned to the 3 diet treatments (completely starved, sugar-starved, or sugar-fed). With the exception of the completely starved treatment, water was provided in all treatments by filling a 0.5-mL microcentrifuge tube with distilled water and threading a cotton string through a hole in the cap of the tube. Water tubes were refilled as needed. For the treatments involving sugar feeding, 25% sucrose solution was smeared on the inside of the Petri dish cover as needed (every other day). In treatments involving host provision, parasitoids were supplied with fresh hosts (~80 fourth-instar larvae of *C. maculatus*) every 5 d. The number of hosts was determined by counting the number of egg plugs of *C. maculatus* on infested cowpea seeds. Only seeds with 1 to 3 *C. maculatus* egg plugs were used. In this and subsequent experiments, Petri dishes were kept at 30 ± 1ºC, 70 ± 5% r.h., and L12:D12, and dishes were checked daily for parasitoid mortality. Twenty parasitoids of each sex were tested for each treatment combination. Data were analyzed by using proportional hazard modeling (SAS Institute, 2003) to test for effects and interactions of diet, host provision, mating, and sex on survivorship. Longevity data for each sex were then square-root ($\sqrt{(x + 0.5)}$) transformed and subjected to analysis of variance (ANOVA) followed by the Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons of means at $P < 0.05$ (JMPIN Version 5.1, SAS Institute Inc., 2003).

Effect of diet on progeny production

This experiment was conducted to determine lifetime progeny production (lifetime fecundity) by *P. cerealellae* females provided different diet treatments. Following the test protocols described in the previous experiment, freshly emerged females were paired with a male (for mating) in a 6-cm diameter plastic Petri dish. Infested cowpea seeds containing ~80 fourth-instar larvae of *C. maculatus* were provided in each Petri dish as hosts for females to oviposit on. The females were then assigned to each of the 3 diet treatments evaluated in Experiment 1. In order to maximize opportunity for oviposition, females were provisioned with new hosts in batches every 5 d throughout their lifetime. Provision of new batches of hosts to females every 5 d (i.e., at ages 1, 6, 11, 16, and 21 d) made it possible to determine any temporal fluctuations in oviposition and timing (age) of peak oviposition. Based on data from Experiment 1, which showed an average longevity of ~25 d for female *P. cerealellae*, this experiment was continued until females were 25 d old, thereby allowing each female to oviposit on 5 successive batches of new hosts (batch 1 = progeny produced at ages 1-5 d, batch 2 = progeny produced at ages 6-10 d, batch 3 = progeny produced at ages 11-15, batch 4 = progeny produced at ages 16-20 d, batch 5 = progeny produced at ages 21-25 d). Each batch of attacked host was incubated separately in a growth chamber until emergence of adult parasitoids. Emerging parasitoids from each batch were sexed and counted to determine progeny production and offspring sex ratio. For each female, the sum of number of progeny produced from successive batches of attacked hosts (5 batches total) was used to calculate cumulative (total) lifetime progeny production. At least 21 females were tested per diet treatment. Progeny production data obtained at each age were square-root

transformed ($\sqrt{(x + 0.5)}$) and analyzed by using two-way factorial ANOVA followed by the Tukey-Kramer HSD test for multiple comparisons of means at ($P < 0.05$) to test for effects of diet, age, and their interactions on progeny production. Cumulative fecundity data were further analyzed by using one-way ANOVA followed by the Tukey-Kramer HSD test ($P < 0.05$). Data on offspring sex ratio were analyzed by using a χ^2 2 × 2 test of independence with Yates' correction for continuity (Parker, 1979) to test for significant deviation ($P < 0.05$) from an expected 1:1 sex ratio of the progeny recorded per diet treatment for each host batch, as well as for the cumulative progeny.

RESULTS

Effects of diet, host provision, and mating on longevity

Proportional hazard analysis showed significant effects of diet ($\chi^2 = 471.03$, $df = 2$, $P < 0.0001$), host provision ($\chi^2 = 21.25$, $df = 1$, $P < 0.0001$), sex ($\chi^2 = 291.52$, $df = 1$, $P < 0.0001$) and mating ($\chi^2 = 12.36$, $df = 1$, $P < 0.0001$) on the longevity of *P. cerealellae* (Table 1). In addition, significant interactions were recorded between mating and host provision ($\chi^2 = 17.70$, $df = 1$, $P < 0.0001$), diet and host provision ($\chi^2 = 49.67$, $df = 1$, $P < 0.0001$), diet and sex ($\chi^2 = 141.49$, $df = 2$, $P < 0.0001$), as well as a significant diet x host provision x sex interaction ($\chi^2 = 43.97$, $df = 2$, $P < 0.0001$), suggesting that longevity is also influenced by multiple interactions among the various factors.

Differences were recorded on the influence of sugar feeding (diet) and host provision on longevity of mated and unmated female *P. cerealellae.* For females, sugar feeding exerted a significant positive effect on longevity when no hosts were provided, but no significant effect of sugar feeding was recorded when females were provisioned

with hosts (Fig. 1), possibly suggesting that females are capable of utilizing host materials for optimum longevity. Sugar feeding also exerted a significant effect on longevity of unmated females, irrespective of whether they were provisioned with hosts (Fig. 1). Similarly, sugar feeding exerted a significant effect on male survival: longevity of sugar-fed males was 3 to 4 times greater than longevity of sugar-starved or completely-starved males, irrespective of their mating status and whether they were provisioned with hosts (Fig. 2). Provision of hosts had a positive effect on longevity of mated males, but had no effect on longevity of unmated males, suggesting that male *P. cerealellae* are incapable of initiating host feeding, but may obtain valuable host materials from punctures made by the females. The effect of mating on male longevity was dependent upon whether or not hosts were provided. A negative impact of mating on longevity was recorded for males provided no hosts: unmated males provisioned with no hosts had a greater longevity than mated males provisioned with no hosts, irrespective of diet treatment (Fig. 2). However, completely starved mated males which were provided hosts had a greater longevity than completely starved unmated males, further confirming that males paired with females provisioned with hosts could obtain host resources from punctures made by females, which could result in increase in male lifespan. In addition, while a negative effect of mating was recorded on longevity of sugar-fed males provisioned with no hosts, no significant effect of mating on longevity was recorded for sugar-fed males provisioned with hosts, suggesting that provision of hosts may lessen the potential negative impact of mating on male longevity. In general, longevity was greater for females than for males, irrespective of the treatment combination. For instance,

average longevity of completely starved unmated female and male *P. cerealellae* provisioned with no hosts was 9.00 ± 0.58 and 5.70 ± 0.25, respectively (Figs. 1 and 2).

Effect of diet on progeny production

Two-way factorial ANOVA showed no significant effects of diet *(F* = 1.83, *df* = 2, *P* = 0.16) but age (batch) (*F* = 54.24, *df* = 4, *P* < 0.0001) on progeny production by female *P. cerealellae.* Diet*age interaction effect was not significantly different (*F* = 0.30, *df* = 8, *P* = 0.96). Progeny production by sugar-fed females on batch 4 hosts (i.e., progeny produced by females at ages 16-20 d) was significantly greater than progeny production by completely starved females at the same age range *(F* = 3.37, *df* = 2, *P* = 0.04, Fig. 3). A similar trend showing numerically greater progeny production by sugar-fed females was recorded for the other batches of hosts (i.e., batches 1, 2, 3 & 5), but this was not significant (Fig. 3). In general, age (batch) had a significant effect on progeny production, irrespective of diet: greater progeny was produced earlier in life than later in life (Fig. 3). Mean (± SE) progeny production by sugar-fed females was significantly greater at ages 1-5 d (37.86 ± 5.93) than at ages 6-10 d (16.67 ± 3.95), or at subsequent age ranges. Approximately 60-70% of the total lifetime progeny were produced within the first 5 days of female life, suggesting early peak oviposition (Fig. 3). The lowest number of progeny was produced on the last batch of attacked hosts (at ages 21-25 days). Similar results were obtained for sugar-starved and completely starved females, suggesting that diet has no effect on the timing of peak oviposition.

Mean cumulative (total) lifetime progeny production (lifetime fecundity) was not significantly different among diet treatments *(F* = 1.15, *df* = 2, *P* = 0.32). Mean (± SE)

lifetime fecundity (total/cumulative progeny) of sugar-fed females (64.24 ± 9.16) was not significantly greater than lifetime fecundity of sugar-starved (44.50 ± 6.81) or completely starved (54.55 ± 9.26) females. Nevertheless, significant effects of diet and age were recorded on sex ratio of progeny. In general, the progeny produced during the first 5 days of female life (at ages 1-5 days) was female-biased, compared to male-biased progeny recorded later in life (Fig. 4). Further chi-square analysis of the lifetime fecundity data showed a significant female-biased offspring for sugar-fed females (53%) compared to the significant male-biased offspring recorded for completely starved (37%) females. However, no significant difference in sex ratio was recorded for females provided water only (sugar-starved) (Fig. 4).

DISCUSSION

Sugar feeding is a major factor influencing adult lifespan of female and male *P. cerealellae*. Sugar feeding increased male lifespan by a factor of 3 to 4 relative to sugar-starved or completely starved males, irrespective of mating status or host provision. However, the magnitude of the impact of sugar feeding on longevity of females is affected by mating status and host provision. Our results suggest the following among others: i) females are capable of obtaining resources from host feeding for increased longevity, ii) sugar feeding represents an alternative food source for females in achieving increased longevity, iii) mating could have a negative impact on longevity of females and males *P. cerealellae* irrespective of whether there are opportunities for host feeding, iv) males are incapable of initiating host feeding, and sugar feeding, therefore, represents a major food source available for males to achieve increased longevity, and v) males paired

with females provisioned with hosts (mated treatments) could benefit from host feeding by obtaining resources from hosts punctured by females.

The positive impact of sugar feeding on adult longevity has been demonstrated for several parasitoids species from different taxa (Heimpel et al., 1997; Olson et al., 2000; Fadamiro and Heimpel, 2001; Lee et al., 2004, Fadamiro and Chen, 2005; Fadamiro et al., 2005; Chen et al., 2005). For some of these species, provision of water was also shown to increase longevity compared to completely starved (no water, no sugar) adults (Fadamiro et al., 2005; Chen et al., 2005). However, no difference in longevity was recorded between water-provided (sugar-starved) and completely starved male and female *P. cerealellae* provisioned with hosts in the current study, suggesting the positive effect of host availability on longevity of starved adults, as reported for *Trichogramma minutum* Riley (Hymenoptera: Trichogrammatidae) (Leatemia et al., 1995). In general, average lifespan was greater for female than for male *P. cerealellae*, as has been reported for several hymenopteran parasitoids (Olson et al., 2000; Fadamiro and Heimpel, 2001).

Our longevity data showed that only females that were not provided hosts benefited significantly from sugar feeding: sugar feeding did not result in significant increase in the lifespan of females provisioned with hosts. These results suggest that female *P. cerealellae* are capable of obtaining resources from host feeding for increased longevity, and that supplemental sugar feeding is beneficial only in the absence of hosts. Host feeding, a widespread phenomenon in female parasitoids (Jervis and Kidd, 1986; Nettles, 1987), has been shown to increase adult longevity, particularly in the absence of non-host food (Narayanan and Mookherjee, 1955), similar to the results of the current study. In addition, we recorded higher longevity for males paired with females (mated

males) provisioned with hosts compared to unpaired males (unmated males) provisioned with hosts suggesting that male *P. cerealellae* are capable of obtaining host materials from punctures made by the females for enhanced survival. Provision of hosts may adversely impact longevity possibly by allowing for oviposition, which may result in the death of females (Lim, 1986). Similar adverse effect of host availability on lifespan was obtained for *P. cerealellae* in the present study.

In general, mating had a negative effect on longevity of female and male *P. cerealellae* in the absence of hosts, but not when hosts were provided. A trade-off between mating and longevity has been reported for several parasitoids (Li et al., 1993; Carpenter, 1995; Jacob and Evans, 2000; Sagarra et al., 2002). A common hypothesis is that mating can negatively impact longevity by stimulating allocation of resources to reproductive activities (e.g., oogenesis and egg maturation in females), thereby reducing resources available for other life processes (Partridge and Farquhar, 1981; Reznick, 1985; Ellers, 1996; Jacob and Evans, 2000). This potential trade-off is more likely to occur when energy resources are limited, or in the absence of a high-quality food, as demonstrated by Jacob and Evans (2000). The authors reported adverse impact of mating on longevity of *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) females only in the absence of a high-quality food source such as honey (Jacob and Evans, 2000).

In the second experiment, we compared lifetime progeny production (lifetime fecundity) of females provided different diet treatments; each female having been supplied with a fresh batch of hosts every 5 d throughout her lifetime. Data from this experiment showed a major effect of age on progeny production with the greatest number

of progeny (> 60% of total progeny produced per female) produced during the first 5 days of life, irrespective of diet treatment, suggesting that peak oviposition by female *P. cerealellae* occurs early in life. In general, sugar feeding had a modest effect on the number of progeny produced on each successive batch of hosts: a significant effect of sugar feeding was recorded only for progeny produced by females at ages 16-20 d (batch 4 hosts). Nevertheless, cumulative total lifetime progeny was not significantly different among diet treatments. However, the higher female progeny recorded for sugar-fed females compared to completely starved females may indicate a potential benefit of sugar feeding in the production of female-biased progeny, an important consideration in the utilization of biological control agents. Sex allocation in many parasitoid species is known to be influenced by host quality (Chow and Heinz, 2005). Our results suggest that diet quality may have an effect on sex allocation, as was reported by Leatamia et al. (1995): the authors recorded 99 female offspring per lifetime of honey-fed female *T. minutum* compared to 65 female offspring per lifetime of unfed females. In addition, our data showed that the progeny produced early in life is female-biased while the progeny progeny later in life is preponderantly male-biased, as observed for *Trichogramma* spp. (Lim, 1986; Leatamia et al., 1995). The results showing a positive impact of sugar feeding on sex allocation by female parasitoids may have an important ramification for their use in biological control programs.

Our results suggest that sugar feeding may increase lifespan of *P. cerealellae*, in particular in the absence of hosts, and may enhance production of female-biased progeny. The data showing similar longevity for water-provided (sugar-starved) and completely starved *P. cerealellae* in the presence of hosts suggests that host fluid may serve as an

alternative to provision of a free water source. Host provision may have an effect on fecundity of female parasitoids as reported by several authors (Jervis and Kidd, 1986; van Lenteren et al., 1987). However, it is difficult to test the effect of host feeding on fecundity of *P. cerealellae* since the females are concurrent host feeders utilizing the same individual host for feeding and oviposition (personal observation). The experimental difficulty in separating the opportunity of a parasitoid to host-feed from that to oviposit was alluded to by Heimpel et al. (1996).

In summary, this study suggests among others, several interesting and sufficiently novel results, including the impact of diet quality on sex allocation and the observation of exploitative host feeding by males paired with females provisioned with hosts. These results offer new insights into parasitoid nutritional ecology and should prove invaluable for the development of efficient mass-rearing system for *P. cerealellae,* a prerequisite to its utilization for biological control of stored-product insects.

ACKNOWLEDGEMENTS

I thank Dr. Li Chen and Elly Maxwell for assisting with insect rearing. This research was supported in part by the Alabama Agricultural Experiment Station and an Auburn University Competitive Research grant to HYF.

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Table 1 Proportional hazard model testing for effects of diet, mating, host provision, sex, and interactions of these variables on longevity of *P. cerealellae*

Source of Variation	d.f.	χ^2	P
Diet	2	471.03	< 0.0001
Host provision	1	21.25	< 0.0001
Sex	1	291.52	< 0.0001
Mating	1	12.36	0.0004
Diet × Host	2	49.67	< 0.0001
Diet × Sex	2	141.49	< 0.0001
Diet × Mating	2	3.70	0.16
Host × Sex	1	0.49	0.48
Host × Mating	1	17.70	< 0.0001
Sex × Mating	1	0.41	0.52
Diet × Host × Sex	2	43.97	< 0.0001
Diet × Sex × Mating	2	0.15	0.93
Host × Sex × Mating	1	1.17	0.28
Diet × Host × Mating	2	3.01	0.22
Mating × Diet × Host × Sex	2	0.80	0.67

Figure 1 Effects of diet and host provision on longevity of female *P. cerealellae*. Figure shows the influence of diet (sugar-fed, sugar-starved, and completely starved) on mean longevity (days ± SE) of mated and unmated females in the presence or absence of hosts.

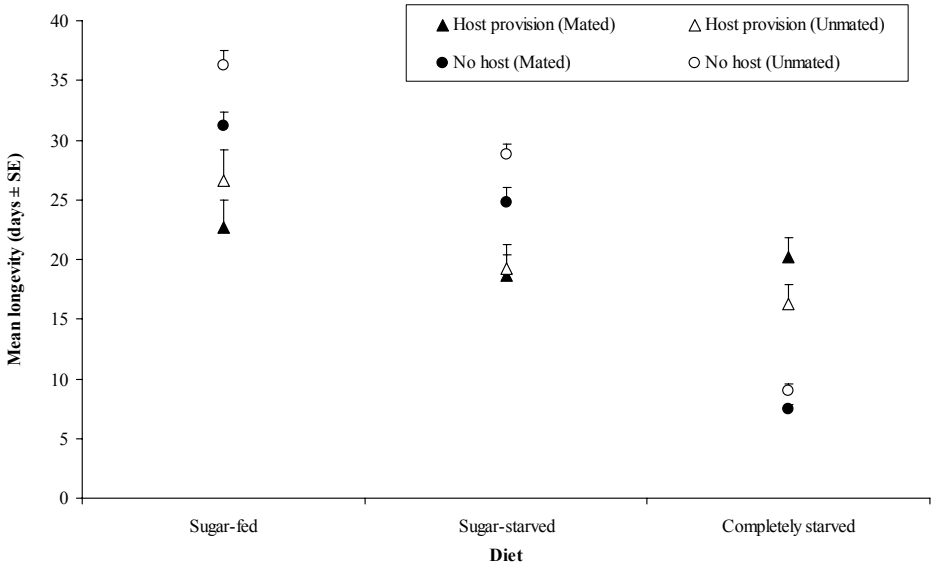


Figure 2 Effects of diet and host provision on longevity of male *P. cerealellae*. Figure shows the influence of diet (sugar-fed, sugar-starved, and completely starved) on mean longevity (days ± SE) of mated and unmated males in the presence or absence of hosts.

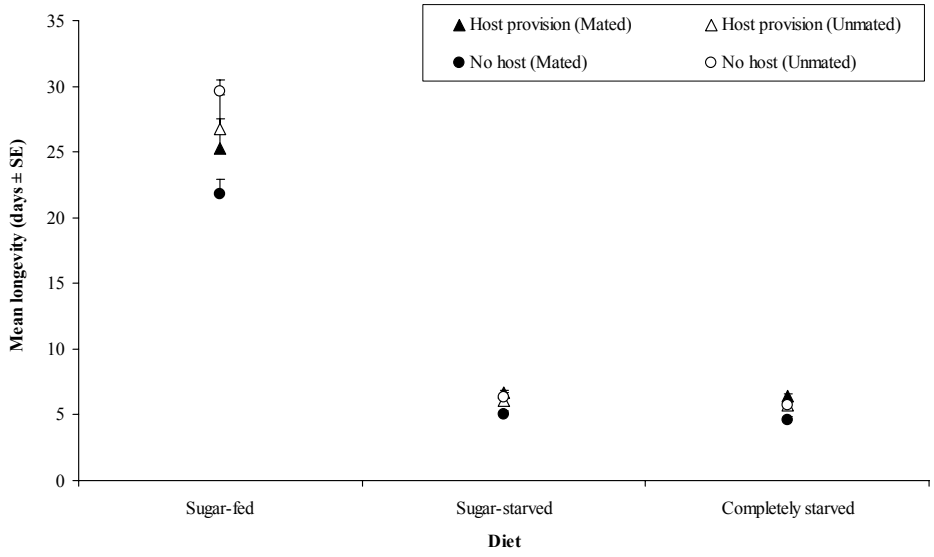


Figure 3 Fecundity of female *P. cerealellae* of different age ranges provisioned with different diet treatments. Figure shows mean (± SE) number of progeny produced per female at different age ranges, and the total cumulative progeny (lifetime fecundity). In this and the next figure, females were provisioned with new hosts (infested cowpea seeds containing ~80 fourth-instar larvae of *C. maculatus*) in batches every 5 d (i.e., at ages 1, 6, 11, 16, and 21 d) throughout their lifetime (~25 d) thereby allowing each female to oviposit on 5 successive batches of new hosts (batch 1 = progeny produced at ages 1-5 days, batch 2 = progeny produced at ages 6-10 d, batch 3 = progeny produced at ages 11-15, batch 4 = progeny produced at ages 16-20 d, batch 5 = progeny produced at ages 21-25 d). Means for the same age range and for cumulative progeny followed by different letters are significant ($P < 0.05$, Tukey HSD test).

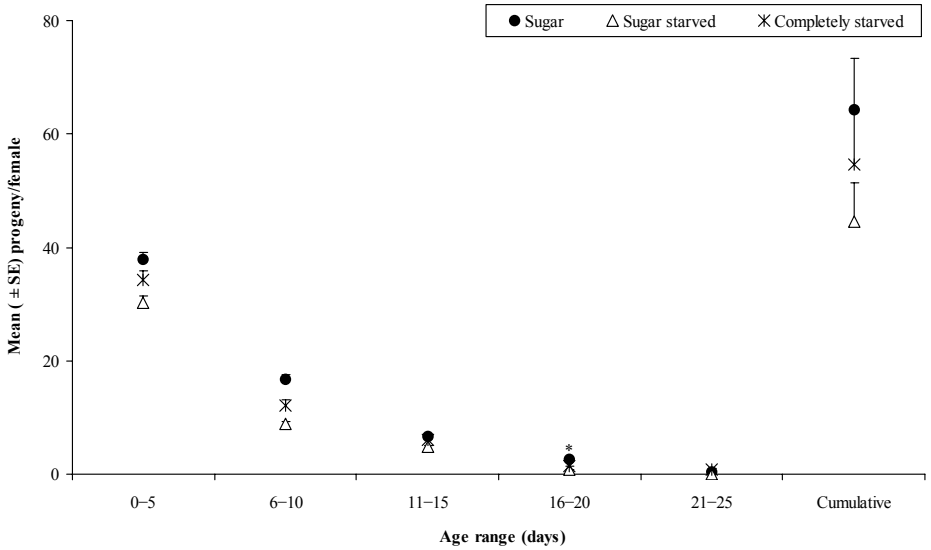
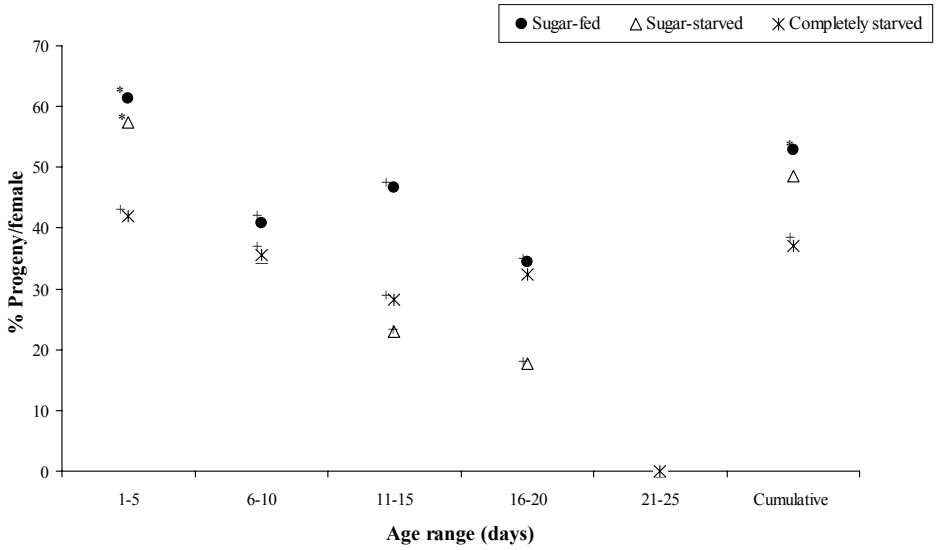


Figure 4 Sex ratio of offspring produced by female *P. cerealellae* of different age ranges provisioned with different diet treatments. Figure shows proportion (percent) of female progeny * = significant female-biased progeny; + = significant male-biased progeny ($P < 0.05$, χ^2 2 × 2 analysis).



CHAPTER 3: ASSESSMENT OF FROZEN LARVAE OF *CALLOSOBRUCHUS MACULATUS* AS HOSTS FOR REARING *PTEROMALUS CEREALELLAE*

INTRODUCTION

Parasitoids are insects that develop as larvae on the tissues of other arthropods, usually insects, which they eventually kill (Hassel and Waage, 1984). They are potentially important regulators of their hosts' populations, and some are commercially produced as biological control agents of various pests (Mills, 1994; Cranshaw et al., 1996; Donnelly and Phillips, 2001; Floate and Spooner, 2002). Mass rearing and release of natural enemies such as parasitoids are critical components of any biological control strategy to suppress pest populations (Rueda and Axtell, 1987; Petersen and Cawthra, 1995, Kaufman et al*.,* 2001; Geden and Hogsette, 2006; Geden and Kaufman, 2007). Typically, parasitoids are reared on living immature hosts and this strategy has many limitations. Use of live (fresh) larvae or pupae as rearing hosts for parasitoids may reduce the window of opportunity for parasitism due to rapid development of the hosts from potentially suitable stages for parasitism to unsuitable stages. Furthermore, rearing of parasitoids on developing live hosts has the inherent risk of accidental releases of unparasitized pest hosts in the field.

The use of killed (frozen or irradiated) hosts to rear parasitoids could potentially mitigate these limitations and may even have some advantages over the use of live hosts in certain applications such as in foreign exploration efforts to established colonies of exotic parasitoids in locales where live hosts are not available (Pickens and Miller, 1978; Geden et al*.,* 2006). Use of frozen (freeze-killed) hosts for maintenance of parasitoid colonies can increase the efficiency of rearing programs. For instance, when hosts are reared in excess of needs, they can be frozen and used when normal supplies are low or can be stockpiled and used later in mass release programs (Klunker and Fabritius, 1992; Geden and Kaufman, 2007). In addition, rearing parasitoids on frozen hosts may reduce the risk of contamination of one population with another because any potential contamination which may occur during host maturation can be eliminated by freeze-killing (Geden and Kaufman, 2007).

Several studies have reported on the ability of some pupal parasitoids to successfully develop on frozen pupal hosts (Richerson and Borden, 1972; Petersen and Matthews, 1984; Rueda and Axtell, 1987; Rivers and Delinger, 1995; Floate and Spooner, 2002). For instance, Pickens and Miller (1978) successfully reared a fly pupal parasitoid, *Pachycrepoideus vindemiae* (Rondani) Hymenoptera: Pteromalidae) on frozen pupae of the housefly, *Musca domestica* Linn (Diptera: Muscidae), and concluded that continued, periodic additions of frozen house fly pupae could increase effectiveness of the parasitoid in chicken houses. Rueda and Axtell (1987) also reported that frozen pupae of *M. domestica* were as suitable as fresh pupae for mass rearing of three pupal pteromalid parasitoids (Hymenoptera: Pteromalidae): *Muscidifurax raptor* Girault and Sanders, *P. vindemiae*, and *Spalangia cameroni* Perkins. Most of the available literature

on the ability of parasitoids to develop on frozen hosts has focused on pupal parasitoids of flies; much less is known about the development larval parasitoids on frozen hosts (Kaschef, 1959).

Pteromalus cerealellae (Ashmead) (Hymeoptera: Pteromalidae) is a solitary larval ectoparasitoid of several pests of stored products, including *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae), *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae), *Lasioderma serricorne* (Fab.) (Coleoptera: Anobiidae), *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), and *Sitophilus* spp. (Coleoptera: Curculionidae) (Ashmead, 1902; Brower, 1991; Howard, 2001; Onagbola et al., 2007). Females of *P. cerealellae* lay eggs in host larvae, which typically are concealed within grain seeds. This parasitoid is presently being considered as having potential for utilization as biological control agents against some of the above stored grain pests (Brower, 1991; Mbata et al., 2005; Onagbola et al., 2007). The ability of *P. cerealellae* or other parasitoids of stored product insects to successfully develop on frozen host larvae has not been previously investigated. This study was therefore conducted to determine if *P. cerealellae* could be reared successfully on frozen larvae of *C. maculatus*, one of its principal hosts. First, the reproductive potential (number and sex ratio of progeny) of female *P. cerealellae* when presented frozen larvae versus live (fresh) larvae of the same stage were compared. Second, to determine how long previously frozen larvae could remain viable as hosts after exposure to ambient conditions, reproductive potential of female *P. cerealellae* was compared on frozen larval hosts which were subsequently allowed to thaw and held at ambient conditions for various lengths of time (0-5 d). Finally, the relative suitability of frozen versus live larvae

was compared by presenting together both host types to female *P. cerealellae* in the same arena.

MATERIALS AND METHODS

Insects: Cowpea bruchids

Callosobruchus maculatus was utilized as host for *P. cerealellae* in this study. The starting culture of *C. maculatus* was initially obtained from Fort Valley State University, Fort Valley, GA, USA (contact: Dr George Mbata), where it has been reared continuously on cowpea seeds (*Vigna unguiculata* Walp.) for several years. *Callosobruchus maculatus* was reared in our laboratory on cowpea seeds (California Black Eyed variety) in 1-L wide-mouthed Mason glass jars. A fresh culture was started every 5 d 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 ºC, 70 ± 5% r.h., and L12:D12 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, which were then provided to *P. cerealellae* for parasitization.

The parasitoid

The original culture of *P. cerealellae* was obtained from Fort Valley State University, Fort Valley, GA, USA, where the parasitoid has been reared continuously for several years. *Pteromalus cerealellae* culture was maintained in our laboratory by transferring about 30 adult pairs onto a glass jar containing *C. maculatus*-infested cowpea

seeds at a stage when most of the bruchid larvae were at the fourth instar. This was determined in a preliminary experiment to occur ~15 d 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 and the attacked *C. maculatus* larvae incubated in a growth chamber at the above environmental conditions until the emergence of adult parasitoids.

Reproductive potential of P. cerealellae on live (fresh) and frozen C. maculatus larvae

The development of *P. cerealellae* was compared on live (< 1-day-old) and frozen (< 1-day-old) fourth-instar larvae of the *C. maculatus* (still contained in the cowpea seeds). Frozen larvae were obtained by freezing infested cowpea seeds containing a fourth-instar larva of *C. maculatus* at -20 ˚C for 48 h (Johnson and Valero, 2003) and thereafter exposed to ambient laboratory conditions (~25 ± 1ºC, 60 ± 5% r.h.) for ~4 h prior to exposure to female parasitoid (frozen larvae were confirmed dead after holding infested seeds at -20 ˚C for 48 h by dissection). Eighty infested cowpea seeds containing live or frozen larvae (1 larva per seed) were placed in a 10-cm diameter plastic Petri dish. A mated 2-d old female *P. cerealellae* was then placed in each Petri dish and allowed to parasitize the larval hosts for 120 h. At the end of the 120-h exposure period, the female parasitoid was removed and the Petri dish containing host larvae was incubated at 30 ± 1ºC, 70 ± 5% r.h., and L12: D12 h until the end of emergence of parasitoid F_1 progeny (~10 d after the start of parasitoid emergence). Each treatment was replicated 20 times (i.e. 20 female parasitoids each exposed to 80 host larvae). The number of offspring produced by each female parasitoid and sex ratio were recorded daily and summed at the

end of the incubation period. Data were analyzed by using the students' *t - test* (JMPIN Version 5.1, SAS Institute, 2003) to determine any significant difference between reproductive potential on live and frozen larvae.

Effects of duration of thawing of frozen host larvae on parasitoid reproductive potential

Having demonstrated the ability of *P. cerealellae* to develop on frozen host larvae in the preceding experiment, a second experiment was conducted to test for possible effects of duration of thawing of frozen host larvae on reproductive potential of female *P. cerealellae*. Infested cowpea seeds containing fourth instar (< 1-d-old) larvae of *C. maculatus* were placed in a 10-cm diameter plastic Petri dish (80 seeds per dish each containing 1 larva) and frozen at -20 ˚C for 48 h as described in the preceding experiment. The Petri dishes were then removed from the freezer (thawed) and subsequently held at ambient laboratory conditions for various lengths of time: 4, 24, 48, 72, 96, and 120 h before exposure to female parasitoids. The control treatment consisted of infested cowpea seeds containing live fourth instar (< 1-d-old) *C. maculatus* larvae (80 seeds per dish each containing 1 larva). A mated 2-day old female *P. cerealellae* was then placed in each Petri dish and allowed to parasitize the larval hosts for 24 h as described in the preceding experiment. At the end of the 5-d exposure period, the female parasitoid was removed and the Petri dish containing host larvae was incubated as described in the preceding experiment. Each treatment was replicated 20 times (i.e. 20 female parasitoids each exposed to 80 host larvae). The number of offspring produced by each female parasitoid and sex ratio were recorded daily and summed at the end of the incubation period. Progeny production data obtained for each treatment were square-root

transformed ($\sqrt{(x + 0.5)}$) and then analyzed with one-way ANOVA followed by Tukey's HSD test ($P < 0.05$). Data on offspring sex ratio were subjected to Students' *t - test* (JMPIN Version 5.1, SAS Institute, 2003) to test for significant deviation ($P < 0.05$) from an expected 1:1 sex ratio of emerged progeny per treatment.

Relative suitability of live and frozen C. maculatus larvae when presented together

The relative suitability of live versus frozen *C. maculatus* larvae as hosts for *P. cerealellae* when presented together in a Petri dish was tested. Infested cowpea seeds containing (< 1-d-old) live or frozen fourth instar larvae of *C. maculatus* (obtained as described above) were marked with Sharpie® permanent marker for later identification. Forty marked seeds containing live larvae and 40 seeds containing frozen larvae were placed together in a 10-cm diameter plastic Petri dish for a total of 80 seeds (mixed live and frozen larvae). A mated 2-d old female *P. cerealellae* was then placed in the Petri dish and allowed to parasitize the larval hosts for a period of 12, 24, 48, 72, 96 or 120 h (parasitism exposure period), as described in the preceding experiments. At the end of each exposure period, the female parasitoid was removed and the Petri dish containing host larvae was incubated as described in the preceding experiments. This experiment was replicated 20 times. The number of offspring produced by each female parasitoid and sex ratio were recorded daily and summed at the end of the incubation period. Data obtained on the total number of progeny produced per female *P. cerealellae* at each exposure period were analyzed by using the students' *t - test* (JMPIN Version 5.1, SAS Institute, 2003) to determine any significant differences in reproductive potential of female parasitoid on live versus frozen larvae. The number of progeny produced on each

host type at the different exposure periods were square-root transformed ($\sqrt{(x + 0.5)}$) and then analyzed with one-way ANOVA followed by Tukey's test to determine any significant effects ($P < 0.05$) of exposure period on progeny production.

RESULTS

Reproductive potential of P. cerealellae on live (fresh) and frozen C. maculatus larvae

No significant differences were recorded in progeny production by female *P. cerealellae* on live versus frozen *C. maculatus* larvae (students' *t* - test: $df = 1$, $t = 0.230$, $P = 0.819$): approximately 42.9 ± 5.0 and 40.8 ± 5.1 (mean ± SE) progeny were produced per female on live and frozen larvae, respectively (Fig. 1). Also, no significant differences were recorded on the sex ratios of progeny on live larvae ($df = 1$, $t = 0.019$, $P = 0.985$) or frozen larvae ($df = 1$, $t = -0.093$, $P = 0.927$).

Effects of duration of thawing of frozen host larvae on parasitoid reproductive potential

One-way ANOVA revealed significant effects of host type on the mean number of progeny produced by female *P. cerealellae* ($F = 3.692$, $df = 6$, $P = 0.002$). Significantly fewer progeny were produced from frozen larvae which had been thawed and held at ambient conditions for 120 h (5 d) compared to those that were held for 4 or 24 h, or to live larvae. Progeny production was, however, not significantly different between live and frozen larvae, which were held at ambient conditions for 4 or 24 h prior to exposure to female *P. cerealellae* (Fig. 2). In general, a gradual decline in progeny production on frozen larvae was recorded with increasing thawing duration. Students' *t* - test analysis showed no significant difference in the sex ratios of progeny produced on

live larvae ($df = 1$, $t = 0.884$, $P = 0.0.382$) or frozen larvae thawed and held for 4 h ($df = 1$, $t = 0.932$, $P = 0.357$), 24 h ($df = 1$, $t = -0.609$, $P = 0.546$), 48 h ($df = 1$, $t = -1.712$, $P = 0.095$), 72 h ($df = 1$, $t = 0.733$, $P = 0.468$), 96 h ($df = 1$, $t = 0.525$, $P = 0.603$), or 120 h ($df = 1$, $t = -0.489$, $P = 0.628$).

Relative suitability of live and frozen host larvae when presented together

When live and frozen hosts were presented together in the same Petri dish to female *P. cerealellae*, significant differences were recorded in mean number numbers of progeny produced on each host type at some exposure periods (Fig. 3). Significantly greater mean number of progeny was recorded on live than on frozen hosts at parasitism exposure periods of 12 h ($F = 5.61$, $df = 1$, $P = 0.023$), 24 h ($F = 16.85$, $df = 1$, $P = 0.0002$), and 48 h ($F = 5.84$, $df = 1$, $P = 0.021$) (Fig. 3). However, no significant differences were recorded in progeny production on the 2 host types at 72 h ($F = 2.21$, $df = 1$, $P = 0.145$), 96 h ($F = 2.83$, $df = 1$, $P = 0.101$) and 120 h ($F = 2.35$, $df = 1$, $P = 0.134$) after exposure. ANOVA showed significant effects of exposure period on progeny production on both live ($F = 20.04$, $df = 5$, $P < 0.0001$) and frozen hosts ($F = 16.46$, $df = 5$, $P < 0.0001$). In general, progeny production on each host type increased with exposure period (Fig. 3).

DISCUSSION

The results of this study, which showed no significant differences in the reproductive potential of female *P. cerealellae* on live versus frozen larvae of *C. maculatus*, suggest that both host types are equally suitable for rearing *P. cerealellae*. Similar results have been reported for other hymenopteran parasitoids, in particular those

in the same family (Pteromalidae) as *P. cerealellae* (Kaschef, 1959; Petersen and Matthews, 1984; Morgan et al*.,* 1986; Rueda and Axtell, 1987; Roth et al*.,* 1991; Rivers and Delinger, 1995; Floate and Spooner, 2002). For example, Kaschef (1959) reported that females of *Lariophagus distinguendus* Först (Hymenoptera: Pteromalidae) successfully parasitized living and CO_2-killed prepupae of *Stegobium paniceum* L. (Coleoptera: Anobiidae). Similarly, several species of pupal parasitoids of flies (Diptera) have been successfully reared on frozen housefly, *M. domestica* pupae including *P. vindemiae*, *S. cameroni* and several *Muscidifurax* spp. (Pickens and Miller, 1978; Petersen and Mathews, 1984; Rueda and Axtell, 1987; Petersen et al*.,* 1992; Petersen and Currey, 1996; Floate and Spooner, 2002; Geden and Kaufman, 2007). Housefly pupae killed by exposure to gamma radiations were suitable for production of *Spalangia endius* Walker (Morgan et al*.,* (1986). Also, frozen hornfly pupae were shown to be suitable hosts for *S. cameroni* (Roth et al*.,* 1991). The ability of *P. cerealellae* to develop on frozen host larvae may be related to its idiobiontic (host killing prior to oviposition) life history strategy (personal observation) as typical of several pteromalid parasitoids including *Cyrtogaster vulgaris* Walker (Askew, 1965), *Trichomalopsis apanteloctena* (Crawford) (Nakamatsu and Tanaka, 2004) and *Nasonia vitripennis* (Walker) (Pexton and Mayhew, 2005). During oviposition, female *P. cerealellae* injects a sting through the ovipositor, which results in paralysis and subsequent death of the host soon thereafter (personal observation). In essence, most part of the developmental period of *P. cerealellae* is spent on a dead host since the parasitized (live) host is likely to be dead by the time of larval hatch and development. The results of this study suggest that *P. cerealellae* will readily develop on dead hosts thereby alleviating any potential

contamination that may occur during host maturation can be eliminated by freeze-killing (Geden and Kaufman, 2007).

The results showing that frozen *C. maculatus* larvae that had been held at ambient conditions for up to 96 h (4 d) produced statistically similar numbers of progeny as live larvae with no significant differences in sex ratios further supports the case for use of frozen larvae for rearing *P. cerealellae*. However, the data showing that progeny production on frozen hosts gradually declined with thawing duration and was significantly less at 120 h is interesting, and should be considered in future applications of this strategy. Similar results showing reduced progeny production on frozen hosts which had been exposed to ambient conditions for 5 d have been reported for other pteromalid wasps including *M. raptor*, *P. vindemia*, *S. cameroni* and *Spalangia endius* Walker (Rueda and Axtell, 1987). The authors attributed the reduced suitability of frozen larvae with thawing duration to faster deterioration of frozen pupae during exposure to thawing temperatures, and the disintegration of insect cells (cytolysis), which normally occurs after slow thawing (Losina-Losinsky, 1967; Rueda and Axtell, 1987). Deterioration and cell disintegration may explain the results of this study showing a gradual reduction in progeny production by female *P. cerealellae* on frozen host larvae which were subsequently exposed to ambient conditions for 3-5 d.

The results of the third experiment in which both live and frozen host larvae were simultaneously presented together to female *P. cerealellae* at different exposure periods showed relatively greater progeny production on live hosts than on frozen hosts at 12, 24 and 48 h of exposure. This pattern may be indicative of the preference of female *P. cerealellae* for live over frozen host larvae or the relatively greater suitability of live host

larvae for parasitoid development. This experiment was not designed to evaluate host preference or host location cues in *P. cerealellae*, but it is likely that these patterns resulted from preference of female *P. cerealellae* for live larvae, given the data from the preceding experiments showing that live and frozen larvae are equally suitable for development of this parasitoid.

Little is known about the cues used by female *P. cerealellae* to locate larval hosts, which typically are concealed within seeds. However, parasitoids of endophytic hosts (i.e. specialized on hosts covered by hard substrate) have been reported to locate hosts by using various cues in hierarchical order (e.g. Godfray, 1994; Hailemichael et al., 1994; Meyhöfer and Casas, 1999; Fischer et al., 2004). In particular, many parasitoids of endophytic/concealed hosts are known to use vibrational cues to locate their hosts (Kaschef, 1964; Sokolowski and Turlings, 1987; Meyhöfer et al., 1997, 1999; Van Dijken and van Alphen, 1998; Fischer et al., 2004). For example, the leaf miner parasitoid *Dapsilarthra rufiventris* (Nees) (Hymenoptera: Braconidae) (Sugimoto et al., 1988a, b) and *Biosteres longicaudatus* Ashmead (Hymenoptera: Braconidae) (Lawrence, 1981) were reported to show preference for live hosts than dead hosts. Also, 2 parasitoids of *Drosophila* spp., *Asobara tabida* Nees (Hymenoptera: Braconidae) and *Leptopilina longipes* Spier (Hymenoptera: Eucoilidae) failed to locate dead larvae in substrate, suggesting use of host vibrational signals for host location (Sokolowski et al., 1987). Larvae of *C. maculatus* are concealed within cowpea seeds and produce vibrational signals during feeding (Kaschef, 1964; Shade et al., 1990). Thus, our results may suggest involvement of host vibrational cues in host location by female *P. cerealellae*. However, the ability of female *P. cerealellae* to parasitize live and freeze-killed hosts suggest that

female parasitoids do not rely exclusively on larval vibrational signals for host location. Ongoing studies on the host location behavior of this parasitoid may provide insight into the relative importance of vibrational cues for host location.

To our knowledge, the study represents the first report on successful development of *P. cerealellae* on frozen host larvae. Our results showing that frozen host larvae are as suitable as live larvae for rearing *P. cerealellae* may have practical implications in the development of efficient mass-rearing systems for *P. cerealellae*, a prerequisite to its future utilization for biological control of stored-product insects. It remains to be determined however, if prolonged freezing or storage of frozen host larvae will support development of this parasitoid.

ACKNOWLEDGEMENTS

This research was supported in part by the Alabama Agricultural Experiment Station and an Auburn University Competitive Research grant to H.Y.F.

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Figure 1 Reproductive potential of female *P. cerealellae* on live and frozen *C. maculatus* larvae. Figure shows the mean number of progeny produced per mated female *P. cerealellae* (n = 20) on live and frozen *C. maculatus* larvae. A female parasitoid was exposed to 80 larvae of each type for 120 h (5 d). White and gray bars indicate female and male progeny, respectively (not significantly different, Students' *t* - test, $P < 0.05$).

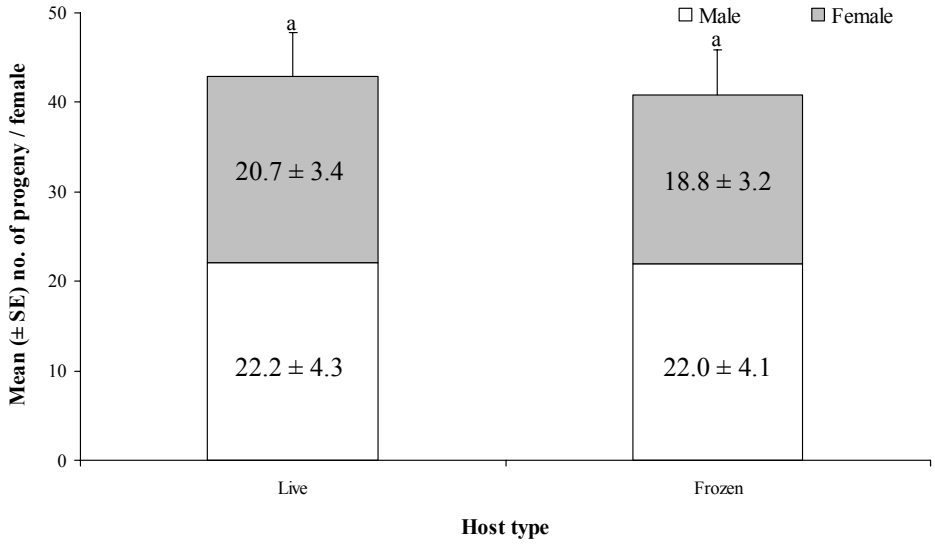


Figure 2 Reproductive potential of female *P. cerealellae* on previously frozen *C. maculatus* larvae which were subsequently thawed and held at ambient laboratory conditions (~25 ± 1 ºC, 60 ± 5% r.h.) for various periods of time. Figure shows the mean number of progeny produced per mated female *P. cerealellae* on live compared to frozen *C. maculatus* fourth-instar larvae thawed and held for 4h (F-4), 24 h (F-24), 48 h (F-48), 72 h (F-72), 96 h (F-96), or 120 h (F-120) prior to exposure to female *P. cerealellae*. A female parasitoid was exposed to 80 larvae of each type for 5 d. White and gray bars indicate numbers of male and female progeny, respectively (not significantly different, Students' *t* – test, $P < 0.05$).

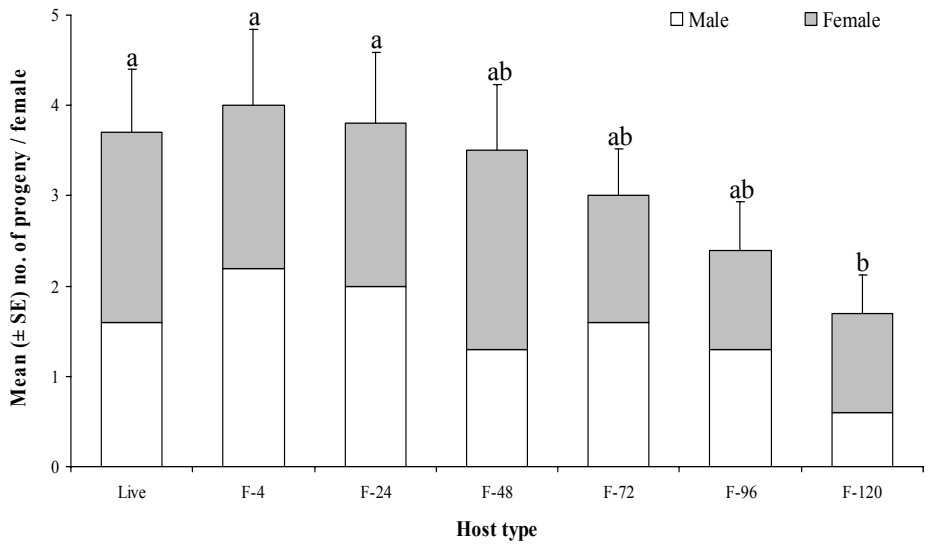
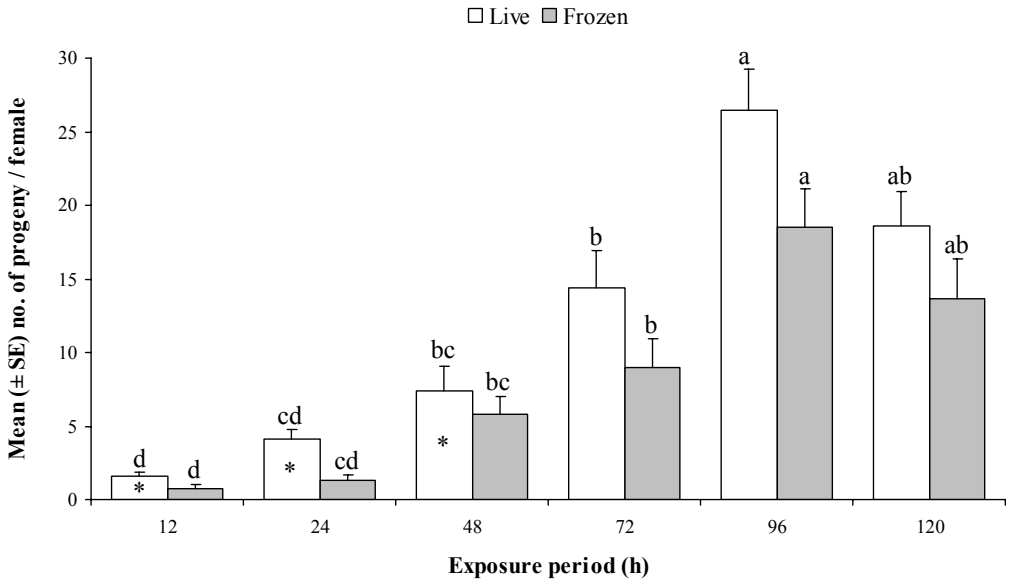


Figure 3 Relative suitability of live and frozen *C. maculatus* larvae when presented together to female *P. cerealellae*. Figure shows mean (± SE) number of progeny per female at various exposure periods: 12, 24, 48, 72, 96 and 120 h. At each exposure period, means followed by asterisks (*) are significantly different between live and frozen larvae (Students' *t* – test, $P < 0.05$). For each host type (live and frozen larvae) means for the different exposure periods having no letters in common are significantly different (Tukey's HSD test, $P < 0.05$).



CHAPTER 4: MORPHOLOGY AND DEVELOPMENT OF *PTEROMALUS CEREALELLAE* ON *CALLOSOBRUCHUS MACULATUS*

INTRODUCTION

Pteromalus cerealellae (Ashmead) (Hymenoptera: Pteromalidae) is a solitary ectoparasitoid of larvae of various stored-product insect pests. Native to West Africa (Southgate, 1978), it has been reported from many parts of the world, including United States (Wen and Brower, 1994). The known hosts of *P. cerealellae* include *Sitotroga cerealella* Olivier (Lepidoptera: Gelechidae) and several stored-product beetles including bruchids (e.g., *Callosobruchus maculatus* (F.), anobiids (e.g., *Lasioderma serricorne* (Fab.), bostrichids (e.g., *Prostephanus truncatus* (Horn), and grain weevils (*Sitophilus* spp.) (Ashmead, 1902; Brower, 1991). However, the cowpea bruchid, *C. maculatus* was reported as the most susceptible stored-product beetle host of *P. cerealellae* (Brower, 1991).

Most studies on *P. cerealellae* have focused on its potential utilization for biological control of pests of stored products (Smith et al*.,* 1995; Wen and Brower, 1994; Mbata et al., 2004; Onagbola et al., 2007). Little information is available on the developmental biology of *P. cerealellae* (although see Noble, 1932; Fulton, 1933).

Developmental biology studies, including morphological characterization of preimaginal life stages, can be important for identification of an insect to the species level before adult emergence, which can simplify quantification of impact of natural enemies in biological control programs (Bellows and Van Driesche, 1999). Little is known about larval morphology in pteromalid wasps (Grassberger and Frank, 2003; Rojas-Gómez and Bonet, 2003), and we are not aware of any published studies to date on the morphological characterization of the immature stages of *P. cerealellae*. Furthermore, the number of larval instars produced by this species has not been conclusively determined.

The number of larval instars produced by an insect species is commonly determined by measuring the widths of the head capsules of the larvae and plotting the frequency distribution of the measurements followed by statistical analyses (Dyar, 1890; Odebiyi and Bokonon-Ganta, 1986; Löhr et al., 1989; Llácer et al., 2005). However, Löhr et al. (1989) cautioned against sole reliance on head capsule size for determination of number of larval instars due to possible overlap of the range of head capsule sizes of different instars. Consequently, it may be necessary to use additional structural characters such as measurements of body length and width, mandible length, and other (non-structural) characters such as presence of exuvia for accurate determination of larval instars (Wright, 1986; Löhr et al., 1989; Llácer et al., 2005). This present study was designed to characterize the developmental biology and morphology of the preimaginal stages of *P. cerealellae*. Several morphological parameters including measurements of width of the head capsule, mandible size, and body length were used in conjunction with other characters to determine the number of larval instars produced by this parasitoid.

MATERIALS AND METHODS

Insects

The host insect, *Callosobruchus maculatus* was reared in our laboratory on cowpea seeds, *Vigna unguiculata* Walp (California Black Eyed variety) in 1-L wide-mouthed Mason glass jars. A fresh culture was started every 5 d by placing ~25 pairs of 3-day-old mated *C. maculatus* in a glass jar containing ~100 g of cowpea seeds held at 30 ± 1ºC, 70 ± 5% r.h., and 12L:12D (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 larvae had reached the fourth instar, which were then provided to *P. cerealellae* for parasitization. Fourth-instar larvae of *C. maculatus* were used as hosts in this study because previous experiments showed that *P. cerealellae* develops better on this than other instars (unpublished data).

The original culture of *P. cerealellae* was obtained from Fort Valley State University, Fort Valley, GA, USA (contact: Dr. George Mbata) where it has been reared continuously on *C. maculatus* for several years. The parasitoid was maintained in our laboratory by transferring ~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 instar (i.e. ~15 d 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 5 d of oviposition. Parasitized host larvae were incubated in a growth chamber at the above environmental conditions until emergence of adult parasitoids.

Morphology and characterization of the preimaginal stages of P. cerealellae

To study the development of immature stages of *P. cerealellae* within the host, ~80 fourth-instar larvae of *C. maculatus* (which were concealed within cowpea seeds) were exposed to 20 freshly emerged (2-d old) mated female *P. cerealellae* in plastic Petri dish (6-cm diameter) for 6 h. In order to obtain adequate parasitized hosts for the experiment the above set-up was replicated 6 times (i.e., a total of 120 female *P. cerealellae* were used). Cowpea seeds containing exposed host larvae were transferred into a growth chamber at the above environmental conditions until dissection. For dissection, cowpea seeds were first carefully cracked open to expose the concealed larvae of *C. maculatus,* which were then grouped into parasitized (dead) or unparasitized (alive). Parasitized larvae were first frozen for ~4 h to arrest development and then dissected to excise the developing stages of the parasitoid. Dissection of parasitized larvae were made in 6-h intervals from 6 to 48 h (after exposure to female parasitoids) and, thereafter, in 12-h intervals until adult parasitoids started to emerge (~11 d after exposure). Parasitized host larvae were dissected in a drop of water under a 20× stereomicroscope scouting for preimaginal stages (eggs, larvae, prepupae and pupae) of the parasitoid. Excised preimaginal stages were immediately transferred into glass vials containing 50% ethyl alcohol. At least 30 parasitized hosts were dissected for each time interval.

Measurements were made of the length and width of *P. cerealellae* eggs and larvae and the width of the larval head capsule (nearest 0.02 mm) under a stereomicroscope (National Microscope, Model DC 3-420, Meiji, Japan) equipped with an ocular micrometer. Separation of the larval instars was done by statistical analyses of the head capsule measurements (width) combined with the use of reliable characters such

as the number of exuviae on the body of parasitoid larvae, cuticular folding, and excretion of the meconium. In addition, maximum lengths of the mandibles (m-length) of larvae (measured from the points of articulation with the head to the pointed tip) belonging to the different instar groupings (as determined from the analyses of the width of the head capsules) were measured to the nearest 0.02 µm under a compound microscope (National Microscope, Model ML2000, Meiji, Japan) at 40× magnification. Micrographs of all stages were taken under the above stereo and compound microscopes fitted with a Nikon Coolpix 4500 camera. Developmental time for each stage was recorded.

Histological methods and scanning electron microscopy

Preimaginal stages of *P. cerealellae* were further processed using clearing techniques similar to those described by Hansson (1996) and Kondo and Williams (2005). Immature specimens were transferred from alcohol vials into heated 10% KOH (150 ºC) for ~5 min. They were then transferred individually into distilled water for ~2 min to remove excess alkali and then into a previously stained heated Essig's fluid (~93 ºC) for ~3 min, and finally into 75% ethyl alcohol for ~2 min to remove excess stain. The specimens were then dehydrated for ~5 min in 99.9% ethyl alcohol. Specimens were cleared in clove oil with spatulated probes leaving them in the oil for ~30 min to further fix the stains. They were then individually removed from the clove oil, placed centrally on clean glass slide and mounted in Canada balsam. All mounted specimens were dried in Model 4 Precision oven (Thelco, Chicago, USA) set at 60 ºC for 4 wk and later observed under the compound microscope for morphological details.

In addition, eggs of *P. cerealellae* were further processed by scanning electron microscopy (SEM). Preparation for SEM was modified from Sukontason et al. (2003). Eggs were removed from alcohol vials after 24 h and subjected to progressive dehydration in graded ethyl alcohol series of 70, 80, 90, 95 and 100% for 4 h in each alcohol concentration. The dehydrated specimens were mounted on aluminum stubs with double-faced silver-based adhesive and subjected to critical-point drying (Baker, 2001). Specimens were sputter coated with gold/platinum mix using Pelco SC-7 (EMS 550X / 250) Sputter coater and observed under the DSM 940 SEM (Zeiss, W. Germany) at 10kV and 15 mm working distance.

Statistical analyses

Data obtained on days 2-8 after oviposition were used in the analyses of larval morphological characters because under our experimental conditions, occurrence of larvae started 36 to 48 h after oviposition while the earliest pupae were recorded on day 7. Data obtained from measurements of the width of the head capsules of larvae obtained on days 2-8 (after oviposition) were pooled and first subjected to students' *t* - test to test for differences in head capsule sizes of developing male and female larvae (determination of sex of larvae was made when they were in the second-instar stage), and then analyzed by using a two-way (width of larval head capsule × sex) factorial analysis of variance (ANOVA) (SAS, 2003) followed by Tukey's HSD test ($P < 0.05$) to separate the means of the larval head capsules.

The data obtained from measurements of the mandible length (m-lengths) of the second-through the fourth-instar larvae were first subjected to *t* - test analysis to

determine significant difference between m-lengths of male and female larvae. For each instar, m-length measurements were obtained from 10 individuals of each sex. No significant effect of sex was recorded on the m-length data, so the m-length data for all the larvae were pooled and analyzed with one-way ANOVA followed by Tukey's HSD test ($P < 0.05$) to determine significant variations in the mandible lengths of larvae of various instars. Pooled data were further analyzed by testing for correlation between m-lengths and number of larval instars as determined using measurements of width of larval head capsules. Data were not transformed prior to analysis since the assumption of normality was generally met. Whenever normality assumption is not met, data will first be subjected to square-root ($x = (\sqrt{(x + 0.5)})$ transformation for normality.

RESULTS

Description of the preimaginal stages of P. cerealellae

Egg

The description of immature insects and classification methods used in this paper were based on the methods of Clausen (1940) and Hagen (1964). Fully developed eggs of *P. cerealellae* are typically hymenopteriform (i.e., with a pointed anterior and posterior tips with enlarged posterio-medial portion) in shape with thin transparent chorion (Figure 1a). The chorion appears smooth when observed under the stereomicroscope at 80×, but SEM revealed the chorion to be sculptured and of the stalked type with warty tubercles (Figure 1b). The egg is approximately 3 times as long as wide, almost round at one end and narrow at the opposite end. The mean (± SE) length and maximum width of the egg are 0.54 ± 0.002 and 0.19 ± 0.002 mm, respectively (n = 15). A single egg is most often

laid by female *P. cerealellae* per host, although a female may occasionally deposit more than one egg in a single host. The egg is nearly transparent when newly deposited (0-6 h) turning opaque-whitish 24-36 h after oviposition. The egg is not encapsulated by the host. Prior to hatching, the eggs become round and increased in size.

First instar

Most of the eggs hatched between 36 and 48 h after oviposition (parasitization) at 30 ± 1 ˚C, 70 ± 5% r.h. and 12L:12D photoperiod, although viable eggs could still be detected 4 d after oviposition (Table 1). The neonate endophytic larva usually moves onto the body surface of the host to complete its development. The first instar has a smooth, transparent cuticle that exposes its developing (whitish) gut (Figure 2a). Shortly after hatching, the first instar measured 0.67 ± 0.02 mm in length and 0.44 ± 0.03 mm in width (Table 1) with the width of the head capsule ranging between 0.30 ± 0.02 mm and 0.32 ± 0.01 mm (Table 1). The head capsule is almost indistinct from the body when viewed at 20× magnification. The larva gradually turns yellowish as it feeds on the tissues of the dead host. The sex of the parasitoid could not be easily determined at this stage, which lasts ~2 d. Observation of cleared first-instar larvae under the compound microscope revealed that the body is made up of 13 segments with peripneustic condition (9 pairs consisting of 1 pair of mesothoracic and 8 pairs of abdominal) of spiracles (Figure 3). The crania of the larvae (Figure 4a) are well developed, with antennal lobes and chitinized, tusk-like mandibles (M, Figure 4b).

Second instar

The second instar resembles the first instar, having smooth cuticle but with a distinct head capsule (Figure 2b), and occurs 2-6 d after oviposition (Table 2). The key characters which separate this instar from the previous instar include the distinct head capsule, yellowish-brown gut, size, and presence of the exuviae of the first instar on its body. An early second instar (n = 1) (Table 1) was excised ~72 h after oviposition. The sex of the developing parasitoid can be determined at this stage using the tip of the abdomen, which is nearly pointed in females and blunt in males. The mean width of the head capsule is ~0.45 ± 0.03 and 0.59 ± 0.01 mm for males and females, respectively. The larva is robust and brownish possibly due to feeding on the necrotic host larva. The head of the larva remained sunk in the body of the host (probably because the mandibles are buried in host tissue) while other parts of the body undulates as it feeds on the host. The tracheal systems (tubes) are visible through the transparent cuticle. The cleared specimen of this instar was not morphologically different from the first instar. However, the mandibles appeared to be more chitinized in the second instar than in the first.

Third instar

The hymenopteriform third instar is present from days 5-11 after oviposition (Table 1). It is far more tracheated than the preceding instars and has cuticular folds (CF, Figure 2c) showing that the cuticle of the third-instar larvae is transparent. Under the stereomicroscope, the larva appears brownish possibly due to meconium (the midgut waste materials). The parasitoid spends nearly 50% of the larval developmental period at this stage. The structures on the head capsules are more visible in the third instar when

observed at 20× magnification. The imaginal eyes (Figure 5a) develop late at this stage. The larva stops feeding and excretes the guts contents, the meconium (Figure 5b) to form the prepupae (Figure 2d). As with the preceding instars, the mandibles of the third instar are simple, tusk-like and with no serrations on its blade (Figure 4b).

Fourth instar (prepupa)

Soon after the third instar begins to expel its meconium, its length shortens to form the prepupa, the fourth-instar larva (Figure 2d). Excretion of the meconium starts after the fifth day post oviposition and some third instar larvae had turned into prepupae by the end of the day 6. The prepupal stage is characterized by narrowed thoracic region, widened abdominal region and presence of the imaginal eyes (Figure 2d). The caudad end of the prepupa is rounded. The prepupal period is short and lasts between 12 and 24 h at 30˚C. The prepupa has clear midgut (Figure 5). Formation of pupa begins towards the end of day 7 after oviposition.

Pupa

Pteromalus cerealellae, like other Hymenoptera, produces exarate pupae with clearly visible mouthparts, antennae and legs (Figure 6 a-d). Pupation occurs on the necrotic host larva and the pupa is not protected by any special cocoon. The newly formed pupa is whitish with no pigmentation (Figure 6a). Within ~12 h of pupation, the pupal cuticle tan and turns yellowish-brown (Figure 6b). Eyes of the pupa become pigmented after ~24 h. The head and the dorsum of the thorax darken (become blackish) shortly thereafter. No morphological difference was observed between the different

stages of pupal development. Emergence of adult *P. cerealellae* (Figure 7) occurs ~5 d after the start of pupation with males (Figure 7a) emerging ~24 h earlier than females (Figure 7b).

Total developmental time

Eggs of *P. cerealellae* hatch into neonate first-instar larvae within 2 d at the environmental conditions stated above. Total larval developmental period is between 5 to 9 d at 30˚C. Male and female individuals spend ~4 and 5 d, respectively, as pupae before eclosion. Thus, complete development of male (Figure 7a) *P. cerealellae* takes ~11 d with the females (Figure 7b) emerging ~1 d later.

Characterization and separation of larval instars

Two-way factorial ANOVA revealed significant effects of age (time period between oviposition and extraction of larva from the host ($F = 74.05$, $df = 6$, $P < 0.0001$) and sex ($F = 3.94$, $df = 1$, $P < 0.0493$) on the width of the head capsules of excised *P. cerealellae* larvae (Table 2). Further analysis using Tukey's HSD tests separated all excised larvae into 4 groups (instars). The width (mean ± SE) of the head capsules of the first instar ranged between 0.30 ± 0.02 and 0.35 ± 0.02 mm (Table 2). Students' *t* - test revealed that the width of the head capsules of female larvae were significantly greater than the head capsule sizes of counterpart male larvae for second (male: 0.45 ± 0.03; female: 0.59 ± 0.01; t = 2.06, $P < 0.0001$) and third instars (male: 0.59 ± 0.02; female: 0.68 ± 0.01; t = 2.04, $P = 0.0002$). However, no significant difference was detected between the head capsule sizes of male and female fourth-instar larvae (male: 0.80 ±

0.01; female: 0.81 ± 0.00) (Table 2). This result suggests that both sexes of *P. cerealellae* develop through the same number of instars.

Two distinct types of mandibles were observed in *P. cerealellae*. The mandibles of the first-through the fourth-instar larvae are simple and tusk-like (M, Figure 4a, b) while those of the pupae and adults are of the conventional toothed types (Figure 8). Students' t - test analysis of the mandible size (m-length) showed no significant difference between the sexes in the first ($F = 0.22$, $df = 1$, $P = 0.64$), second ($F = 0.55$, $df = 1$, $P = 0.47$) third ($F = 2.0$, $df = 1$, $P = 0.17$) and fourth ($F = 0.07$, $df = 1$, $P = 0.79$) larval stages (instars). However, the m-length of the different instars were significantly different ($F = 232.8$, $df = 3$, $P < 0.0001$). The mean (± SE) m-lengths of first-, second-, third-, and fourth-instar larvae were 26.28 ± 0.24, 28.96 ± 0.75, 35.02 ± 0.18 and 42.16 ± 0.45 µm, respectively. Analysis of the pooled m-length data using Tukey's HSD test resulted in four larval groupings (Figure 9), further confirming that *P. cerealellae* undergoes 4 larval instars. Significant positive correlations were recorded between m-lengths and instar groupings (Figure 10), and m-lengths and widths of the head capsules (Figure 11a, b), suggesting that larval mandible length is a good predictor of the number of instars in *P. cerealellae*.

DISCUSSION

The morphology and biology of the family Pteromalidae is poorly known, making it difficult to compare our results with those reported for other members of the family. However, many of the characteristics observed in the preimaginal stages of *P. cerealellae* are common among the Chalcidoidea superfamily. Fully developed eggs of *P. cerealellae*

are hymenopteriform, which is typical of chalcidoids (Clausen, 1940; Llácer et al., 2005; Kazimirova and Vallo, 1999). The eggs appeared to have smooth and transparent chorion when observed under stereomicroscope. However, detailed observation of the eggs using SEM revealed the chorion to have warty tubercles. The presence of warty tubercles on the egg chorion has not been commonly observed in chalcidoids and may be a distinctive feature of *P. cerealellae* and other pteromalids. The function of the warty tubercles is unclear, but may serve to aid movement of the egg during oviposition (Austin, 1985) or to protect the chorion.

Female *P. cerealellae* typically lays a single egg per host, which can be deposited in any body region of the host. Upon hatching, the first-instar larvae moved onto the surface of the host to complete its development, as reported for some other ectoparasitoids such as *Galeopsomyia fausta* LaSalle (Hymenoptera: Eulophidae) (Llácer et al., 2005). Four larval instars were observed in *P. cerealellae*. All instars are typically hymenopteriform with 13-segmented body and peripneustic conditions of spiracles, and have simple tusk-like mandibles similar to those described for *Spilomicrus hemipterus* Marshall (Hymenoptera: Diapriidae) (Hoffmeister, 1989) and *Coptera occidentalis* Mues (Hymenoptera: Diapriidae) (Kazimirova and Vallo, 1999).

Internal parasitoids typically undergo significant morphological changes as they develop through various instars, which could range considerably from 2 to 5 instars (Clausen, 1940). For example, 5 larval instars were reported for *Encyrtus saliens* Prinsloo and Annecke (Hymenoptera: Encyrtidae) (Wright, 1986) and *Epidinocarsis lopezi* (DeSantis) (Hymenoptera: Encyrtidae) (Odebiyi and Bokonon-Ganta, 1986), 4 reported for *Encarsia formosa* (Hymenoptera: Aphelinidae) (Hu et al., 2002), and 3 for *C.*

occidentalis (Kazimirova and Vallo, 1999). The first instar is usually regarded as the most distinctive immature stage of parasitoids with diverse structures (Hagen, 1964). However, no distinctive diverse structures were observed on the first instar of *P. cerealellae*. Separation of this from the second instar was based on the presence of a distinct head capsule in the second instar and the presence of the exuviae of the first instar on the body of the second instar. Wright (1986) utilized the presence of distinct head capsule to discriminate the second from the first instars of *E. saliens*, while the presence of the exuviae was used by Löhr et al. (1989) to separate the second instar of *E. lopezi* from the first instar. The third-instar larvae appeared ~5 d after oviposition and are highly tracheated with folded cuticle. Late third-instar larvae excrete meconium to transform into prepupae (fourth instar). The prepupae of *P. cerealellae* have narrowed thoracic region, widened abdominal region and presence of imaginal eyes, typical of parasitoid prepupae (e.g., Kazimírova and Vallo, 1999; Wilk and Kitayama, 1981). No morphological difference was observed in the head capsule (crania) of the first, second and early third instars of *P. cerealellae*. However, bulging imaginal eyes appeared in the late third instar, as reported for *C. occidentalis* (Kazimírova and Vallo, 1999). In addition, the levels of chitinization of the tusk-like mandibles increased in progressive instars.

Measurements of the widths of larval head capsules have been used by many authors to characterize larval instars in parasitoids (Dyar, 1890; Odebiyi and Bokonon-Ganta, 1986; Wen et al., 1995). In the current study, we observed considerable overlap in the range of the widths of the head capsules of third and fourth instars of *P. cerealellae*, as reported also for *E. lopezi* (Löhr et al., 1989). Due to this overlap, this character could

not be used alone to separate the last two larval instars of *P. cerealellae*. Additional characters such as the length of the mandibles (m-length) (Tschinkel et al., 2003), and reliable non-structural characters were necessary to determine the number of larval instars in this parasitoid. Thus, our results suggest that the use of the width of head capsule alone to determine number of larval instars in parasitoids is unreliable and may explain some contrasting results reported by different authors for the same species. For instance, Wright (1986) reported that members of the parasitoid genus *Encyrtus* develop through 5 instar stages, whereas Löhr et al. (1989) reported only 4 instars for *E. lopezi.* Our results showed that mandible lengths correlated positively to head capsule sizes and instar groupings. This suggests that mandible length, which has been traditionally used in morphometric studies (Kawano, 2000; Manzoor and Akhtar, 2006) may be a reliable character for determining the number of instars in parasitoids.

Pupae of *P. cerealellae* are exarate and are not protected by any cocoon. Exarate pupae have also been reported in several other chalcidoids (Wright, 1986; Llácer et al*.,* 2005). The pupal stage of *P. cerealellae* has many of the features that characterize the imago, as reported for some other parasitoids (Schauff et al., 1998; Llácer et al., 2005). The mandibles of the pupae and adults are of the conventional toothed types, compared to the tusk-like type observed in the larvae. At the conditions of this study, life cycle was completed in ~11 d in males and 12 d in females. Male parasitoids are known to emerge earlier than conspecific females, probably to ensure sexual maturity upon female emergence (Thornhill and Alcock, 1983; Doyon and Boivin, 2006). In summary, our results showed that *P. cerealellae* undergoes 4 larval instars. It is hoped that this study

will provide much needed information on the morphology and biology of immature stages of pteromalid wasps.

ACKNOWLEDGEMENTS

I thank Dr. Michael L. Williams of the Entomology Department, Auburn University for technical assistance with histological techniques. This research was supported in part by the Alabama Agricultural Experiment Station and an Auburn University Competitive Research grant to HYF.

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Table 1 Measurements (mean ± SE; in mm) of the body sizes of the immature stages of *P. cerealellae* at specified intervals after parasitization.

Age (days)	First instar			Second instar			Third instar			Prepupa			Pupa		
	N	Length (mean±SE)	Width (mean±SE)	*N*	Length (mean±SE)	Width (mean±SE)	*N*	Length (mean±SE)	Width (mean±SE)	*N*	Length (mean±SE)	Width (mean±SE)	*N*	Length (mean±SE)	Width (mean±SE)
2	7	0.67±0.02	0.44±0.03	1	0.71±0.00	0.42±0.00									
3A	7	1.10±0.15	0.57±0.04	1	1.61±0.00	0.68±0.00									
3B	2	1.20±0.27	0.66±0.02	3	1.82±0.03	0.71±0.00									
4A	2	1.20±0.02	0.54±0.00	1 1	2.25±0.18	0.82±0.04									
4B	1	1.22±0.00	0.54±0.00	8	2.44±0.15	0.87±0.04	3	3.05±0.06	0.99±0.05						
5A				1 1	2.17±0.08	0.90±0.02	1 1	2.81±0.09	1.00±0.03						
5B				5	2.22±0.14	0.96±0.05	6	3.05±0.17	1.14±0.04						
6A				6	2.14±0.02	0.98±0.02	9	2.99±0.14	1.11±0.02						
6B							9	2.90±0.10	1.14±0.06	3	2.84±0.34	1.26±0.04			
7A							5	2.52±0.12	1.06±0.04	1	2.54±0.00	1.12±0.00	8	2.58±0.10	1.10±0.05
7B							4	2.52±0.23	1.05±0.09				6	2.29±0.13	0.92±0.07
8A										2	2.27±0.37	1.09±0.06	10	2.53±0.07	0.97±0.03
8B							3	2.51±0.02	0.95±0.04	1	1.93±0.00	0.81±0.00	10	2.41±0.10	0.94±0.02
9A										1	2.44±0.00	0.93±0.00	13	2.39±0.07	0.92±0.02
9B										2	2.78±0.01	1.04±0.04	9	2.33±0.08	0.88±0.02
10A							1	1.88±0.00	0.88±0.00				11	2.65±0.10	0.96±0.02
10B							2	2.09±0.04	0.87±0.04				8	2.52±0.08	0.91±0.03

"A" indicates the first 12 h and "B" the last 12 h of each day.

Table 2 Body sizes of male and female *P. cerealellae* larvae of different instars as categorized using Tukey HSD analysis of the widths of head capsules of larvae

Sex	Age after oviposition	BL	BW	HC	Instar grouping
Male	2	0.68 ± 0.02d	0.47 ± 0.01c	0.30 ± 0.02d (a)	1
	3	1.28 ± 0.17cd	0.59 ± 0.05c	0.32 ± 0.02d (a)	1
	4	1.58 ± 0.09c	0.66 ± 0.03c	0.45 ± 0.03c (b)	2
	5	2.09 ± 0.04b	0.90 ± 0.02b	0.59 ± 0.02b (b)	3
	6	2.42 ± 0.09a	1.02 ± 0.03a	0.68 ± 0.02a (b)	4
	7	2.27 ± 0.08ab	0.96 ± 0.03ab	0.67 ± 0.01ab (a)	3,4
	8	2.40 ± 0.13ab	1.03 ± 0.07ab	0.80 ± 0.01a (a)	4
Female	2	0.67 ± 0.03d	0.41 ± 0.04e	0.32 ± 0.01d (a)	1
	3	1.43 ± 0.16c	0.65 ± 0.03d	0.35 ± 0.02d (a)	1
	4	2.75 ± 0.07b	0.93 ± 0.02c	0.59 ± 0.01c (a)	2
	5	2.89 ± 0.07ab	1.05 ± 0.02b	0.68 ± 0.01b (a)	3
	6	3.17 ± 0.06a	1.21 ± 0.03a	0.74 ± 0.01a (a)	4
	7	2.78 ± 0.10ab	1.16 ± 0.03ab	0.69 ± 0.03ab (a)	3,4
	8	2.67 ± 0.11ab	1.00 ± 0.04bc	0.81 ± 0.00a (a)	4

BL = body length; BW = body width; HC = width of the head capsules. Means (± SE) within each column for the same sex followed by the same letters are not significantly different ($P < 0.05$). Similarly, for each column letters in parenthesis indicate significant difference between male and female larvae of the same instar (Students' *t - test*, $P < 0.05$).

Figure 1 Hymenopteriform eggs of *P. cerealellae* as observed at different magnifications. Egg with smooth chorion as seen under 80× objective stereomicroscope (a); and stalked and sculptured egg with warty tubercles on the surface of the chorion as observed under SEM at 250× (b)

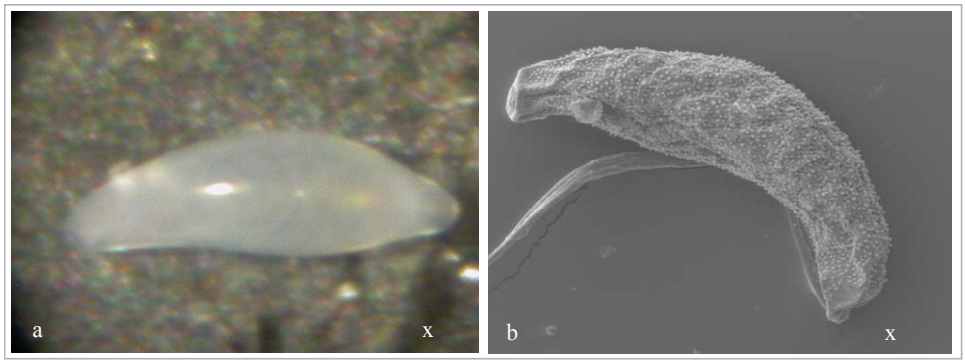


Figure 2 Larvae of *P. cerealellae*. Neonate, first instar (L) on *C. maculatus* host (a). Figure shows larva with smooth and transparent cuticle revealing its whitish gut systems as observed stereomicroscope at 20×; Second instar on the host (b). Figure shows a larva feeding on the necrotic host with its head firmly attached to the body of the host; Third instar actively feeding on the necrotic host (c). Figure shows a third instar with folded cuticle (CF) on the host; and the prepupa (fourth instar) (d)

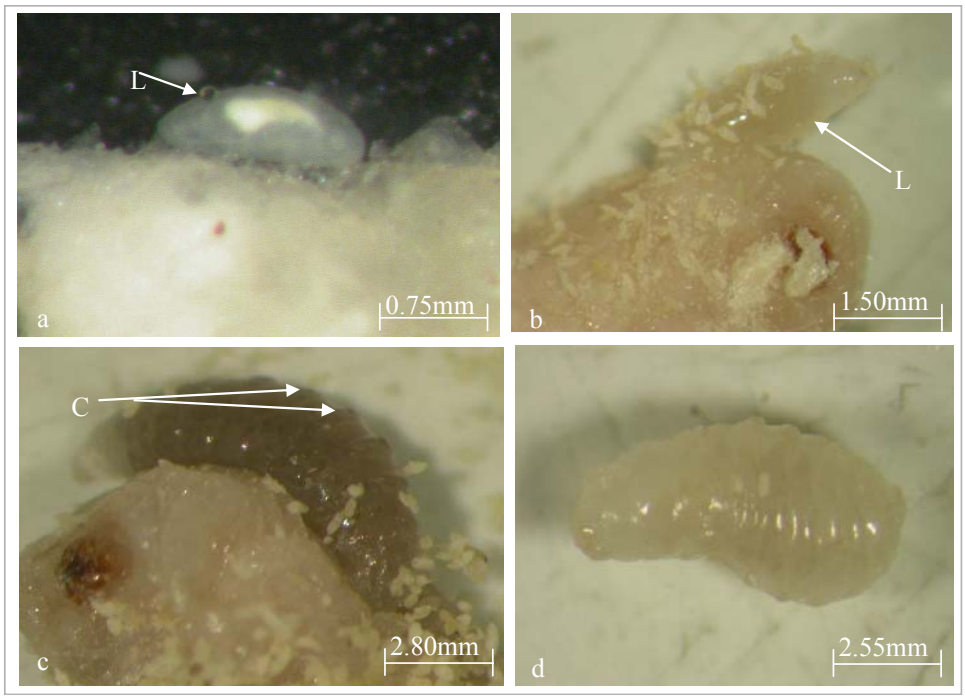


Figure 3 Photomicrograph of a cleared larval specimen showing the body segmentation and the peripneustic condition (1 pair of mesothoracic and 8 pairs of abdominal) of spiracles of *P. cerealellae*. A_1 = first abdominal segment, A_2 = second abdominal segment, A_1S = first abdominal spiracles, A_8S = eighth abdominal spiracles, Ep = epiproct, HC = head capsule, Pp = paraproct, T_1 = prothorax, T_2 = mesothorax, T_2S = mesothoracic spiracles, and T_3 = metathorax.

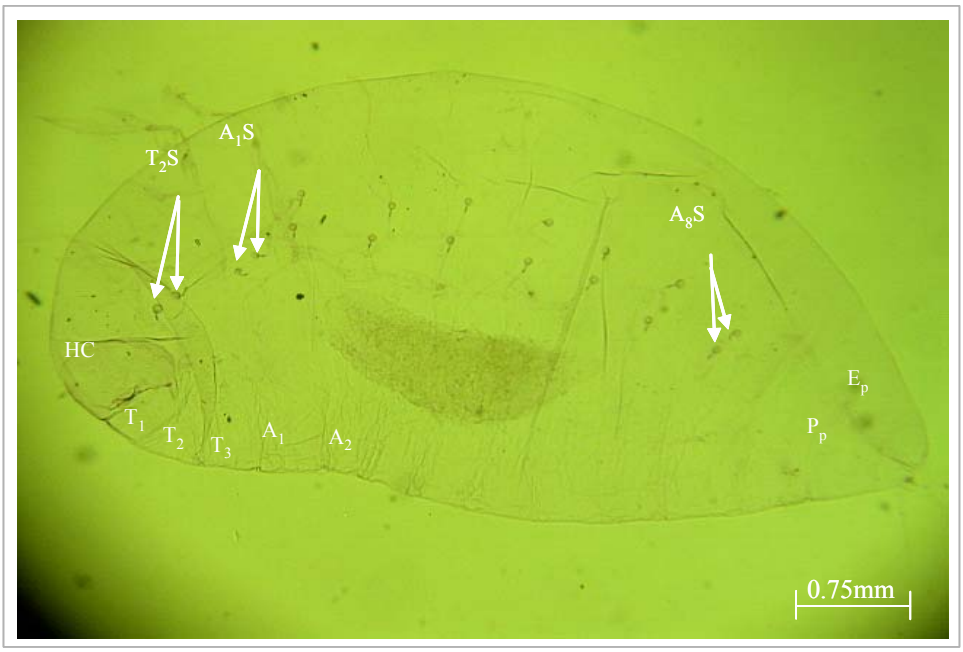


Figure 4 The head capsule of a cleared larval specimen of *P. cerealellae*. Figure shows the short antennal lobes (AL) and the simple, tusk-like mandibles (M) as observed under a compound microscope at 40× (a); and the magnified tusk-like mandibles (b).

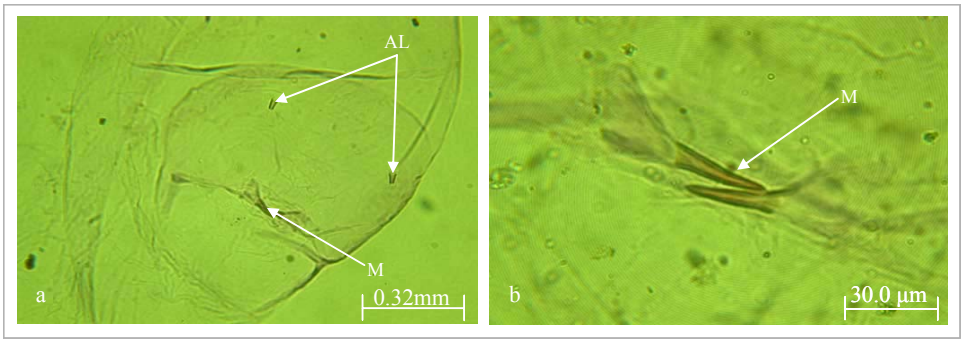


Figure 5 Photomicrographs of third instar larvae of *P. cerealellae*. Figure shows a third instar showing the folded cuticle and the bulging imaginal eye on the head capsule (a); and a late third instar excreting meconium (b).

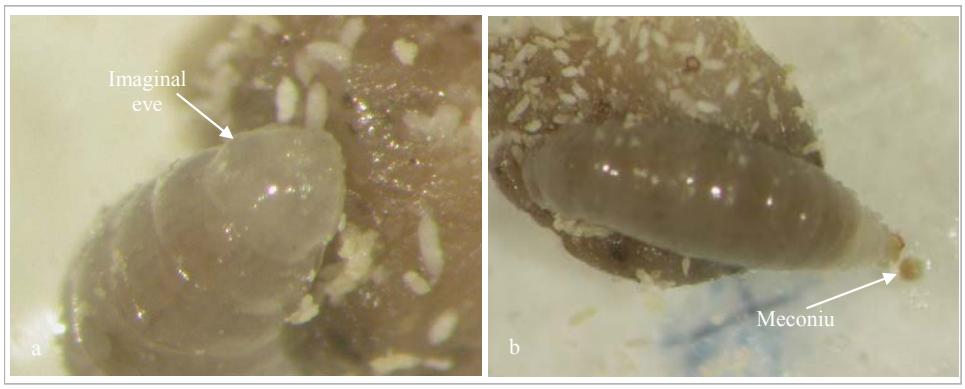


Figure 6 Pupae of *P. cerealellae*. Figure shows a newly formed (a); a tanned (b); a darkened male (c); and a darkened female (d) pupa of *P. cerealellae* as observed under the stereomicroscope at 20×. Note the pointed tip of female abdomen.

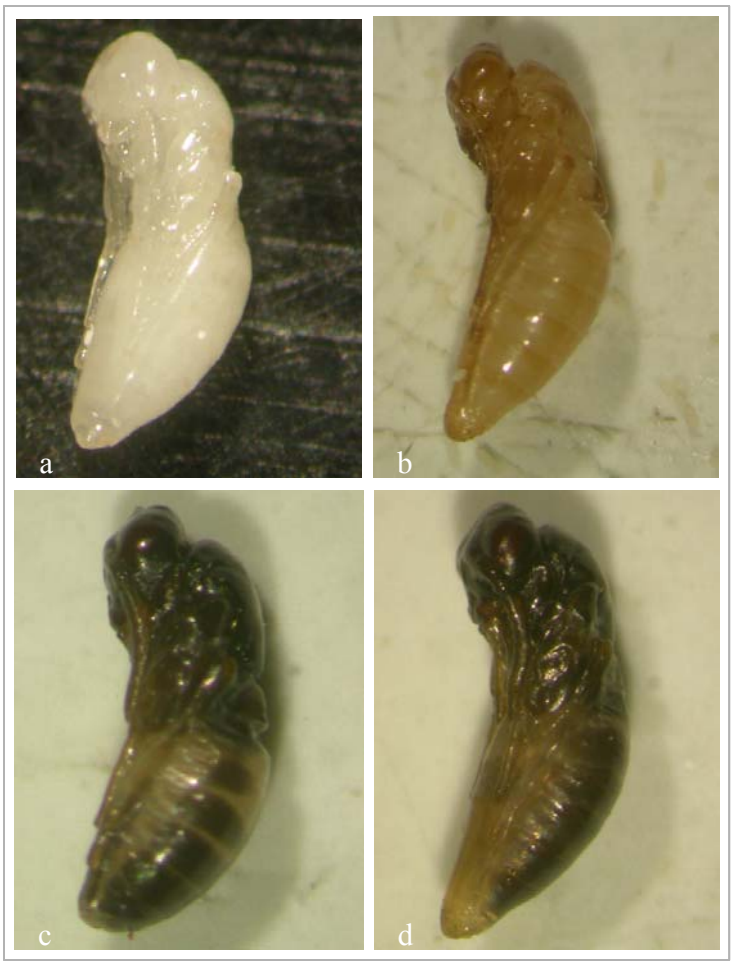


Figure 7 Adult *P. cerealellae* as observed under the stereomicroscope at 20×. A foraging male (a); and a foraging female feeding on the body fluid of an excised host (b).

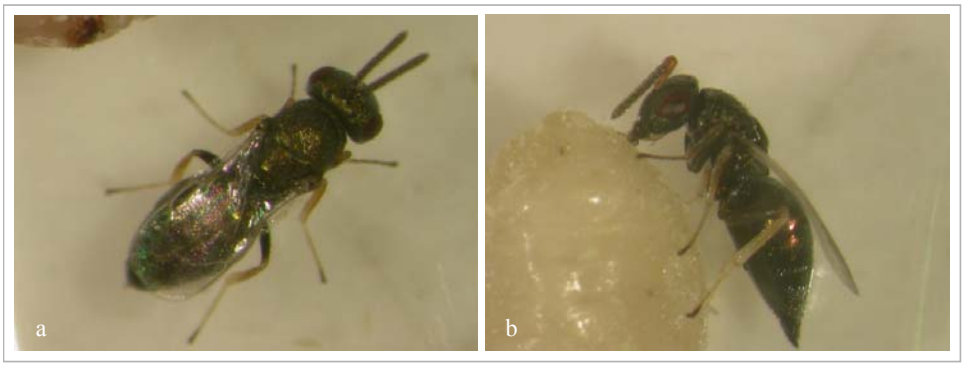


Figure 8 Scanning electron micrographs of the conventional type of mandibles found in pupae and adults of *P. cerealellae*. Figure shows dorsal (DS) and ventral (VS) views of the mandibles as observed under the SEM at 250×.

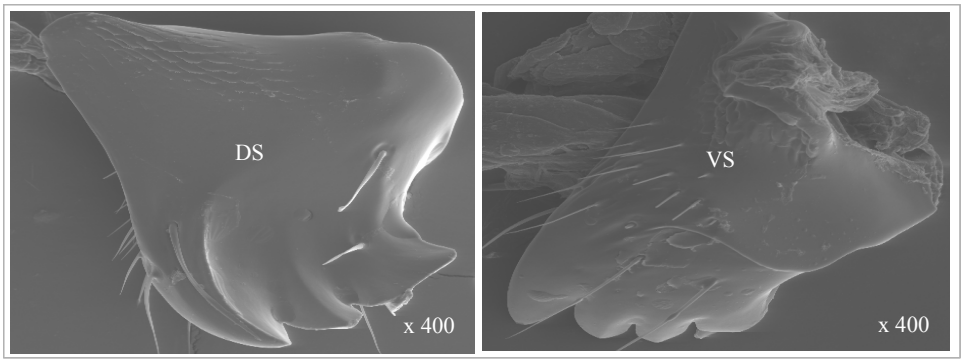


Figure 9 Significant differences in the mean (± SE) mandible lengths (m-length) of the four larval instars of *P. cerealellae*. Means having different letters are significantly different (Tukey's HSD test, $P < 0.05$).

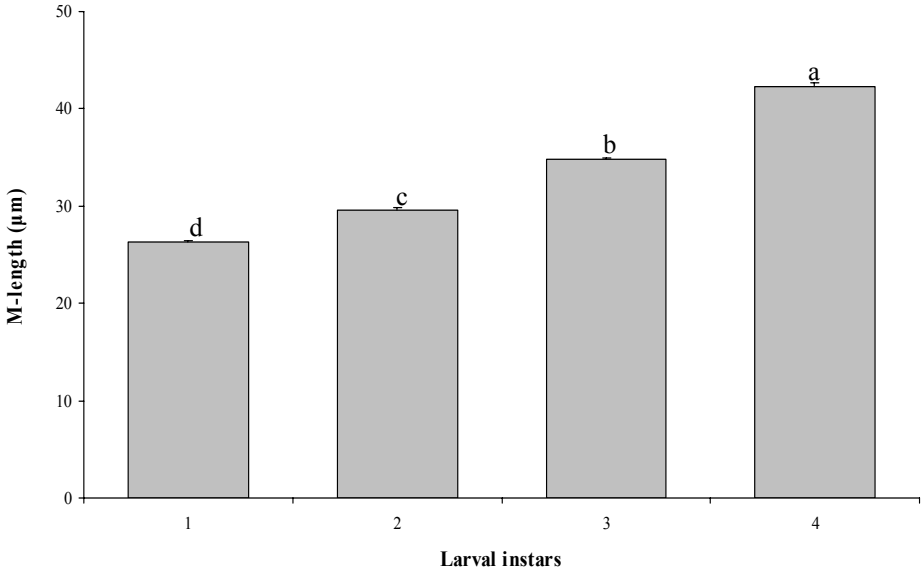


Figure 10 Positive correlation between mandible lengths (m-length) and instar groupings of *P. cerealellae* larvae. Numbers 1 to 4 indicate the four larval groupings.

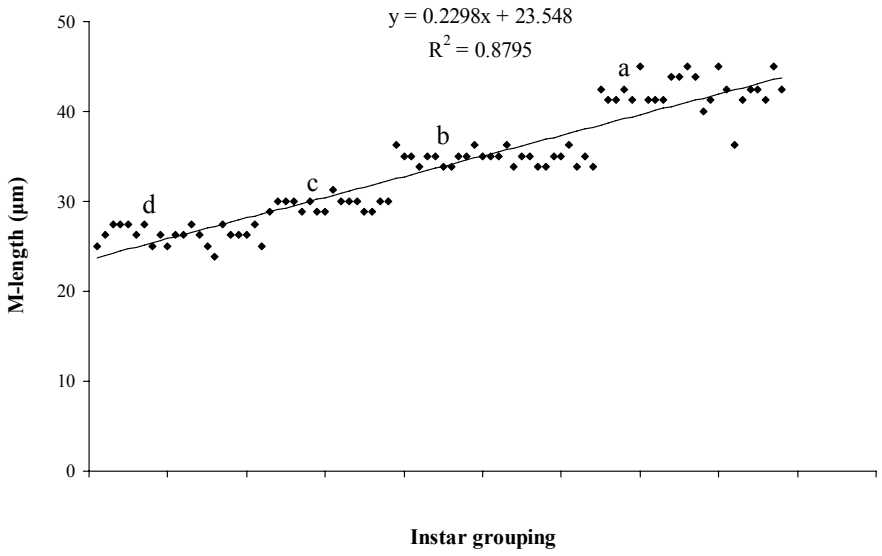
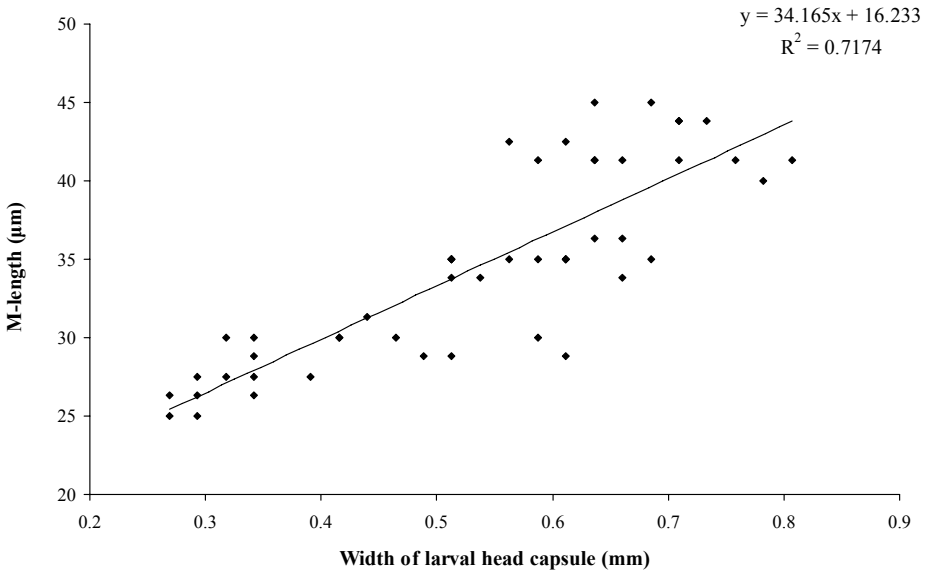
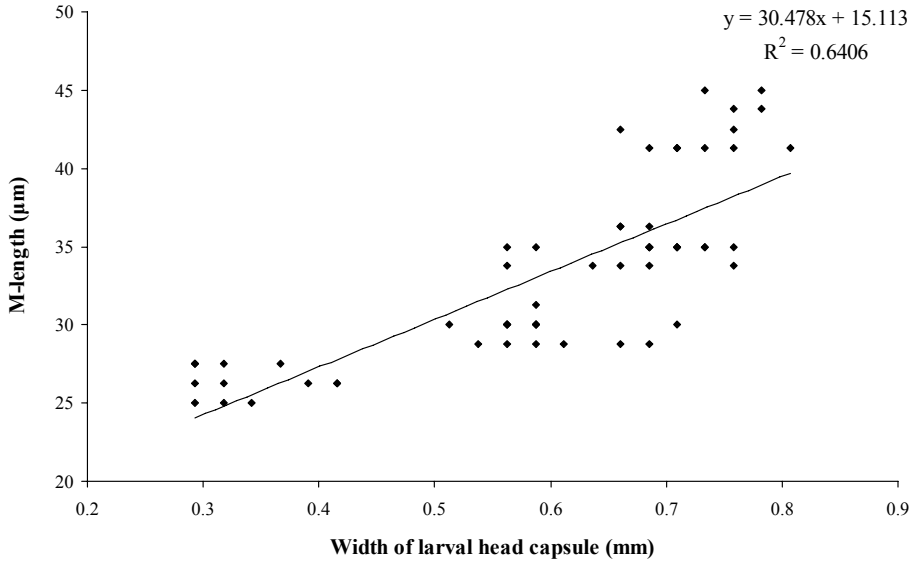


Figure 11 Positive correlation between mandible lengths (m-length) and sizes (widths) of head capsules of *P. cerealellae* male (a) and female (b) larvae.


y = 34.165x + 16.233
R2 = 0.7174
M-length (µm)
Width of larval head capsule (mm)
50
45
40
35
30
25
20
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

(a)


y = 30.478x + 15.113
R2 = 0.6406
M-length (µm)
Width of larval head capsule (mm)
50
45
40
35
30
25
20
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

(b)

CHAPTER 5: SCANNING ELECTRON MICROSCOPY STUDIES OF ANTENNAL SENSILLA OF *PTEROMALUS CEREALELLAE*

INTRODUCTION

Antennae of insects, in particular parasitic Hymenoptera, are important sensory structures involved in various behaviors including habitat searching, host location, host examination, host recognition, host acceptance, oviposition, host discrimination and courtship and mating behavior (Weseloh, 1972; Vinson et al., 1986; Bin et al., 1989; Isidoro et al., 1996). Several studies have characterized the antennal sensilla of various species of parasitic wasps using electron microscopic techniques (Norton and Vinson, 1974; Barlin and Vinson, 1981; Barlin et al., 1981; Wibel et al., 1984; Navasaro and Elzen, 1991; Olson and Andow, 1993; Baaren et al., 1996; Isidoro et al., 1996; Amornsak et al., 1998; Ochieng et al., 2000; Pettersson et al., 2001; Bleeker et al., 2004). Many of these studies reported strong sexual dimorphism in structure and types of antenna1 sensilla (Wibel et al., 1984; Navasaro and Elzen, 1991; Amornsak et al., 1998; Bleeker et al., 2004), whereas other authors reported little or no distinct sexual differences in the antennal sensory system (Ochieng et al., 2000; Pettersson et al., 2001).

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). Females lay eggs in host larvae, which typically are concealed within seeds. Little is known about host location mechanisms in *P. cerealellae* or related wasps. To detect their concealed hosts, female *P. cerealellae* presumably use certain host location cues such as physical/mechanical cues (e.g., host vibration) and chemical cues, which could originate from the host insect or host environment (i.e. host-seed).

In an ongoing research on the host location mechanisms of *P. cerealellae*, we demonstrated antennal response of both sexes to host-related odor (unpublished results). To provide requisite background information to further this research, we investigated the external morphology of the antennal sensilla of the parasitoid using scanning electron microscopy (SEM). In this paper, we describe the morphology, location, abundance, and distribution of the different sensilla present on the antennae of male and female *P. cerealellae*. The possible roles of the sensilla types in the behavioral ecology of the parasitoid are also discussed.

MATERIALS AND METHODS

Insects

Pteromalus cerealellae was reared in our laboratory on the larvae of the cowpea bruchid, *Callosobruchus 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 ºC, 70 ± 5% r.h., and L12:D12 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, which were then provided to *P. cerealellae* for parasitization. The parasitoid was maintained by transferring about 30 adult pairs onto 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 d 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 5 d of oviposition. Parasitized host larvae were incubated in a growth chamber at the above environmental conditions until the emergence of adult parasitoids.

Scanning electron microscopy (SEM)

Freshly emerged adult parasitoids were first anaesthetized in a freezer (-20 ˚C) for 5 min after which the heads were removed. Antennae of *P. cerealellae* were carefully excised from the antennal sockets with fine forceps (*Fisher* Scientific) at 20× under a stereomicroscope (National Microscope, Model DC 3-420, Meiji, Japan). Antennae were

first kept in 70% ethanol for 24 h and then dehydrated in a graded alcohol series of 75, 80, 85, 90 and 99.9% (Baaren et al., 1996) in each case for 1 h. Antennae were individually mounted on aluminum stubs with double-sided sticky tapes. Antennae were mounted with ventral or dorsal sides on the sticky tape. The dehydration processes was followed by air drying in a drying cabinet conditioned to 25 ± 1 °C and 10 ± 1% r.h. for 5 d. The antennae were then sputter coated with gold/palladium (40:60) in a Polaron E 5400 high-resolution sputter coater. The specimens were examined in a DSM 940 SEM (Zeiss, W. Germany) set at 10 kV and 15 mm working distance. Micrographs were taken of the antennae, antennomeres, and sensilla. Abundance and the distribution of the sensilla on the antennae were compared between males and females. The length, width and the diameter of the pits in which sensilla are recessed (where applicable) were measured. A total of 12 antennae per sex were viewed under SEM.

Statistical analysis

Sensilla on the dorsal and ventral surfaces of the antennae of *P. cerealellae* were identified, counted and measured. Measurements (μm) obtained from photomicrographs of at least 15 individual sensilla of the same type were used to calculate the means. Data obtained on the distribution and abundance of different types of sensilla on male and female antennae were analyzed using Students' *t - test* to determine any significant sexual differences ($P < 0.05$).

RESULTS

Terminology

The literature on morphology of insect antennae is somewhat inconsistent and confusing with different names and terminologies assigned to sensilla types despite similarity in form and distribution. To overcome this inconsistency in terminology, we define and classify antenna1 sensilla in this study on the basis of their external appearance when viewed under a scanning electron microscope (SEM) and followed the nomenclature of Chapman (1982; 1998), Wibel et al. (1984), Isidoro et al. (1996); Amornsak et al. (1998), Pettersson et al. (2001), Ryan (2002), and Bleeker et al. (2004). The description of sensilla types is based largely on our inference from the published photomicrographs, using morphological characters such as presence and positions of pores as bases for identification.

General description of antennae of P. cerealellae

The antennae of *P. cerealellae* are geniculate in shape and consist of the basic segments: scape with radicula, pedicel, and flagellum. The radicula (*Rd*) is completely separated from the scape, has a different type of sensilla and, thus, may be considered as a separate segment. The radicula fits into the antennal socket where it functions as the fulcrum to the antenna. It constitutes ~4% of the total antennal length and is 50 ± 0.8 µm (mean ± SE) µm and 55 ± 0.6 µm long in males and females, respectively (Table 1). The cylindrical scape (*Sc*) is about 5 times as long as wide (length: 355 ± 6.0 µm in males and 355 ± 6.0 µm in females) constituting ~30% of total antennal length. The pedicel (*Pd*) is a short, barrel-shaped segment measuring 14 ± 1.8 µm long in males and 106 ± 1.2 µm long in females and constitutes ~8% of total antennal length. The radicula, scape, and pedicel are each composed of one antennomere. In this study, we define antennomeres as

individual separate segments or sub-segments and number them consecutively from the radicula (1st antennomere = A1) to the last claval antennomere (15th antennomere = A15). The elongate flagellum (*F*) constitutes ~60% of the total antennal length and measures 798 ± 13.4 μm and 780 ± 9 μm long in males and females, respectively (Table 1). The flagellum is differentiated into the basal ring-like sub-segments, anelli (*An*), mesal funicle (*Fn*) and distal club-shaped clava (*Cl*) (Fig. 1). The anelli, funicle, and clava consist of 2, 6, and 4 antennomeres respectively, making the whole antenna of *P. cerealellae* to be composed of 15 antennomeres. The anelli antennomeres are small and ring-like while the funicular antennomeres are cylindrical. The first 3 claval antennomeres are barrel-shaped, whereas the last forms a flattened plate that covered the preceding barrel-shaped antennomere (Fig. 2; *Cl*). Each of the anelli antennomere is about twice as wide as long (Fig. 2; *An*), whereas each funicular antennomere (e.g. Fn_6, Fig. 2) is nearly twice as long as wide. The enlarged antennal clava tapers to a circular, flattened point (the last antennomere), which faces ventrally down on the antennal tip. The surfaces of the antennae of both sexes appear similar, but abundance and distribution of the different sensilla differ.

Sensilla types

Eight morphologically different types of sensilla were recorded on the antennae of male and female *P. cerealellae*. These include 4 types of sensilla trichodea (types I, II and IV are aporous, while type III is multiporous), uniporous chaetica sensilla,

multiporous placoid sensilla, basiconic capitate peg sensilla, and coeloconic sensilla. Sensilla trichodea are long, slender and hair-like with or without pores on the shaft, and have been categorized on the basis of external structure and size. They are distinguished from chaetica sensilla on the basis of presence of grooves on the surface of the thick-walled shaft which tapers to a blunt tip (Ryan, 2002). Basiconic capitate peg sensilla have longitudinal ridges and are recessed in shallow pits differentiating them from coeloconic sensilla, which are recessed in deep cuticular pits (Ryan, 2002). The multiporous placoid sensilla are the largest and most distinct sensilla type on the antennae of parasitic wasps (e.g., Isidoro et al., 1996; Pettersson et al*.,* 2001). The distribution of the different sensilla types on each antennal segment is shown in Table 2.

Aporous type 1 sensilla trichodea (ST1-AP)

Sensilla trichodea type I occur on distal ends (on the last antennomere in males and on the last two antennomeres in females) of the antennal flagellum. They have small conical basal sockets with slender and strongly bent shafts (Fig. 3, A; *ST1-AP*). The number and distribution of the *ST1-AP* is shown in Tables 2 (whole antennae) and 3 (focus on the flagellum). The *ST1-AP* range from 29.5 ± 0.2 µm to 48.0 ± 0.4 µm in length and 3.0 ± 0.1 µm and 3.5 ± 0.1 µm in basal width, depending upon location on the antennae (Table 4). Female *P. cerealellae* have greater number of *ST1-AP* than males (Table 2).

Aporous type 2 sensilla trichodea (ST2-AP)

The *ST2-AP* are elongated and widely distributed on the scape, pedicel, anelli, funicle (F1 - F5), and the first 2 claval (*Cl*1 – *Cl*2) antennomeres of male and female antennae. They are tapered and show deep basal insertion into sockets terminating in pointed apices (Fig. 3, B; *ST2-AP*). The length and basal socket characteristics of *ST2-AP* vary according to location, but the morphology of the shafts does not vary; shafts have flutted surfaces (Fig. 3, B). The *ST2-AP* is the most abundant sensilla type on the female antennae numbering ~490 on each antenna, with relatively fewer numbers on the male antennae (Table 2). The *ST2-AP* is morphologically similar to the *ST1-AP* in external appearance and cuticular insertion into sockets but differs in shape of the shafts. *ST1-AP* tapers to a fine tip on a bent shaft (Fig. 3A), whereas the shaft of *ST2-AP* is straight (Fig. 3, A, B).

Multiporous type 3 sensilla trichodea (ST3-MP)

The *ST3-MP* are long and tapering and distributed on all the funicular antennomeres and on the first 3 - claval antennomeres of male and female antennae; are the most abundant sensilla on the antennae of male *P. cerealellae* (Tables 2 and 3). Each sensillum gradually curves distally arising directly from the cuticle (no socket is present). They have smooth cuticle covered by small pores (Fig. 3, C; *ST3-MP*). *ST3-MP* is slightly bulbous at the base (Fig. 5, A, C; *ST3-MP*) with a mean length and basal diameter of 88.9 ± 0.4 µm and 4.6 ± 0.3 µm, respectively (Table 4). Multiple pores can be seen on the walls of the *ST3-MP* occurring at a density of 2.8 ± 0.12 per μm^2 (mean ± SE, $n = 5$) at half length.

Aporous type 4 sensilla trichodea (ST4-AP)

The aporous sensilla trichodea type IV (*ST4-AP*) occur at 2 locations: on the basal portion of the radicula (Figs. 2, *Rd*; 3, D; *ST4-AP*) and on the pedicel (Fig. 2, *Pd*; *ST4-AP*). Each sensillum is a triangular peg-like structure with smooth cuticle that tapers to a blunt apex. The *ST4-AP* are short measuring 16.7 ± 0.9 µm in length and a basal width of 3.5 ± 0.1 µm (Table 4); are inserted into large pits of 8.3 ± 0.3 µm in diameter. Twenty *ST4-AP* sensilla occur on the radicula of male and female *P. cerealellae* arranged in groups of 3 to 5, whereas only 1 cluster of 3 sensilla occurs on the basal part of the pedicel (Table 2; Fig. 2, *Pd*).

Uniporous chaetica sensilla (ChS-UP)

Uniporous chaetica sensilla occur on the distal ends of the antennae of both sexes. This type of sensilla is characterized by grooved surfaces (Fig. 4, A; *ChS-UP*) and projects slightly more perpendicularly with respect to the axis of the antennal surface than does the *ST1-AP* type. The *ChS-UP* measures 39.6 ± 1.6 µm and 3.5 ± 0.1 µm in length and basal diameter, respectively (Table 4). The length, orientation, and the blunt tip with apical pore of the *ChS-UP* (Fig. 4, A; *ChS-UP*) may suggest a contact chemoreception function (Ryan, 2002).

Multiporous placoid sensilla (MPS)

Multiporous placoid sensilla are the largest and the most conspicuous sensilla type on antennae of *P. cerealellae*. Each sensillum arose from an elevated cuticular rim and it tapers apically. The *MPS* occur on all funicular (A6-A11) and claval (A12-A15)

antennomeres forming a ring-like distribution. They have a mean length and width of 112.8 ± 0.4 μm and 11.6 ± 0.0 μm, respectively (Table 4), and are more numerous on antennae of females (Tables 2 and 3). Multiporous placoid sensilla are generally aligned parallel with the antennal axis. They are sausage-shaped and are between the rows of *ST2-AP* and *ST3-MP*. *MPS* sensilla are elongate, plate-like sensory organs with shafts containing numerous pores (Fig. 4, B; *MPS*). As estimated at half length, multiple pores occur on the walls of the *MPS* at a density of 31.6 ± 1.61 per μm^2 (mean ± SE, $n = 5$).

Basiconic capitate peg sensilla (BCPS)

Basiconic capitate peg sensilla (*BCPS*) are distinguished by their external structure, shape and distribution (Figs. 4, C, D; *BCPS*). *BCPS* are bulb-like structures, each set into a shallow cuticular depression of 13.2 ± 0.1 μm in width. Each BCPS measures 8.4 ± 0.2 μm in length and 4.5 ± 0.2 μm in width (Table 4). They possess a rounded capitate peg on a distinct stalk. An obvious ring of wrinkled cuticle (Fig. 4, D; *WC*) surrounds the stalk, which is set in a distinct cuticular depression. A total of 6 *BCPS* occur on the flagellar antennomeres A8, and A10-A14 on the male antenna and 9 on flagellar antennomeres A6-A9 on the female antenna (Table 3). On both male and female antennomeres, *BCPS* are located on the distal portions of flagellar antennomeres (Fig. 4, C, E; *BCPS*).

Coeloconic sensilla (CS)

Coeloconic sensilla are recessed in deep pits (Ryan, 2002). They are stump-like pegs with no grooved trunk, and nearly as long as wide with blunt but ridged tip (Fig. 4,

E, F; *CS*). Male *P. cerealellae* have 2 *CS* located on the 3rd (A8) and 5th (A10) funicular antennomeres, whereas females have only one on the 3rd funicular antennomere.

Abundance and distribution of sensilla on antennae of male and female P. cerealellae

Major differences were recorded in distribution and abundance of some sensilla types on the antennae of male and female *P. cerealellae* (Table 2). The *ST1-AP* occur on the antennal apices of both sexes and are significantly more abundant on the female than on the male antennae ($F = 75.9$, $df = 1$, $P < 0.0001$; Tables 2 and 3). Similarly, the long, hair-like *ST2-AP* occur in significantly greater numbers on the female antennae ($F = 5398$, $df = 1$, $P < 0.0001$; Tables 2 and 3). The *ST2-AP* occur on the scape (A1), pedicel (A2), anelli (A3-A4), funicle (A5-A11) and on the first 2 claval antennomeres (A12-A13). In contrast, the *ST3-MP* are absent on the scape, pedicel and anelli but widely distributed on the funicular (A6-A11) and the first three claval antennomeres (A12-A14) (Tables 2 and 3). The *ST3-MP* are significantly more abundant on the antennae of male*s* compared to females ($F = 25259$, $df = 1$, $P < 0.0001$; Tables 2 and 3). The distribution of the *ST4-AP* is restricted to the cavities of the antennal socket (on the radicula) and the ventral surface of pedicel at the scape-pedicel elbow joint (Table 2; Fig. 2, A; *Pd*), and no differences were recorded between sexes on abundance and distribution of this sensilla type. The distribution of the *MPS* is also similar on the antennae of the two sexes. However, Students' *t - test* analysis revealed significant difference in the abundance of these sensilla on the antennae of male and female wasps ($F = 10057$, $df = 1$, $P < 0.0001$; Tables 2 and 3), with the females having approximately twice the number recorded in the males (Tables 2 and 3). The abundance of the *ChS-UP*, *BCPS* and *CS* were not

significantly different between sexes (Tables 2 and 3). The *ChS-UP,* like the *ST4-AP,* occur together with the *ST1-AP* and, like the *ST4-AP,* are distributed only on distal ends of the antennal flagellum in males (A15) and females (A14 -A15) (Table 3; Fig. 5 A, B). *BCPS* occur on the proximal and distal antennomeres of the antennal flagellum of the females and males respectively (Table 3). The abundance of *ST2-AP*, *ST3-MP* and *MPS* and the distribution of *BCPS* may indicate sexual dimorphism in the antennae of *P. cerealellae.*

DISCUSSION

The external morphology, types and distribution of sensilla on the antennae of male and female *P. cerealellae* recorded in this study are largely conform with those reported for other parasitoid species (Wibel et al*.,* 1984; Olson and Andow, 1993; Isidoro et al., 1996; Pettersson et al*.,* 2001; Bleeker et al*.,* 2004). The antennae of insects have been typically described as consisting of basic 3 segments, scape with radicula, pedicel, and flagellum (e.g., Chapman, 1998; Isidoro et al., 1996). The radicula of *P. cerealellae* antennae can actually be considered as a separate segment since it is completely separated from the scape and has a different type of sensilla. However, since the term "segment" is usually reserved for parts with their own musculature (Chapman, 1998), further studies are necessary to confirm the radicula of *P. cerealellae* as a separate segment.

Our study revealed 8 morphologically different types of sensilla on the antennae of male and female *P. cerealellae*, similar to those described for other hymenopteran wasps including pteromalid species, such as *Nasonia vitripennis* (Walker) (Slifer, 1969;

Wibel et al., 1984), *Rhopalicus tutela* (Walker) (Pettersson et al., 2001) and non-pteromalid wasps like *Trichogramma nubilale* (Ertle and Davis) (Hymenoptera: Trichogrammatidae) (Olson and Andow, 1993). It should be noted that different nomenclatures were used in some of these studies to describe the antennal sensilla.

Major sexual differences were recorded in the distribution and abundance of 5 sensilla types on the antennae of *P. cerealellae,* similar to the report on another pteromalid, *N. vitripennis* (Wibel et al., 1984). In general, a greater number of antennal sensilla occur on the males compared to the females. For instance, the aporous type II sensilla trichodea *(ST2-AP)* occur in greater numbers (almost twice) in females than in males. The *ST2-AP* appear similar to the "aporous sensilla trichodea B" described on the antennae of *T. nubilale* (Olson and Andow, 1993) and to the tactile sensilla (mechanosensory bristles) described on *R. tutela* antennae (Pettersson et al., 2001). The *ST2-AP* have been described in many Hymenoptera belonging to different families as having putative mechanoreceptive functions, such as in the perception of mechanosensory stimuli (Amornsak et al., 1998; Olson and Andow, 1993; Isidoro et al ., 1996; Baaren et al., 1996, 1999; Pettersson et al., 2001; Roux et al., 2005; Marques-Silva et al., 2006). Isidoro et al., (1996) suggested that these sensilla may be involved in host examination and host discrimination because most parasitoids usually examine the host by drumming its surface with the apicoventral part of the antennal club, which bears these sensilla. It is probable that the *ST2-AP* play a similar role in the behavior of *P. cerealellae* given that the parasitoid has been observed to exhibit antennal drumming behavior (unpublished data).

The multiporous type III sensilla trichoidea (*ST3-MP*) have previously been described with different names including multiporous sensilla trichodea with wall pores (Wibel et al*.,* 1984; Pettersson et al*.,* 2001; Ryan, 2002; Bleeker et al*.,* 2004), sensilla basiconica type B in *M. croceipes* (Navasaro and Elzen, 1991), multiporous pitted (MPP) sensilla trichodea C in *T. nubilale* (Olson and Andow, 1993), and basiconica type *I* sensilla (Ochieng et al*.,* 2000). The *ST3-MP* are also similar in morphology and distribution to the "thin-walled chemoreceptors" described on antennae of *N. vitripennis* (Wibel et al*.*, 1984), "sensilla trichodea" on the antennae of *R. tutela* (Pettersson et al*.*, 2001), and to "sensilla trichodea C" of *T. nubilale* (Olson and Andow, 1993). In general, antennal sensilla trichodea are presumed to function as olfactory receptors in many insects (Steinbrecht, 1987, 1997; Bleeker et al*.,* 2004), and electrophysiological studies have confirmed a sex pheromone receptor function for trichoid sensilla of *Neodiprion sertifer* Geoffroy (Hymenoptera: Diprionidae) (Hansson et al., 1991). Also, Pettersson et al*.* (2001) proposed a pheromone receptor function for the sensilla trichodea of *R. tutela*. The greater abundance of the *ST3-MP* on antennae of male *P. cerealellae* relative to the females may indicate a probable role in mate location, possibly for detection of female sex pheromones, as reported for some other parasitoids (Barlin et al*.,* 1981; Chapman, 1982; Bleeker et al*.,* 2004). *ST3-MP* are found in large numbers and have an elongated shaft, so they may also provide a large surface area to receive the stimuli from the females of this species.

The type IV sensilla trichodea (*ST4-AP*) described in the current study are similar to trichoid sensilla described on antennae of the scelionid *Gryon boselli* Mineo and Szabo (Villa and Mineo, 1990) and *Trichogramma australicum* (Schmidt and Smith, 1987;

Amornsak et al., 1998). These authors proposed that this sensilla type may play a role in measurement of antennal curvature and thus act as proprioceptors. Chapman (1998) also described the hairs that occur at the joints between segments or on sclerites as trichoid sensilla because they taper from base to tip and, also, due to lack of pores. The author suggested that these hairs monitor the position of one cuticular element relative to another and that they are positioned so that the flexing of one part of the cuticle with respect to another will cause the hairs to bend. Our SEM of *ST4-AP* revealed no obvious pores suggesting that the sensilla may be aporous. The occurrence of the *ST4-AP* only on the radicula, which fits into the antennal socket, and at the scape-pedicel elbow joint in *P. cerealellae* may suggest their role as proprioceptors, such as in the measurements of the antennal curved surface and the position of one cuticular element relative to another.

The elongate multiporous placoid sensilla (*MPS*) are widely distributed on antennae of parasitic wasps (Richerson et al., 1972; Barlin and Vinson, 1981; Wibel et al., 1984; Olson and Andow, 1993; Amornsak et al., 1998; Hallberg and Hansson, 1999; Pettersson et al., 2001). *MPS* are generally arranged in alternate rings around the funicular antennomeres (Barlin and Vinson, 1981; Pettersson et al., 2001). Their multiple pores suggest an olfactory function (Barlin and Vinson, 1981; Ochieng et al., 2000; Bleeker et al., 2004; Roux et al., 2005; Marques-Silva et al., 2006). The greater abundance of *MPS* in female *P. cerealellae* than in males is in contrast to the reports for several other Hymenopteran species (Baaren et al., 1999; Navasaro and Elzen, 1991; Ochieng et al., 2000; Bleeker et al., 2004), and may indicate their role in host location, possibly by detection of host-related semiochemicals.

The uniporous chaetica sensilla (*ChS-UP*) are different from the other sensilla types observed in *P. cerealellae,* being characterized by their grooved surfaces. Their morphology and location is similar to some previously described sensilla in many insects including the "thick-walled chemoreceptors" of the pteromalid, *N. vitripennis* (Sliffer, 1969; Wibel et al., 1984), the "uniporous pit pore sensilla trichodea D" in *T. nubilale* (Olson and Andow, 1993), the "uniporous gustatory sensilla" described on antennae of many parasitic wasps (Isidoro et al., 1996), and the "curved trichoid formation with an apical pore" described in *Opius concolor* by Barbarossa et al*.,* (1998). *ChS-UP* have also been described as "fluted basiconic sensilla" in the braconid *Cardiochiles nigriceps* Vireck (Norton and Vinson, 1974) "aporous, socketed hairs" in the eulophid *T. hagenowii* (Barlin et al*.,* 1981), and as "tapering fluted setae" in the eulophid *Melittobia australica* Girault (Dahms, 1984). The *ChS-UP* have also been described in other wasps as having a terminal pore, suggesting that they may be involved in contact chemoreception (Altner and Prillinger, 1980; Olson and Andow 1993; Pettersson et al*.* 2001), including gustatory function (Isidoro et al*.,* 1996; Barbarossa et al*.,* 1998). *ChS-UP* are confined to the apices of antennae of *P. cerealellae* and other pteromalids, further suggesting their role as contact chemoreceptors (Sliffer, 1969; Wibel et al., 1984; Pettersson et al*.*, 2001) and probable involvement in host recognition and host acceptance (Weseloh, 1972; Borden et al*.* 1973).

The basiconic capitate peg sensilla (*BCPS*) described in the current study resemble the "multiporous pegs sensilla" on the eulophid *T. hagenowii* (Barlin et al*.,* 1981), "basiconic capitate peg" on antennae of the pteromalid *N. vitripennis* (Wibel et al., 1984), "peg-like sensillum" or "sensillum coeloconicum" in the eulophid *Sympiesis*

sericeicornis Nees (Hymenoptera: Eulophidae) (Meyhofer et al*.,* 1997), and the type-I coeloconic sensillum on antennae of *Cotesia spp.* (Bleeker et al*.,* 2004). Studies by Baaren et al*.,* (1996) and Olson and Andow (1993) revealed that pores are present in the furrows on the bulbous distal end of *BCPS,* suggesting they may play a role in olfaction (Baaren et al*.,* 1996; Steinbrecht, 1997, Keil, 1999, Bleeker et al*.,* 2004) or as hygro-, thermo- and mechanoreceptors (Wibel et al*.,* 1984; Wcislo, 1995; Pettersson et al*.,* 2001). In contrast, Meyhofer et al*.,* (1997) reported no evidence of pores on this antennal sensilla type in *S. sericeicornis,* similar to our results for *P. cerealellae. BCPS* are proximally distributed on the flagellum of the female *P. cerealellae,* whereas they are more distally distributed on the flagellum of antennae of males. The grooves on bulbous tips of *BCPS* on antennae of *P. cerealellae* are without punctations suggestive of a thermo- or hygroreceptive function (Halberg, 1979; Altner et al*.,* 1983; Wibel et al*.,* 1984).

Coeloconic sensilla (*CS*) are the least abundant sensilla type on *P. cerealellae,* and have been previously described as "pit organs" because they are recessed into deep pits (Wcislo, 1995), and as coeloconic sensilla type II (Bleeker et al ., 2004). The specific function of the *CS* sensilla is difficult to assess. Altner et al., (1983) and Bleeker et al. (2004) suggested that *CS,* like *BCPS*, may be involved in thermo-hygro perception. *CS* may play a similar role in *P. cerealella* given the absence of sexual differences in their abundance and absence of pores on them.

Female *P. cerealellae* may use both mechanosensory and olfactory cues to locate concealed stored product larval hosts. In addition to identifying key mechanosensory sensilla trichodea types I and II, this study has identified and characterized the

distribution of 2 sensilla types, the multiporous type-III sensilla trichodea *(ST3-MP)* and the multiporous placoid sensilla (*MPS*). *ST3-MP* and *MPS* sensilla are likely involved in olfactory communication in *P. cerealellae*, thus providing necessary background information for our ongoing studies of host location mechanisms in this species, including behavioral and electrophysiological studies of olfaction. The density of *MPS* on the flagellum of *P. cerealellae* is much less than that of the *ST3-MP*, but the relatively greater pore density of the *MPS* may suggest its equal importance in olfaction in *P. cerealellae*. Future studies on the functional morphology of the antennal sensilla of *P. cerealellae* using transmission electron microscopy coupled with electrophysiological recordings will likely confirm the functions of the sensilla identified in this study.

ACKNOWLEDGEMENTS

I gratefully acknowledge Dr. Samuel A. Ochieng (Tennessee State University, Institute of Agricultural and Environmental Research, McMinnville, Tennessee, USA) for technical assistance. I also thank Dr. Michael Miller (Auburn University, Auburn, Alabama) for assistance with the use of the electron microscopes. This research was supported in part by the Alabama Agricultural Experiment Station and an Auburn University Competitive Research grant to HYF.

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Table 1 Mean (± SE) length of antennal segments in male and female *P. cerealellae* (*n* = 12)

Antennal segments	Male	Female
Radicula*	50 ± 0.8	55 ± 0.6
Scape	355 ± 6.0	386 ± 4.4
Pedicel	104 ± 1.8	106 ± 1.2
Flagellum	798 ± 13.4	780 ± 9.0
Total	1308 ± 21.9	1328 ± 15.2

* Further studies are necessary to confirm the radicula as a separate segment. Values show mean length (μm ± SE).

Table 2 Abundance and distribution of different sensilla on the antennae of male and female *P. cerealellae*

Sensilla	Sex	Antennal segment				Total
		Radicula	*Scape*	*Pedicel*	*Flagellum*	
ST1-AP	Male	-	-	-	56 ± 1.4	56 b
	Female	-	-	-	69 ± 0.5	69 a
ST2-AP	Male	-	159 ± 1.2	51 ± 0.8	47 ± 1.6	257 b
	Female	-	228 ± 1.5	68 ± 1.6	193 ± 1.2	489 a
ST3-MP	Male	-	-	-	709 ± 1.4	709 a
	Female	-	-	-	373 ± 1.5	373 b
ST4-AP	Male	20 ± 0.0	-	3 ± 0.0	0 ± 0.0	23
	Female	20 ± 0.0	-	3 ± 0.0	0 ± 0.0	23
MPS	Male	-	-	-	44 ± 0.3	44 b
	Female	-	-	-	96 ± 0.4	96 a
ChS-UP	Male	-	-	-	18 ± 0.3	18
	Female	-	-	-	15 ± 0.3	16
BCPS	Male	-	-	-	6 ± 0.0	6
	Female	-	-	-	9 ± 0.0	9
CS	Male	-	-	-	2 ± 0.0	2
	Female	-	-	-	1 ± 0.0	1

Values are mean (± SE) number of different types of sensilla on each antennal segment (n = 12 antennae per sex). *ST1-AP*: aporous type I sensilla trichodea; *ST2-AP*: aporous type II sensilla trichodea; *ST3-MP*: multiporous type III sensilla trichodea; *ST4-AP*: aporous type IV sensilla trichodea; *MPS*: multiporous placoid sensilla; *ChS-UP*: uniporous chaetica sensilla; *BCPS*: basiconic capitate peg sensilla and *CS*: coeloconic sensilla. Total numbers for each sensilla type having different letters are significantly different between male and female (*t* - test, $P < 0.05$).

Table 3 Approximate number and distribution of sensilla on flagella of antennae of male and female *P. cerealellae*

Sensilla	Sex	Antennomeres (A)												Total
		4	5	6	7	8	9	10	11	12	13	14	15	
ST1-AP	Male	-	-	-	-	-	-	-	-	-	-	-	56	56
	Female	-	-	-	-	-	-	-	-	-	-	22	47	69
ST2-AP	Male	9	17	18	-	1	-	1	-	1	-	-	-	47
	Female	15	18	39	21	14	21	19	17	15	14	-	-	193
ST3-MP	Male	-	-	85	88	87	90	83	88	88	65	35	-	709
	Female	-	-	29	59	59	70	63	58	22	13	-	-	373
ST4-AP	Male	-	-	-	-	-	-	-	-	-	-	-	-	0
	Female	-	-	-	-	-	-	-	-	-	-	-	-	0
MPS	Male	-	-	2	5	4	6	4	4	6	8	5	-	44
	Female	-	-	8	1-	1-	14	11	13	13	13	4	-	96
ChS-UP	Male	-	-	-	-	-	-	-	-	-	-	-	18	18
	Female	-	-	-	-	-	-	-	-	-	-	7	9	16
BCPS	Male	-	-	-	-	1	-	1	1	1	1	1	-	6
	Female	-	-	2	3	2	2	-	-	-	-	-	-	9
CS	Male	-	-	-	-	1	-	-	-	-	-	-	-	1
	Female	-	-	-	-	1	-	1	-	-	-	-	-	2

Values are approximate number of different types of sensilla on each flagellar antennomere. *ST1-AP*: aporous type I sensilla trichodea; *ST2-AP*: aporous type II sensilla trichodea; *ST3-MP*: multiporous type III sensilla trichodea; *ST4-AP*: aporous type IV sensilla trichodea; *MPS*: multiporous placoid sensilla; *ChS-UP*: uniporous chaetica sensilla; *BCPS*: basiconic capitate peg sensilla and *CS*: coeloconic sensilla.

Table 4 Sizes of the antennal sensilla of *P. cerealellae*.

Sensilla	Length (μm)	Basal width (μm)	Pit diameter (μm)
ST1-AP	29.5 ± 0.2	3.0 ± 0.1	NA
ST2-AP	48.0 ± 0.3	3.5 ± 0.1	NA
ST3-WP	88. 9 ± 0.4	4.6 ± 0.3	NA
ST4-AP	16.7 ± 0.9	3.5 ± 0.1	8.3 ± 0.3
MPL	112. 8 ± 0.4	11.6 ± 0.0	NA
ChS-UP	39.6 ± 1.6	3.5 ± 0.1	NA
BCPS	8.4 ± 0.2	4. 5 ± 0.2	13.2 ± 0.1
CLCN	2.9 ± 0.1	2.9 ± 0.4	5.3 ± 0.1

Values are mean (μm ± SE). Measurements obtained from a total of 30 sensilla per type from antennae of males and females (15 per sex). *ST1-AP*: aporous type I sensilla trichodea; *ST2-AP*: aporous type II sensilla trichodea; *ST3-MP*: multiporous type III sensilla trichodea; *ST4-AP*: aporous type IV sensilla trichodea; *MPS*: multiporous placoid sensilla; *ChS-UP*: uniporous chaetica sensilla; *BCPS*: basiconic capitate peg sensilla; *CS*: coeloconic sensilla; and NA: not applicable (i.e. the sensilla are not recessed in pits).

Figure 1 Antenna of *P. cerealellae*. Figure shows the scanning electron micrograph of an excised geniculate antenna of *P. cerealellae* male showing the radicula (*Rd*), scape (*Sc*), pedicel (*Pd*), anelli (*An*), funicle (*F*) and the clava (*Cl*). The female antenna is similar in shape and morphology.

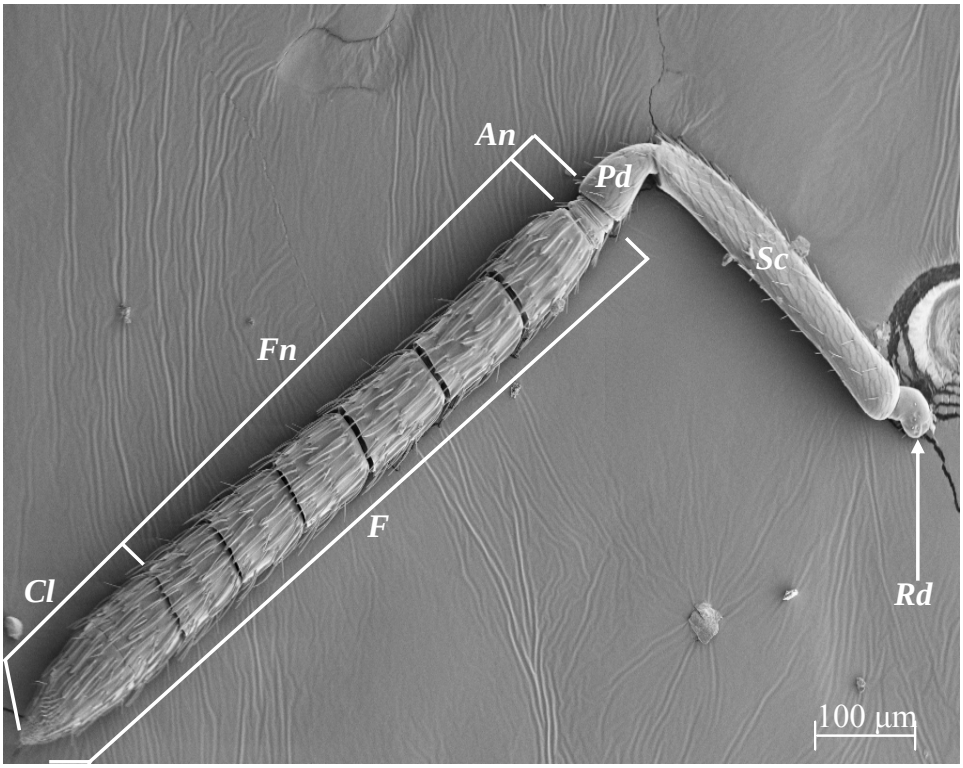


Figure 2 Scanning electron micrographs of the antennal segments and antennomeres of *P. cerealellae*. Figure shows the basal radicula (*Rd*), elongate scape (*Sc*), barrel-shaped pedicel (*Pd*), the ring-like anelli (*An*), the 6th antennomere of the antennal funicle (Fn_6) and the club-shaped clava (*Cl*)

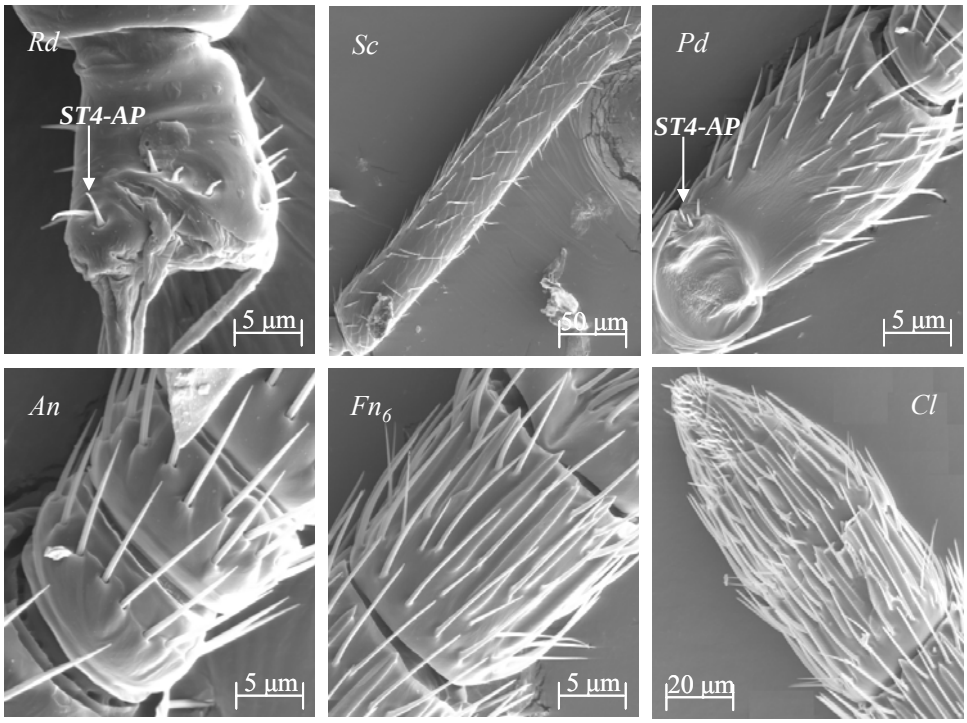


Figure 3 Sensilla trichodea types recorded on the antennae of *P. cerealellae*. Figure shows scanning electron micrograph of type I (*ST1-AP*) on the last claval antennomere (a); type II (*ST2-AP*) on pedicel (b); type III (*ST3-WP*) on funicle antennomere (c), and type IV (*ST4-AP*) on radicula (d)

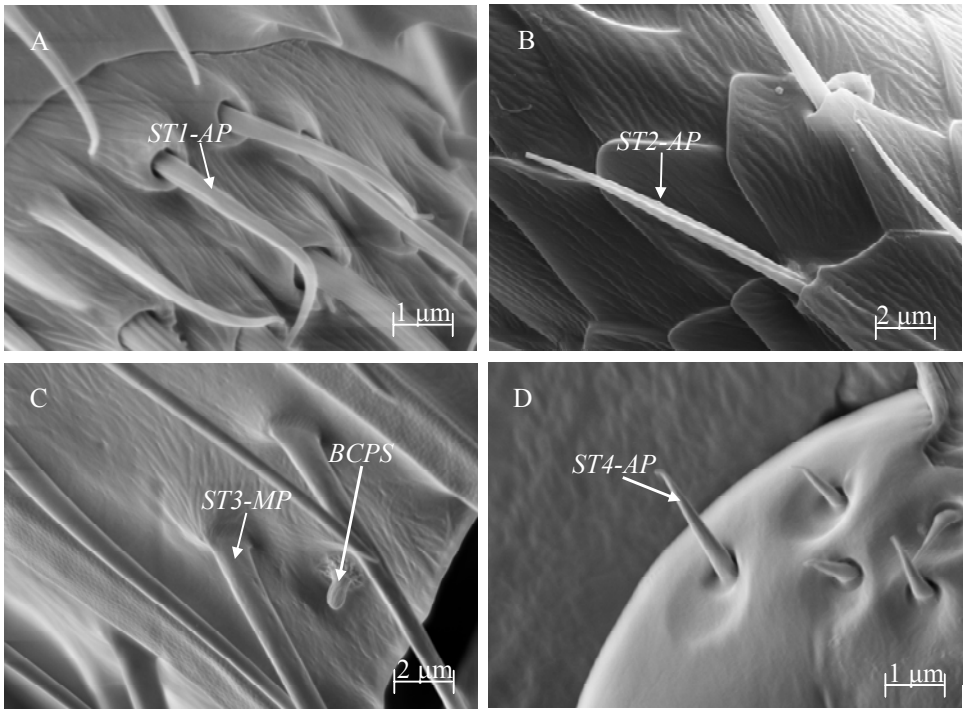


Figure 4 Other sensilla types recorded on antennae of *P. cerealellae*. Figure shows scanning electron micrographs of the uniporous chaetica (*ChS-UP*) (a); multiporous placoid (*MPL*) (b); basiconic capitate peg (*BCPS*) (c) and (d, showing wrinkled cuticle, *WC*); and coeloconic (*CS*) sensilla (e, f) on different antennomeres of *P. cerealellae*

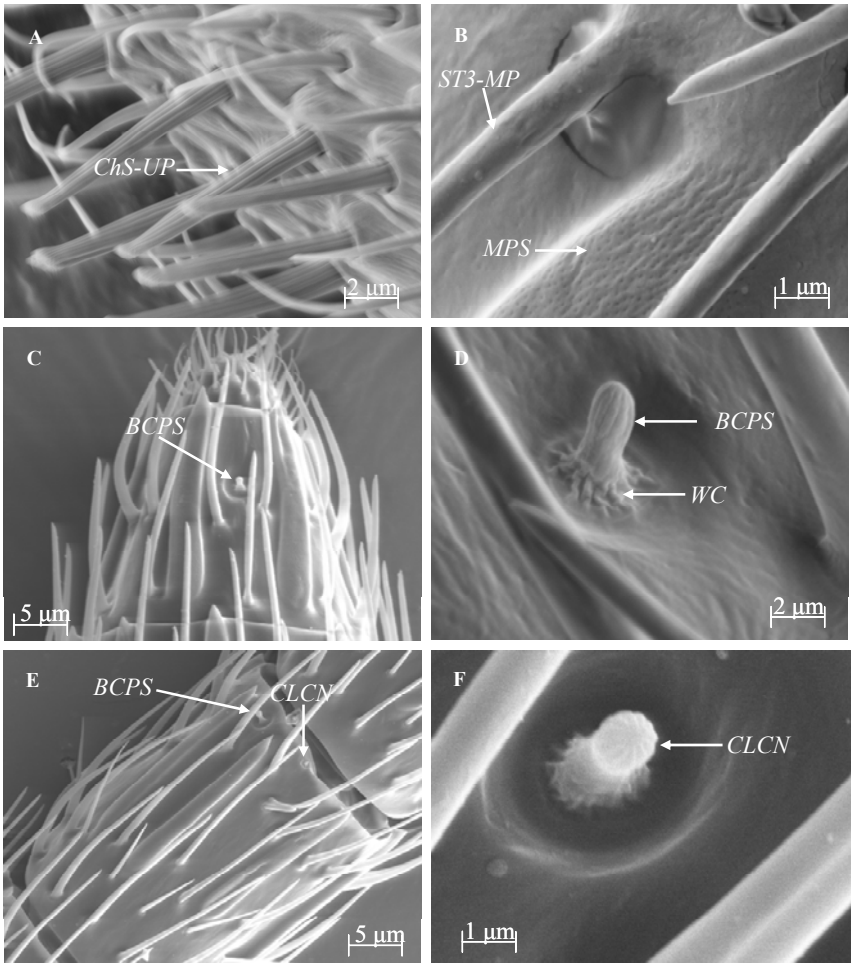
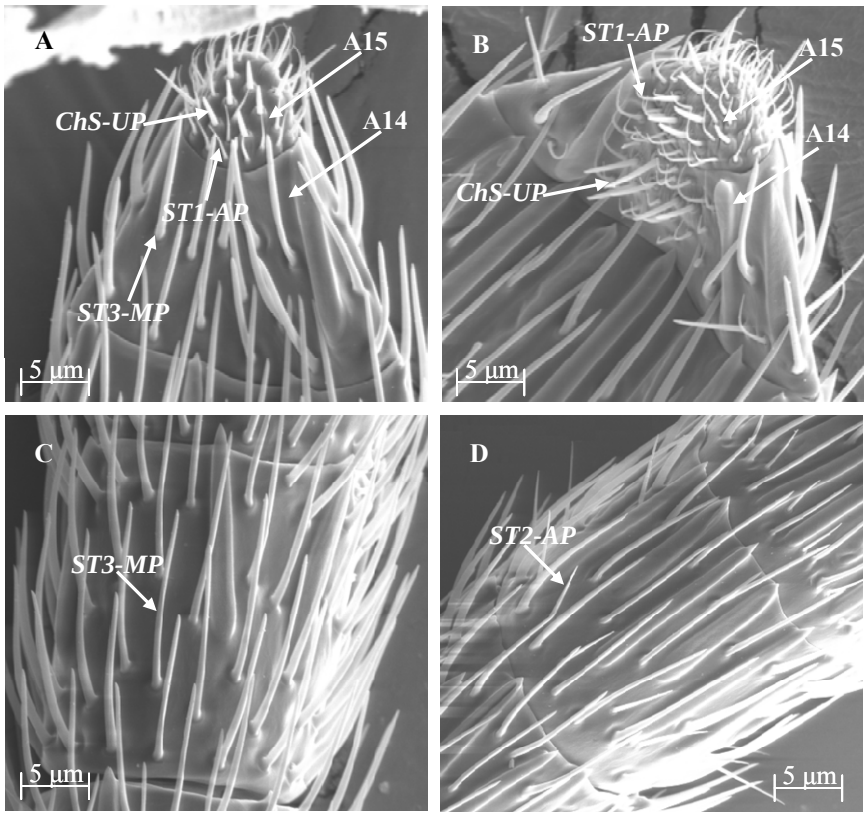


Figure 5 Sexual differences in the abundance and distribution of some key sensilla types on the antennae of *P. cerealellae*. Figure shows scanning electron micrographs of the ventral surfaces of tips of antennae of male (a) and female (b) and the dorsal surfaces of the 6th funicular antennomeres (A11) of male (c) and female (d). The *ST1-AP* and *ChS-UP* which are distributed on the last two antennomeres (A14 and A15) on the female antennae (Fig. 5, a) are only found on the terminal antennomere (A15) in the males (Fig. 5, b). The *ST2-AP* is present on the 6th funicular antennomere (A11) of females (Fig. 5, d), but absent on the same antennomere in males (Fig. 5, c). *ST1-AP*: aporous type I sensilla trichodea; *ST2-AP*: aporous type II sensilla trichodea; *ChS-UP*: uniporous chaetica sensilla



CHAPTER 6: MATING BEHAVIOR OF *PTEROMALUS CEREALELLAE* AND EFFECT OF PREVIOUS MATING EXPERIENCE ON COURTSHIP DURATION AND REPRODUCTIVE PERFORMANCE

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). 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 its mating behavior and sexual communication strategy.

Studies on mating behavior and intraspecific communication in parasitoids are important and may aid their use as biological control agents of agricultural pests. For instance, characterization of the mating behavior of a species can aid its precise identification (Mathews, 1975) and the design of effective mass-rearing programs (Cheng

et al., 2004). In addition, knowledge of sexual communication in parasitoids may enhance their utilization in biological control programs (De Freitas et al., 2004). Mating behavior has been described for several species of hymenopteran parasitoids (Vinson, 1978; van den Assem and Werren, 1994; Jachmann and van den Assem, 1996). Many studies have reported the involvement of sexual attractants in the mating behavior of parasitoids from several families (Mathews 1975; Weseloh, 1976; Vinson, 1978; Simser and Coppel, 1980; Leal et al., 1997). In most species, courtship is initiated through release of female-produced volatile chemicals, which serve to attract males from long-range (Vinson, 1972; Eller et al., 1984; Reed et al., 1994; Cheng et al., 2004). The involvement of male-produced chemicals in close-range courtship behaviors has been reported for some species including *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) (van den Assem, 1986) and *Laelius utilis* Cockerell (Hymenoptera: Bethylidae) (Howard, 1992). Owing to the lack of published studies on the mating behavior of *P. cerealellae* or related species, this study was conducted to provide a quantitative description of the mating behavior of the parasitoid, and to test the possible effects of previous mating experience on courtship duration and reproductive performance (progeny production and offspring sex ratio).

MATERIALS AND METHODS

Insects

Pteromalus cerealellae was reared in our laboratory on the larvae of the cowpea bruchid, *C. maculatus*. The host insect (*C. maculatus*) 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 5 d by placing ~25 pairs of 3-day-old mated *C. maculatus* in a glass jar containing ~100 g of cowpea seeds held at 30 ± 1ºC, 70 ± 5% r.h., and L12: D12 (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, which were then provided to *P. cerealellae* for parasitization. The parasitoid was maintained by transferring about 30 adult pairs onto 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 d after infestation of cowpea seeds under our rearing conditions). 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 d after parasitization at the above conditions). Parasitized hosts (still contained in the infested seeds) which bear the wasps' pupae were immediately transferred into 100 x 15 mm disposable Petri dishes to collect parasitoids immediately upon emergence.

Courtship behavior of P. cerealellae

A mating pair consisting of one male and one female (freshly emerged, unmated) *P. cerealellae* was introduced into a 6-cm diameter plastic Petri dish (i.e. mating arena). The mating arena was then placed on the floor of an observation chamber for observation of mating behavior. The chamber consisted of a 40 x 40 x 70 cm open-roofed fiber board fitted with a centrally positioned, incandescent light of ~250 lux. The floor and the walls

of the chamber were lined with white paper for uniform light diffusion. One side of the chamber was cut open for observation and access. The courtship behavior (including pre- and post-copulatory behaviors) of each mating pair of *P. cerealellae* was observed and recorded using a JVC digital video camera Model GR-D244U (Victor Company of Japan, Malaysia) attached to a stereomicroscope (National Microscope, Model DC 3-420, Meiji, Japan). Detailed behavioral transitions and related timing were determined through slow-motion video playback. Behavioral transitions of male and female wasps were tabulated and an ethogram of mating behavior was generated. Eighteen mating pairs were observed and recorded. Data collected on the durations of each behavioral transition were subjected to one-way multivariate analysis of variance (MANOVA) followed by Tukey's HSD test ($P < 0.05$; JMPIN Version 5.1, SAS Institute Inc., 2003) to determine significant differences in time taken to complete the different behavioral transitions.

Effect of previous mating experience on courtship duration and reproductive performance

In order to determine the effect of prior mating experience on courtship duration and reproductive performance (progeny production and offspring sex ratio) of *P. cerealellae*, inexperienced (virgin) and experienced (recently mated) males and females were paired in a 6-cm plastic Petri dish (mating arena), resulting in 4 pair-wise treatments: i) inexperienced male (IM) and inexperienced female (IF), ii) inexperienced male (IM) and experienced female (EF), iii) experienced male (EM) and inexperienced female (IF), and iv) experienced male (EM) and experienced female (EF). Virgin individuals were obtained by removing parasitoids from the culture immediately after

emergence. Experienced parasitoids were allowed to copulate once in rearing jars and then immediately placed in the mating arena. Virgin and experienced parasitoids were of the same age (~12 h) when paired. Paired parasitoids were observed in the mating arena until copulation had occurred. At the end of copulation, the female was removed immediately and placed in a 6-cm plastic Petri dish containing hosts (fourth instar larvae of *C. maculatus*; $n = 20$) and allowed to parasitize the hosts for 48 h. This was done to check mating success. A successful mating should result in the production of a mixed-sex progeny since *P. cerealellae*, like other hymenopteran parasitoids, is haplodiploid (Charnov, 1982; Godfray, 1994). Parasitized hosts were incubated in a growth chamber conditioned to 30 ± 1˚C, L12: D12 and 70 ± 5% r.h. until parasitoid emergence (F_1 progeny). Eighteen male and female pairs for each pair-wise combination were observed and recorded. Courtship duration (defined as time between when a male and a female pair was introduced into the mating arena to the time the male dismounted the female after copulation), copulation latency (defined as the time between introduction of male and female pair into the mating arena and commencement of copulation) and copulation length (defined as the time between when a male parasitoid inserts his aedagus into the genital orifice of a female and when the aedagus was removed) were determined for the 4 pair-wise treatments. The total numbers and sex ratios of the progeny produced by female wasps in each paired treatment were also determined. Data obtained on courtship duration, copulation length and progeny production were first normalized by using square-root ($x = (\sqrt{(x + 0.5)})$ transformation and then subjected to one-way multivariate ANOVA followed by Tukey's HSD ($P < 0.05$; JMPIN Version 5.1, SAS Institute Inc., 2003)**.** In order to determine the effects of previous mating experience on courtship

duration, copulation lengths and progeny production, data were further subjected to Students' *t* - test ($P < 0.05$) to compare data between experienced and inexperienced wasps of each given sex**.** Progeny sex ratio data was analyzed also with Students' *t* - test ($P < 0.05$; JMPIN Version 5.1, SAS Institute Inc., 2003) to determine possible effect of previous mating experience on sex ratio.

RESULTS

Courtship behavior of P. cerealellae

Descriptions of the various behavioral sequences involved in mating behavior of *P. cerealellae* are shown in Tables 1 (male) and 2 (female). An ethogram of the mating behavior of *P. cerealellae* is shown in Fig. 1, while the sequences associated with behavioral transitions of mating behavior of *P. cerealellae* are illustrated in Fig. 2. When a parasitoid pair was released into the mating arena, both male and female were initially immobile (Fig. 2a, b) for a brief period (~10 s). The female was the first sex to initiate courtship by exhibiting calling behavior during which she waved her antennae, swayed her abdomen and fluttered her wings (Fig. 2b). This calling behavior probably results in release of a chemical stimulus (pheromone) for male attraction (unpublished data). During this period, the male began to exhibit a series of "alert-related" behaviors including antennation, swaying of abdomen, and fanning of wings, probably in response to the female-released pheromone. The female may continue to exhibit calling behavior at the same spot in the mating arena until she was approached by a male. Alternatively, if she was not approached by a male, the calling female may walk directionless around the mating arena until she was closer to the male (indicated by dotted lines in Fig. 1). Once

the male was located, the female exhibited “approach” behavior drumming her antennae on the male’s body (head or thorax) and then retreating and walking away from the male (Fig. 1). While the female was exhibiting “approach” behavior, the male usually remained stationary waving his antennae into the air, probably in response to the female-released pheromone. The male then began to exhibit “alert”, “trail” and “follow” behaviors (Figs. 1; 2c) towards the female**.** Upon approaching the female, the male became agitated (excited) (Fig. 1) and briskly drummed his antennae on the body of the female (Figs. 1; 2e). The male then mounted the dorsum of the female (Fig. 1). After mounting, the male exhibited a series of behavioral transitions including “advance”, “retract” (moving backward to hold female abdomen), and “probe” (extending aedagus to touch the female’s genital orifice) behaviors (Fig. 2f). This series of male behaviors usually triggered “receptive” behavior in the female, ultimately resulting in copulation (Figs. 1; 2g). During copulation, male assumed the posterior-dorsal positions. Typically, male and female are oriented (faced) in the same direction in copula. After a period of copulation (~12 s, range: 5-17 s), the male dismounted (withdrew his aedagus; Fig. 2h) and was separated from the female (Fig. 2i). After copulation, the female typically remained stationary (at the same spot where she had copulated) and exhibited “avoidance” behavior (i.e. refusing male’s pre-copulatory behaviors). Females may later become receptive to males, but only after a latency period of about 1165 s (range: 710-1912 s).

The mean durations (s ± SE) of the different types of behavioral transitions exhibited by males during courtship were 1899 ± 298.0 (alert, including antennation, swaying and fanning), 226.1 ± 77.9 (trail), 489.7 ± 160.3 (follow), 38.6 ± 19.8 (agitate),

14.4 ± 5.3 (mount), 51.9 ± 33.5 (antennal touch), 11.7 ± 0.7 (copulate), and 13.4 ± 0.8 (dismount) (Fig. 3). One-way multivariate ANOVA showed significant difference in mean durations of the various behaviors ($F = 27.7$, $df = 7$, $P < 0.0001$): the duration of alert behavior (including antennation, swaying and fanning) was significantly longer than the duration of each of the other behavioral transitions. Average cumulative courtship duration (time between when a male and a female pair was introduced into the mating arena to the time the male dismounted the female at the end of copulation) in freshly-emerged inexperienced *P. cerealellae* was ~2744 s (range: 547-7539 s) (Fig. 3).

Effect of previous mating experience on courtship duration and reproductive performance

Significant differences were recorded in cumulative courtship durations among the 4 mating pair combinations of inexperienced and experienced male and female *P. cerealellae* ($F = 23.8$, $df = 3$, $P < 0.0001$). Cumulative courtship duration was significantly greater for the mating pair combination of experienced male (EM) and experienced female (EF) (EM × EF) than for the combinations of experienced male (EM) and inexperienced female (IF) (EM × IF) or inexperienced male (IM) and inexperienced female (IF) (IM × IF) (Fig. 4). Courtship duration for the IM × EF mating combination was also significantly greater than for the EM × IF combination, but not significantly different from the EM × EF and IM × IF combinations (Fig. 4). Further data analysis revealed a significant effect of previous mating experience on courtship duration of males ($t = 4.97$, $df = 1$, $P < 0.0001$; Students' t - test). Experienced (previously mated) males concluded mating in 472.9 ± 16.9 (s ± SE) compared to 2743.9 ± 456.6 for inexperienced

males. Taken together, these results suggest that female experience and male experience may have disparate effects on courtship duration. Female experience significantly increased courtship duration, whereas male experience served to reduce courtship duration. Pairing of experienced male with inexperienced female thus resulted in the least courtship duration of ~473 s (Fig. 4).

Previous mating experience also had a significant effect on progeny production ($F = 5.26$, $df = 3$, $P = 0.0025$). Significantly greater number of progeny was produced by the IM × EF mating pair compared to the other 3 mating pair combinations (Fig. 5). The progeny from the IM × EF combination was significantly male-biased ($t = -2.14$, $df = 1$, $P = 0.0025$; Student t - test analysis), whereas progeny from the other pair-wise combinations did not deviate significantly from an expected 1: 1 (male: female) ratio (Fig. 5). Copulation length varied considerably among individuals (5-17 s) but was not significantly different among mating pair combinations ($F = 0.12$, $df = 3$, $P = 0.948$; Fig. 6).

DISCUSSION

Courtship behavior of P. cerealellae

The mating behavior of *P. cerealellae* is generally similar to that described for other pteromalids (van den Assem, 1970; 1996; van den Assem and Werren, 1994; Ruther et al., 2000) and several non-pteromalid parasitoid species (Isidoro and Bin 1995; van den Assem, 1996; Isidoro et al., 1996; Bin et al., 1999; Ruther et al., 2000; Cheng et al., 2004). Our observations suggest that courtship in *P. cerealellae* is initiated by the female, possibly through the release of a pheromone that triggers alert-related response in

the male. Nevertheless, the male appears to play the most active role in the courtship behavior of this species, exhibiting most of the observable behaviors.

Courtship in *P. cerealellae* consists of a series of behavioral transitions, which can be broadly categorized into pre-mounting, mounting and post-mounting behaviors. During pre-mounting, an immobile female begins to exhibit "calling" behavior during which she antennates, sways her abdomen and flutters her wings, probably to release and disperse a sex pheromone. An immobile but alerted male, several centimeters away from the female, begins to antennate, sways his abdomen and fans his wings. The specific function of male wing fanning in the courtship behavior of parasitoids is unknown, but may be a form of male courtship signal towards the females or may help to spread male pheromones (Ruther et al., 2000). Exhibition of "alert" behavior by male *P. cerealellae* may suggest the involvement of a female-released sexual attractant in the mating behavior of this species, as observed in another study (unpublished data). Involvement of female-produced sex pheromones in courtship behavior of parasitic wasps is a common phenomenon. For example, females of *Lariophagus distinguendus* Först (Hymenoptera: Pteromalidae) (van den Assem, 1970), *Anisoptera calandrae* (Howard) (Hymenoptera: Pteromalidae) (Yoshida, 1978), *Brachymeria intermedia* (Nees) (Hymenoptera: Chalcididae) (Leonard and Ringo, 1978; Simser and Coppel, 1980), *B. lasus* (Walker), *Cotesia liparidis* (Bouche) (Hymenoptera: Braconidae) (Weseloh, 1976; Vinson, 1978), and *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) (Weseloh, 1976; Vinson, 1978) have been reported to produce volatile attractants (sex pheromones) that aid in mate finding by conspecific males.

Upon becoming "alerted" the male typically starts to exhibit "trail" behavior in search of the calling female (attractant source). A trailing male typically antennates while running around in the mating arena and then starts to exhibit "follow" behavior (directional movement) towards the female, once the volatile source has been detected. The calling female is not entirely passive at this stage, but, rather, reciprocates by walking towards the male, a behavior that brings both sexes close together. Upon encountering the female, the male becomes agitated and briskly drums the body of the female with his antennae, probably to determine the sexual maturity or mating status of the female (Cheng et al., 2004). Exhibition of agitation behaviors by males when conspecific females are encountered may suggest involvement of a female-released close range contact chemicals for recognition of species, as reported for several other parasitoid species (Howard, 1992; Singer, 1998). The exhibition of "antennal drumming" behaviors by females when in close proximity to males may suggest involvement of male-produced close range contact chemicals, which probably functions for species recognition, as have been reported for other pteromalid wasps (e.g. Steiner et al., 2006). Further studies are needed to confirm the involvement of sex pheromones and contact semiochemicals in the courtship behavior of *P. cerealellae*.

Once the male has mounted on a female, he makes antennal contact with the females' antennae probably to stimulate and increase her sexual receptivity. Antennal touch behavior has been observed in other parasitoids (van den Assem et al., 1980; van den Assem, 1996; Isidoro et al., 1996; Bin et al., 1999; Ruther et al., 2000; Cheng et al., 2004). The adaptive function of antennal touch behavior in *P. cerealellae* is unknown, but previous studies on similar parasitoids indicated that male may transfer sex

pheromones to the female's antennae during antennal contact to induce female's sexual receptivity (van den Assem et al., 1980; Isidoro and Bin, 1995; Isidoro et al.,1996).

In *copula*, most *P. cerealellae* males assume posterior dorso-lateral position, as reported also for *C. tarsalis* (Cheng et al., 2004). Males are relatively passive during copulation: they typically keep their antennae and wings in a fixed position and do not exhibit the rhythmical contractions of the body that have been reported for many parasitoid species (Eberhard, 1994; Cheng et al., 2004). The duration (length) of copulation is short in *P. cerealellae* (~12 s). Short copulation durations typically lasting 10 to 20 s have also been recorded for other parasitoids species (van den Assem, 1986; Cheng et al., 2003; 2004). Chen et al. (2004) proposed that short copulation duration may be selectively advantageous to parasitoids by minimizing the risk of exposure to natural enemies or male competition, which is likely to increase with increasing copulation length. Similar to our results on *P. cerealellae,* Cheng et al. (2003) also reported no significant effect of copulation duration on total offspring production and progeny sex ratio. Once copulation has occurred, males dismount and walk away from the female and do not exhibit post-copulatory behaviors, in contrast to males of other parasitoid species including *N. vitripennis* (van den Assem and Feuth de Bruijn, 1977) and *L. distinguendus* (Ruther et al., 2000).

As has been reported for several female parasitoids (van den Assem, 1986; Ruther et al., 2000), *P. cerealellae* females were relatively passive during copulation becoming active only after copulation. Female *P. cerealellae* typically remains at the spot where she had copulated for ~20 s before she starts to walk around in the mating arena. Shortly after copulation had occurred, females began to exhibit avoidance behavior by running

away from males and remain sexually unreceptive to males (for ~1200 s), as reported for some other parasitic wasps (e.g. van den Assem et al., 1984; Ruther et al., 2000; Cheng et al., 2004).

Effect of previous mating experience on courtship duration and reproductive performance

The results of the second experiment suggest possible occurrence of multiple mating in *P. cerealellae*. Multiple mating is a widespread phenomenon among various insect species. Among parasitic hymenopterans, solitary species tend to be monandrous (i.e. mate only once), whereas gregarious species are typically polyandrous (Riley, 1993). It is unclear whether or not *P. cerealellae* mates multiply in nature. However, Ridley (1993)'s categorization should predict *P. cerealellae*, which is a solitary species, to be monandrous in nature.

The recorded significant effect of previous mating experience on courtship duration indicates that male and female *P. cerealellae* could differentiate between virgin and previously mated individuals. The results that showed courtship duration to increase with female mating experience but decrease with male mating experience is intriguing, and may suggest that mating experience sensitizes males to females, whereas previously mated females are less receptive to males. Evolutionarily, one of the ways male parasitoids increase their fitness (Thornhill and Alcock, 1983; Roitberg et al., 2001) is through the number of mating achieved throughout their lifetime (Cohen, 1973; Parker, 1984). Our results showing that male mating experience reduced courtship duration may be an attempt by male *P. cerealellae* to mate as quickly as possible to maximize fitness.

Copulation has been shown to decrease receptivity of female parasitoids (van den Assem, 1986; Godfray, 1994; West et al., 1997; Damiens and Boivin, 2005). For instance, mated females of *N. vitripennis* were shown to be less receptive to males after mating (van den Assem, 1986). In some insect species, males manipulate female reproduction by preventing her from remating (Roth, 1964). For example, in *Nauphoeta cinerea* (Blattidea: Blaberidae), males insert spermatophores in the bursa copulatrix of females to inhibit sexual receptivity center in female brain (Roth, 1964). It is not known whether the increased courtship duration recorded for experienced female *P. cerealellae* in the current study was due to induced reduced receptivity imposed by males or biochemical effects of sperms deposited in females, as has been reported for other insect species (Roth, 1964). Further studies are necessary to investigate why female experience increased courtship duration in this species.

The data showed a trend for increased progeny production by experienced females compared to inexperienced females, although this result was not always significant. The highest number of offspring was produced by the unmated male ×mated female pair and this progeny was significantly male-biased. These results may indicate that experienced females attack more hosts than their inexperienced counterparts, as reported for some other parasitoid species (Avidov et al., 1967; Antolin, 1989; Michaud, 1994).

Mated females of several species of parasitoids manipulate the sex ratio of their offspring in response to local conditions to produce offspring with variable sex ratios, which can range from slightly female-biased (Donaldson and Walter, 1984; Godfray, 1994; Tillman, 1994), unbiased (Boling and Pitre, 1970), to slightly male-biased

(Kunnalaca and Mueller, 1979). In most parasitoids' species, mated females lay a high proportion of fertilized eggs immediately after mating (King, 1987; Brodeur and McNeil, 1994; Tillman, 1994). The reason for male-biased progeny recorded for experienced females is unclear, but it may relate to the observed relatively greater total progeny production by experienced females compared to inexperienced females. The data showing relatively greater progeny for females paired with inexperienced males compared to those paired with experienced males may indicate some cost of multiple mating in males of this species.

Copulation duration was not affected by previous mating experience and had no significant effect on reproductive performance in *P. cerealellae.* In contrast, Cheng et al. (2003) reported significant effect of previous mating experience on copulation duration in *Cephalonomia tarsalis* (Ashmead) (Hymenoptera: Bethylidae). However, copulation duration had no effect on progeny production (Cheng et al., 2003).

In summary, the courtship behavior of *P. cerealellae* is, generally similar to those of other parasitoid species. Our results implicate possible involvement of a female-produced sex pheromone, as well as female and male contact semiochemicals in the mating behavior of *P. cerealellae*. Further studies are necessary to isolate and identify these semiochemicals, and to determine their roles in the behavioral ecology of this parasitoid species.

ACKNOWLEDGEMENTS

This research was supported in part by the Alabama Agricultural Experiment Station and an Auburn University Research grant to H.Y.F. I would like to thank Drs. Art Appel, Kathy Flanders (Auburn University) and Micky Eubanks (Texas A & M University) for helping to review this manuscript.

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Table 1 Description of the behavioral transitions involved in the courtship behavior of male *P. cerealellae*

Behavior	Description
Immobile	Remained still (no movements of any body parts) in the Petri dish
Antennate	Waving (vibration) of antennae into the air
Alert	Momentary stoppage of antennation probably due to perception of female-released stimulus (pheromone)
Swaying	Swaying of abdomen possibly in response to perception of female-released stimulus
Fanning	Fanning of wings possibly in response to perception of female-released stimulus
Trail	Walking and antennating around in the mating arena in search for the stimulus source (female)
Follow	Running immediately behind the female
Agitate	Sudden stop near a female with antennae still and wings raised in a fixed position
Antennal drum	Antennae tapping female's body (head or thorax)
Mount	Getting on the female in a dorsal riding position beginning with the front legs climbing on from anterior, posterior, or sides of female
Advance	Move anteriorly to make antennal contact with female
Antennal touch	Antennae contacting with female's antennae
Retract	Move backward to hold the abdomen
Hold	Grasping the female's abdominal dorsum with legs on the thorax
Probe	Extending aedagus to touch female's genital orifice (underneath the abdomen)
Insert	Putting aedagus into female's genital orifice while holding the female's abdomen
Copulate	Copulating with female by assuming posterior dorsal or side position with the tip of abdomen stooped underneath the female's abdomen; antennae lowered down or raised and wings raised in stationary position; fore and mid legs on dorsum of female's abdomen with mouth touching the dorsum of the female
Dismount	Getting off the female
Stationary but antennate	Standing still but waving antennae into the air
Run	Moving rapidly around the mating arena
Seek	Searching the arena for a female

Table 2 Description of the behavioral transitions involved in the courtship behavior of female *P. cerealellae*

Behavior	Description
Immobile	Remained still (no movements of any body parts) in the Petri dish
Antennate	Waving (vibration) of antennae into the air
Antennate and swaying	Simultaneously waving the antennae into the air and swinging the abdomen back and forth
Fanning	Continuously flapping the wings (probably to disperse volatile stimulus)
Approach*	Walk up to a male after having called (antennated, swayed and fanned) for a long time without getting a response
Antennal drum*	Antennae tapping the male's body (head or thorax)
Retreat*	Move back and away from male
Receptive	Accepting the mounted male by remaining stationary and lowering the antennae for antennal touch
Copulate	Copulating with male while remaining stationary with antennae lowered down
Separated	Uncoupling herself from the male after copulation
Stationary and antennate	Standing still in the mating arena and waving antennae into the air after copulating
Run	Moving rapidly around the mating arena
Avoidance	Running and keeping away from male (possibly to avoid re-mating), refusing antennal touch and preventing probing

* - indicates uncommon (alternative) behaviors exhibited by females during courtship.

Figure 1 Flow sequence of courtship behavior in *P. cerealellae* (based on 18 successful courtships). Figure shows the three (pre-mounting, mounting and post-mounting) phases (indicated in rectangles with dash lines) and the various behavioral transitions involved in mating behavior of *P. cerealellae*. Arrows with solid lines indicate the regular courtship behavioral transitions; arrows with dash lines indicate uncommon (alternative) behavioral transitions exhibited by female wasps; double-headed arrows indicate alternating behavioral transitions.

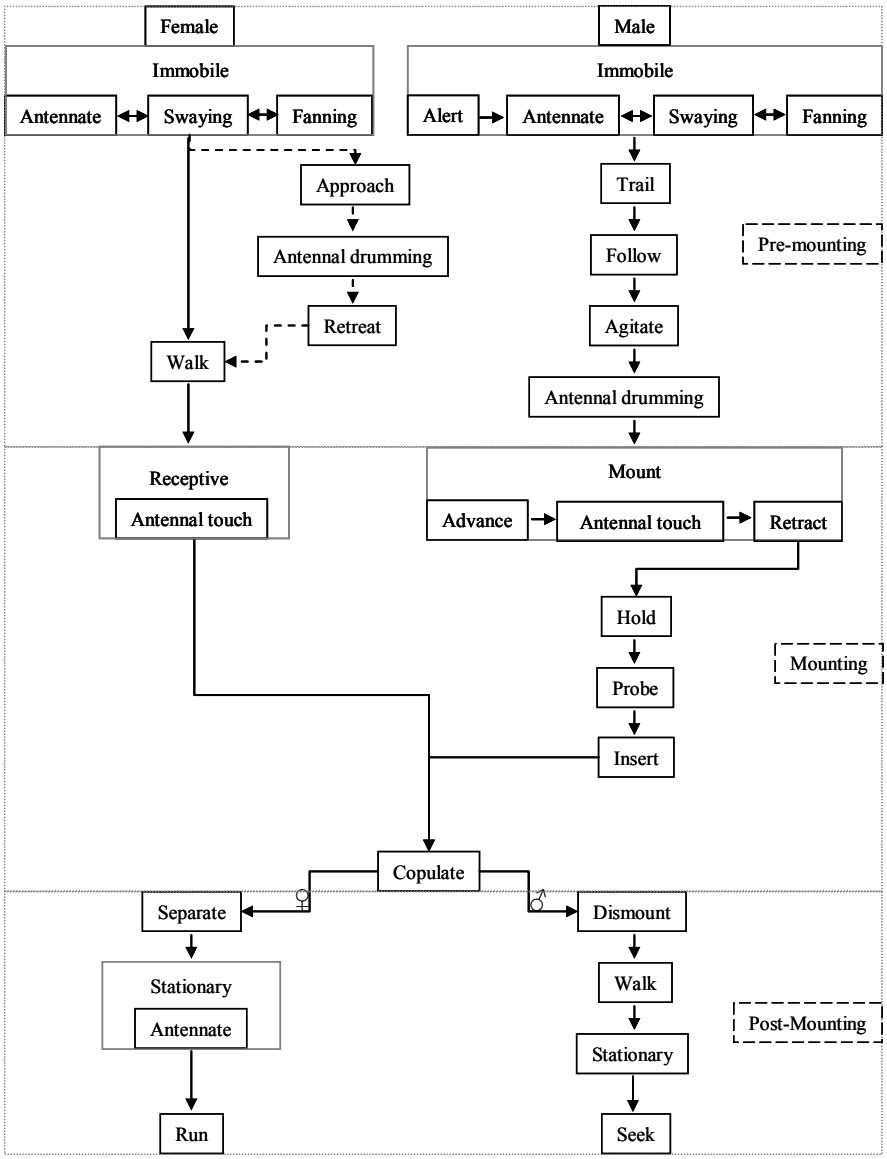


Figure 2 Key sequences in the mating behavior of *P. cerealellae*. Figure shows photo-micrographs taken with a JVC GR-D244U digital video camera (Victor Company of Japan, Malaysia) under a stereomicroscope (National Microscope, Model DC 3-420, Meiji, Japan) of male or female *P. cerealellae* at different instances of courtship behavior. male at rest (a); a calling female exhibiting antennation (b); a male exhibiting follow behavior (c); agitated (excited) male - not clearly seen in the picture (d); a mounting male jumping on the dorsum of a female (e); a male probing the genital orifice of a female (f); a male copulating with a female (g); a male withdrawing his adaegus from female at the end of copulation (h); male separated from female after copulation (i).

Figure 3 Typical courtship sequence and related duration of mating in inexperienced male *P. cerealellae*. Figure shows mean (± SE) and cumulative (± SE) durations (s) of the various behaviors transitions exhibited by male *P. cerealellae* during courtship. Behavioral transitions followed by similar letters are not significantly different in mean duration ($P < 0.05$, Tukey's HSD test).

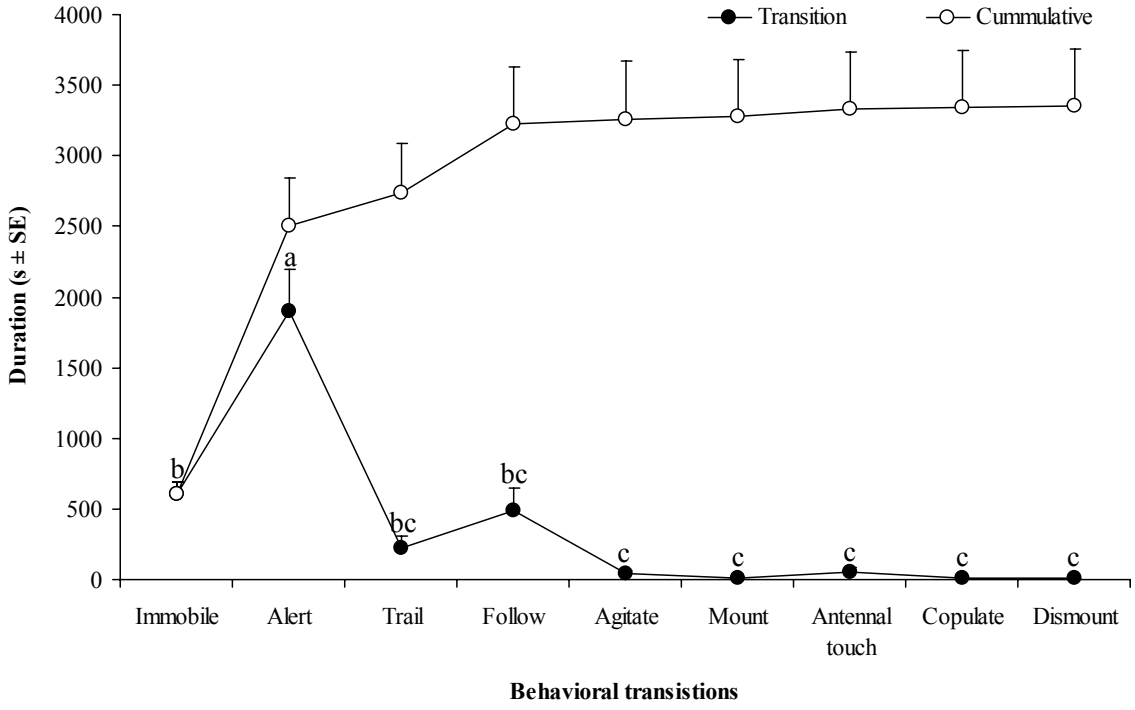


Figure 4 Influence of previous mating experience on courtship duration of *P. cerealellae*. Figure shows mean (± SE) courtship duration (s) of different mating pair combinations of inexperienced and experienced male and female *P. cerealellae*, inexperienced male (IM) × inexperienced female (IF); inexperienced male (IM) × experienced female (EF); experienced male (EM) × inexperienced female (IF), and experienced male (EM) × experienced female (EF) treatments. Means followed by similar letters are not significantly different ($P < 0.05$, Tukey's HSD test).

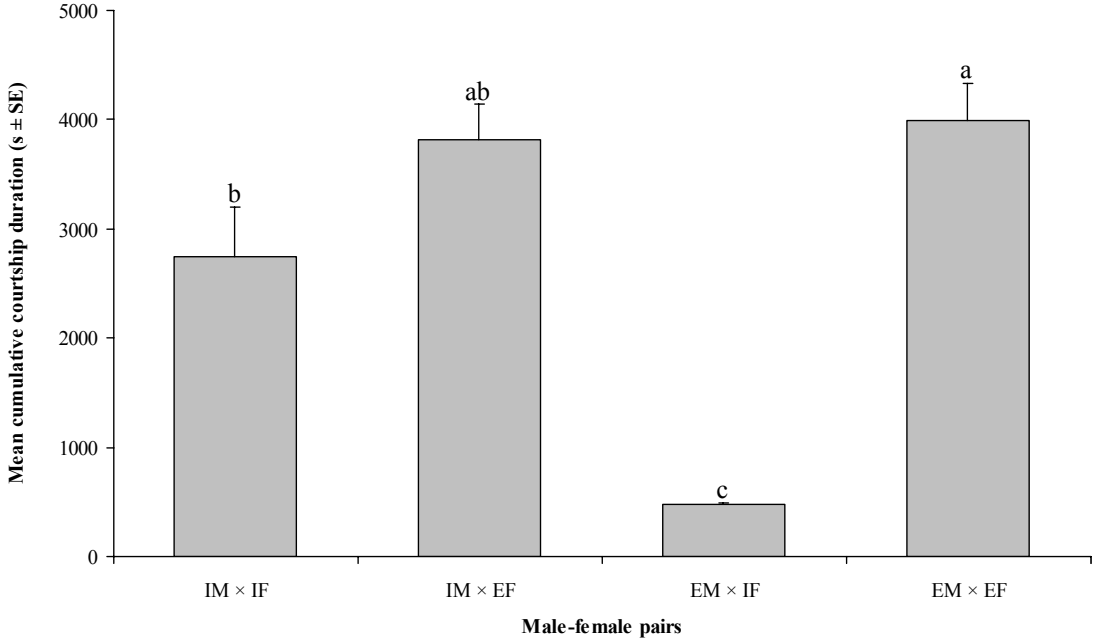


Figure 5 Influence of previous mating experience on progeny production by female *P. cerealellae*. Figure shows mean (± SE) total number of F_1 progeny and proportion of male and female progeny (% male, female proportion) produced by different mating pair combinations of inexperienced and experienced male and female *P. cerealellae*, inexperienced male (IM) × inexperienced female (IF); inexperienced male (IM) × experienced female (EF); experienced male (EM) × inexperienced female (IF), and experienced male (EM) × experienced female (EF) treatments. Means followed by similar letters are not significantly different ($P < 0.05$, Tukey's HSD test). Treatment bars with an asterik (*) indicate significant differences in the proportion of male and female progeny ($P < 0.05$, Students' *t* - test).

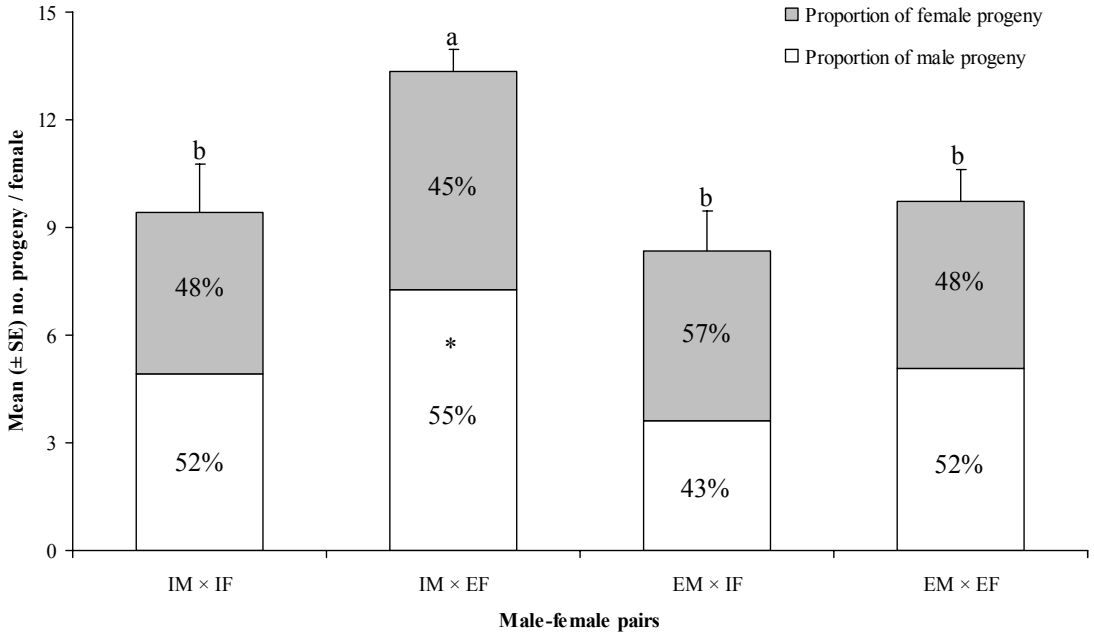
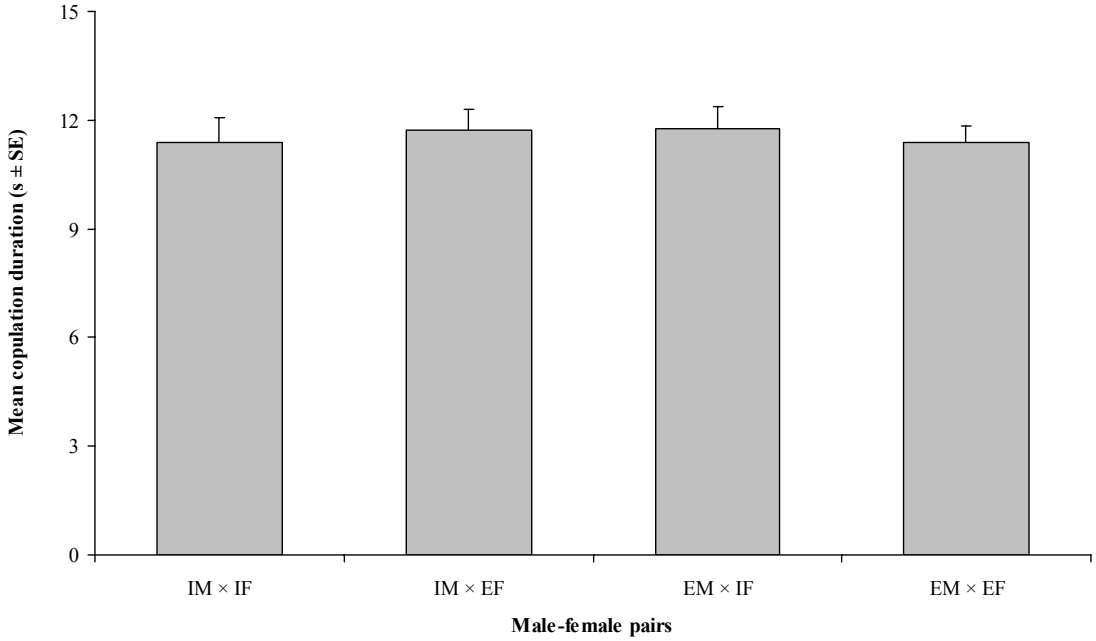


Figure 6 Influence of previous mating experience on copulation duration in *P. cerealellae.* Figure shows mean (min ± SE) copulation durations of different mating pair combinations of inexperienced and experienced male and female *P. cerealellae*, inexperienced male (IM) × inexperienced female (IF); inexperienced male (IM) × experienced female (EF); experienced male (EM) × inexperienced female (IF), and experienced male (EM) × experienced female (EF) treatments.



CHAPTER 7: ELECTROANTENNOGRAM AND BEHAVIORAL RESPONSES OF *PTEROMALUS CEREALELLAE* TO CONSPECIFIC ODOR: EVIDENCE FOR MALE- AND FEMALE-PRODUCED PHEROMONES?

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 (Mathews 1975; Weseloh, 1976; Vinson, 1978; Simser and Coppel, 1980; Leal et al., 1997; Pompanon et al., 1997; Steiner et al., 2006). Most 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 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: Bethylidae) (Howard, 1992) have 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; Singer, 1998; Howard, 2001; Steiner et al., 2006). Cuticular hydrocarbon components of *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) (Syvertsen et al*.,* 1995), *N. vitripennis* (Steiner et al*.,* 2006), *Roptrocerus xylophagorum* (Ratzeburg) (Hymenoptera: Pteromalidae) (Sullivan, 2002) and *Lariophagus distinguendus* (Först) (Hymenoptera: Pteromalidae) (Steiner et al., 2005) were reported to function in attracting parasitoids to mates.

In a recent study of mating behavior of *P. cerealellae*, we obtained results implicating possible involvement of male- and female-produced semiochemicals in mediating courtship in this species (Onagbola and Fadamiro, Chapter 6). First, 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 females sexual receptivity. Based on these result, 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 adult males and adult females to airborne volatiles from live male and female conspecifics and to whole body extracts of both sexes. The chemical profiles of whole body extracts of male and female parasitoids were then characterized by gas chromatography to detect possible quantitative or qualitative differences in their chemical compositions.

MATERIALS AND METHODS

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, 70 ± 5 % r.h., and L12: D12 (Mbata et al., 2005; Onagbola et al., 2007). Beetles were allowed to lay eggs on the seeds for 24-hours after which they were removed with an aspirator. Infested seeds were incubated at the conditions specified above until larvae had reached the fourth instar, which were then provided to *P. cerealellae* for parasitization. The parasitoid was

maintained by transferring ~30 adult pairs onto 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 d after infestation of cowpea seeds under our rearing conditions). Jars were held at the environmental conditions stated above for *C. maculatus*. Adult *P. cerealellae* were removed from the jars after 5 d of oviposition. Parasitized host larvae were incubated in a growth chamber at the above environmental conditions until the adult parasitoids started to emerge (~11 d after parasitization at the above conditions). Pupae of the parasitoids (still within parasitized hosts) were immediately transferred into 100 x 15 mm disposable Petri dishes to collect adult parasitoids immediately after emergence.

Solvent extraction whole body of adult male and female 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 10 times for a total of 3000 adults per sex. Extracts were stockpiled in organic glass vials and concentrated under Nitrogen (N_2) to obtain a concentration of ~1 insect equivalent per 0.2 µl (5 insects per µl of extract). Each extract was made in 3 replicates, dehydrated in ~1 g of anhydrous Sodium Sulfate for ~12 h and stored in a freezer at -20°C until used. Hexane extracts were kept in the freezer until they were used for experiments.

Electroantennogram responses of P. cerealellae to volatile extracts of conspecifics

Electroantennogram (EAG) techniques used in this study were similar to those previously described by Chen and Fadamiro (2007 a, 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. Ten-µL 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-µL aliquot of hexane. The tip of the pipette was placed ~3 mm into a small hole in the wall of a metal tube (13 cm long, 8 mm diameter), which was oriented towards 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 was allowed between successive stimulations for antennal recovery.

EAG response of antennae of virgin (6 to 12 h - old) *P. cerealellae* of both sexes was tested to the following treatments: (i) whole body extracts (WB) of conspecific males, (ii) WB of conspecific females, and (iii) 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 was 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 detect sexual differences in EAG responses to each treatment ($P < 0.05$).

Behavioral (Y-tube olfactometer) bioassays

A Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) was used to test the attraction of virgin (6- to 12 h - old) adult male and female *P. cerealellae* to airborne volatiles from live male and female conspecifics and to WB 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 inlayed 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 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 300 male or female parasitoids 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 (180º). 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 24 males or females were tested per choice test. All bioassays were conducted at 25 ± 1˚C and 60 ± 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 WB of conspecific males and females. WB of male or female parasitoids was delivered as a 10-µL 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-µL 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 24 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.

Gas chromatographic (GC) analyses

Whole body solvent (hexane) extracts of male and female *P. cerealellae* were analyzed in a gas chromatograph (GC, Shimadzu GC 17A, Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector (FID) set at 320 ºC. The GC column used was a non-polar Restek® Rtx-IMS capillary column (30 m × 0.25 mm ID; 0.25-µm film thickness). The temperature conditions were 2 min at 80 ºC, increased at a rate of 5 ºC/min to 280 ºC, and held at this temperature for 18 min. The injector temperature was set at 275 ºC and operated in splitless mode throughout the analyses.

The carrier gas was Helium (purity 99.96%) with a flow rate of 1.5 mL/min and at a constant pressure of 100 KPa. Approximately 2 µL of each extract was injected into the GC for analysis. The retention time (RT) of the recognizable peaks (compounds) was determined and compared between the treatments.

RESULTS

Electroantennogram responses of male and female P. cerealellae to whole body extracts of their conspecifics

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 treatments ($F = 37.44$, $df = 2$, $P < 0.0001$), sex ($F = 46.45$, $df = 1$, $P < 0.0001$), and a 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 ($F = 4.71$, $df = 2$, $P = 0.016$), and females ($F = 47.20$, $df = 2$, $P < 0.0001$). WB 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 WB of males (df = 1, $t = 4.32$, $P = 0.0002$), WB of females (df = 1, $t = 4.93$, $P < 0.0001$), and hexane control (df = 1, $t = 3.56$, $P = 0.001$), with females showing greater EAG response than males in all cases (Fig. 1).

Behavioral (olfactometer) bioassays

Responses of adult male and female 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 ($\chi^2 = 7.26$, $df = 1$, $P = 0.007$; 74%) and live females ($\chi^2 = 16.13$, $df = 1$, $P < 0.0001$; 87%). Similarly, female parasitoids also showed significant attraction to airborne volatiles from live males ($\chi^2 = 4.80$, $df = 1$, $P = 0.029$; 70%) and live females ($\chi^2 = 13.44$, $df = 1$, $P = 0.0002$; 81%).

Responses of adult male and female P. cerealellae to whole body extracts of conspecifics

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

GC quantification

GC analyses revealed major qualitative and quantitative differences in the chemical profiles of WB of male and female *P. cerealellae*. The total numbers of recognizable peaks in extracts of male and female wasps were 50 and 65, respectively (Fig. 4; Table 1). The different recognizable peaks could be categorized into male-specific (x, $n = 29$), female-specific (x, $n = 44$) or shared ($\boldsymbol{x}$, $n = 21$) peaks. Observed

qualitative and quantitative differences as revealed from the GC analyses may explain our results, which showed attraction of both sexes of *P. cerealellae* to live and WB of conspecific females. For example, peak numbers 1-6 are female-specific peaks. These peaks (peak 1, RT = 553.1 s; 2, RT = 607.8 s; 3, RT = 653.4 s; 4, RT = 689.9 s; 5, RT = 781.0 s; and 6, RT = 987.7 s) have very low retention times, suggesting them to be highly volatile (Fig. 4, Table 1). Similarly, some of the 29 male-specific peaks could account for the attractiveness of WB of males.

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 stimuli, as recorded for *P. cerealellae* in the present study, may 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 (intersexual attraction), suggesting presence of volatile attractants in the effluvia of both sexes, which may function in species recognition similar to other parasitoids (Howard, 1993; Singer, 1998; Beukeboom and van den Assem, 2001, Cheng et al., 2004). Attraction of male parasitoids to conspecific females has been reported by many authors. 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. Our GC analyses revealed the presence of 21 shared peaks in the chemical profiles of whole body extracts of both sexes, some of which may provide the basis for the recorded intrasexual and intersexual attractions.

In contrast to the generally attractive airborne volatiles from live males and females, whole body extracts of male and female *P. cerealellae* were generally not as 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 may somewhat be repellent to the parasitoids, as have been reported in studies involving other

insect species (Teal et al., 1986; Parrilla and Guerrero, 1994; Howse et al., 1997; Bau et al., 1999). For example, Teal et al. (1986) reported differences in the chemical profiles of pheromone gland extracts of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) versus airborne volatiles collected from calling females. An important pheromone component ((Z)-9-tetradecenal) which was present in the airborne volatiles from calling females of *H. virescens* was absent in the extracts of pheromone glands and hair pencils (Teal et al., 1989). In some cases, some of the compounds contained in the whole body extracts of insects may be analogues of pheromones and may function as inhibitors of pheromone perception (Parrilla and Guerrero, 1994; Bau et al., 1999).

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 hydrocarbon 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, may account for 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 hydrocarbon that 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, Chapter 6), probably to stimulate and induce sexual receptivity in females, as have 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). Some of the 29 male-specific compounds obtained in our GC analyses of male extracts may constitute the unidentified male-produced pheromones of *P. cerealellae*.

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 (Mathews 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 that 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, in review). It was also observed in the same study (Onagbola and Fadamiro, Chapter 6), 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, as has been reported for other parasitoid species (Howard, 1992; Singer, 1998; Ruther et al., 2000). The 6 female-specific peaks identified in the GC analyses of the extracts have very low retention times (suggesting them to be highly volatile) and, thus, may constitute the unidentified components of the sex pheromone and close range contact chemicals of female *P. cerealellae*.

In summary, the results of this study provides 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 these chemicals and determine their roles in the behavioral ecology of *P. cerealellae*.

ACKNOWLEDGEMENTS

I would like to thank Drs. Art Appel and Kathy Flanders (Auburn University) and Micky Eubanks (Texas A & M University) for reviewing this manuscript. Dr. Li Chen (Auburn University) is thanked for technical assistance. This research was supported in part by the Alabama Agricultural Experiment Station and an Auburn University Research grant to HYF.

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Table 1 Retention time (s) of compounds analyzed from the whole body extracts of male and female *P. cerealellae*

Peak No.	Retention time (s)	Peak specificity	
		Male	Female
1	553.1		*x*
2	607.8		*x*
3	653.4		*x*
4	689.9		*x*
5	781.0		*x*
6	987.7		*x*
7	1033.3	x	
8	1043.9		*x*
9	1139.7		*x*
10	1586.4	x	
11	1311.4	x	
12	1610.7	x	
13	1625.9		*x*
14	1346.3		*x*
15	1519.5		*x*
16	1543.9		*x*
17	1587.9		*x*
18	1613.8		*x*
19	1619.8		*x*
20	1641.1		*x*
21	1671.5	x	
22	1726.2	x	
23	1741.4		*x*
24	1817.4	x	
25	1823.4		*x*
26	1907.5	***x***	***x***
27	2005.0	x	
28	2028.1	x	
29	2050.3		*x*
30	2080.4	***x***	***x***
31	2116.6		*x*
32	2164.8	***x***	***x***
33	2178.9	***x***	***x***
34	2214.1	***x***	***x***
35	2231.2		*x*
36	2267.3	x	
37	2287.4		*x*
38	2324.6	***x***	***x***
39	2331.7	x	

40	2357.8		*x*
41	2386.9		*x*
42	2396.0	x	
43	2417.1		*x*
44	2456.3	x	
45	2474.4		*x*
46	2490.5	x	
47	2502.5	***x***	***x***
48	2522.6		*x*
49	2524.6	x	
50	2544.7		*x*
51	2576.9	***x***	***x***
52	2602.0		*x*
53	2623.1		*x*
54	2643.2		*x*
55	2646.2	x	
56	2670.4		*x*
57	2679.4	x	
58	2705.5		*x*
59	2715.6	***x***	***x***
60	2789.9	***x***	***x***
61	2796.0	***x***	***x***
62	2831.2		*x*
63	2864.3		*x*
64	2889.4		*x*
65	2891.5	x	
66	2911.6		*x*
67	2904.5	***x***	***x***
68	2936.7	***x***	***x***
69	2976.9	***x***	***x***
70	3020.1		*x*
71	3053.3		*x*
72	3070.4	***x***	***x***
73	3101.5	***x***	***x***
74	3113.6	x	
75	3143.7		*x*
76	3155.8	***x***	***x***
77	3200.0	***x***	***x***
78	3220.1	x	
79	3240.2	x	
80	3259.3	x	
81	3316.6	x	
82	3366.8		*x*
83	3377.9	x	
84	3417.1	***x***	***x***
85	3429.1	x	

86	3477.4		*x*
87	3521.6	***x***	***x***
88	3535.7	***x***	***x***
89	3577.9	x	
90	3622.1	x	
91	3859.3	x	
92	3893.5		*x*
93	3959.8	x	
94	4002.0		*x*

Table shows adult male-specific peaks (x), adult female-specific peaks (*x*) and peaks which are common to both males and females (***x***).

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Figure 1 Electroantennogram (EAG) responses of *P. cerealellae* to whole body (WB) extracts of conspecifics. Figure shows absolute EAG (-mV ± SE) responses of males (grey bars) and females (white bars) to WB extracts of male and female conspecifics and hexane (control). Treatment bars followed by similar letters are not significantly different (Tukey's HSD test; $P < 0.05$).

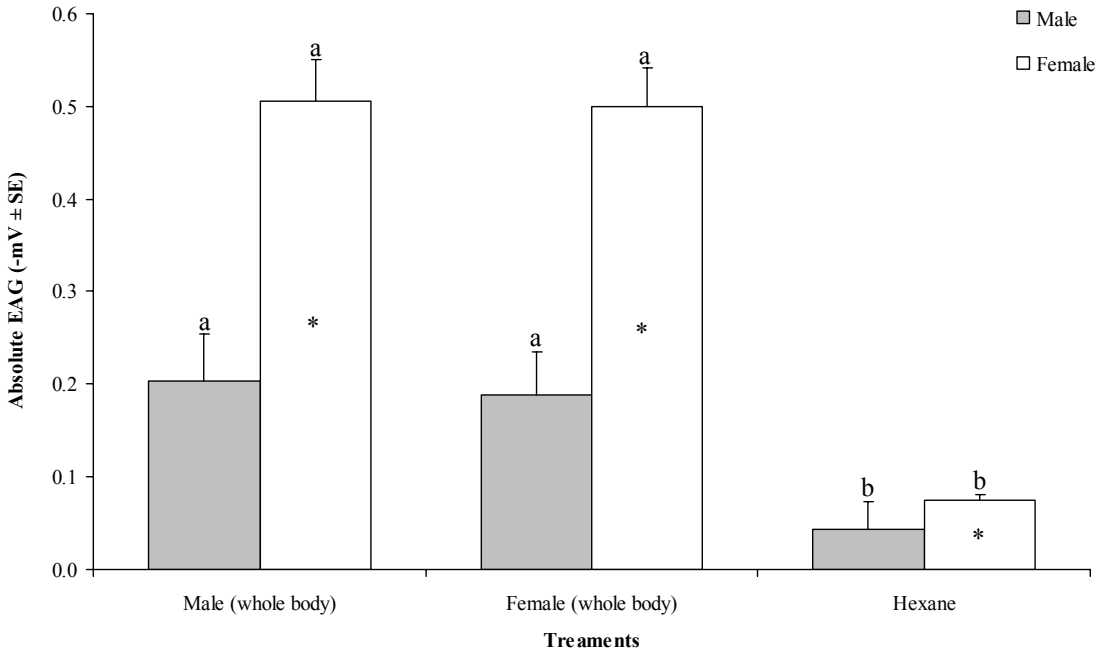


Figure 2 Responses of male and female *P. cerealellae* in a Y-tube olfactometer when given a choice between humidified and purified laboratory air (control) and airborne volatiles from conspecific males or females. Grey bars indicate the percentage of male or female wasps responding to the control, while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

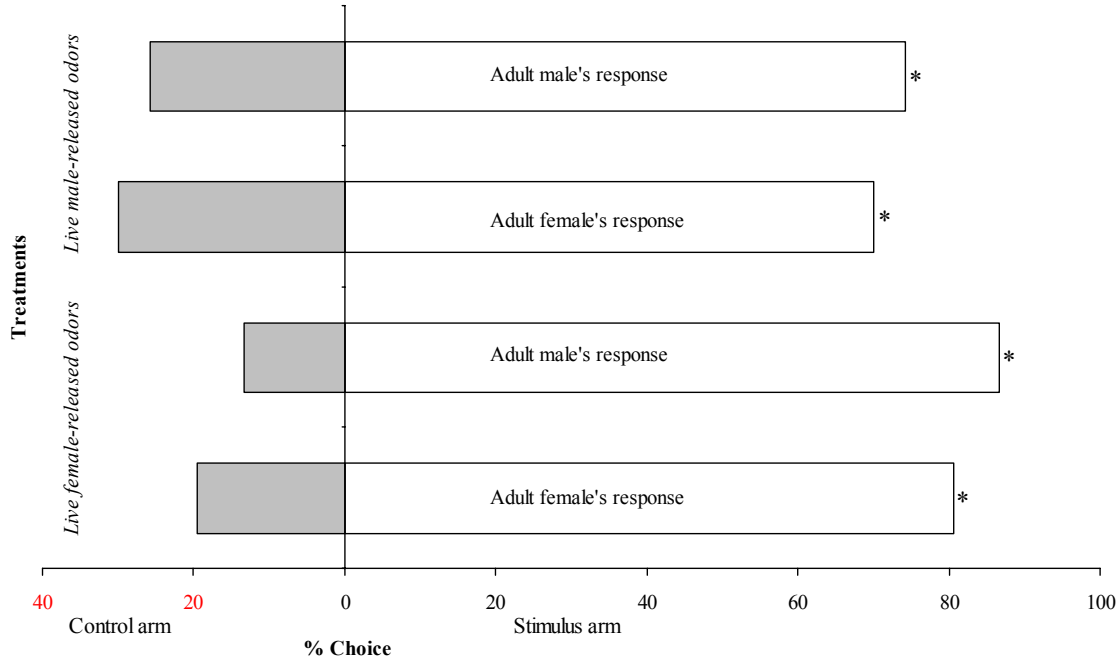


Figure 3 Responses of male and female *P. cerealellae* in a Y-tube olfactometer when given a choice between filter paper impregnated with hexane (control) and filter paper impregnated with 10-µL aliquot of whole body hexane extracts of male or female *P. cerealellae*. Grey bars indicate the percentage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

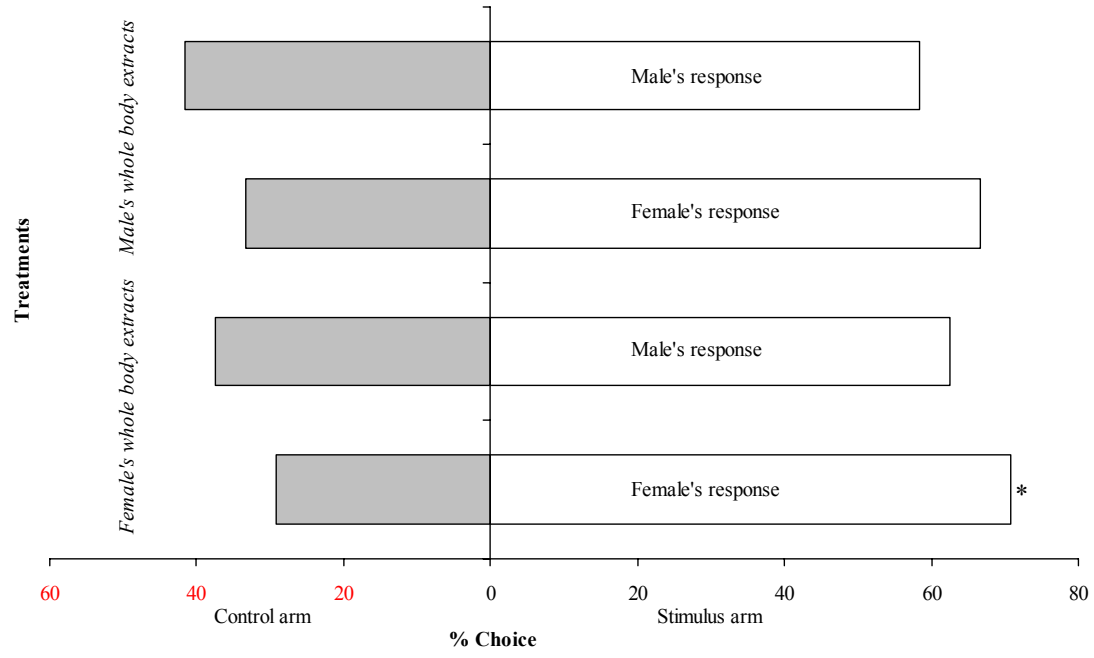
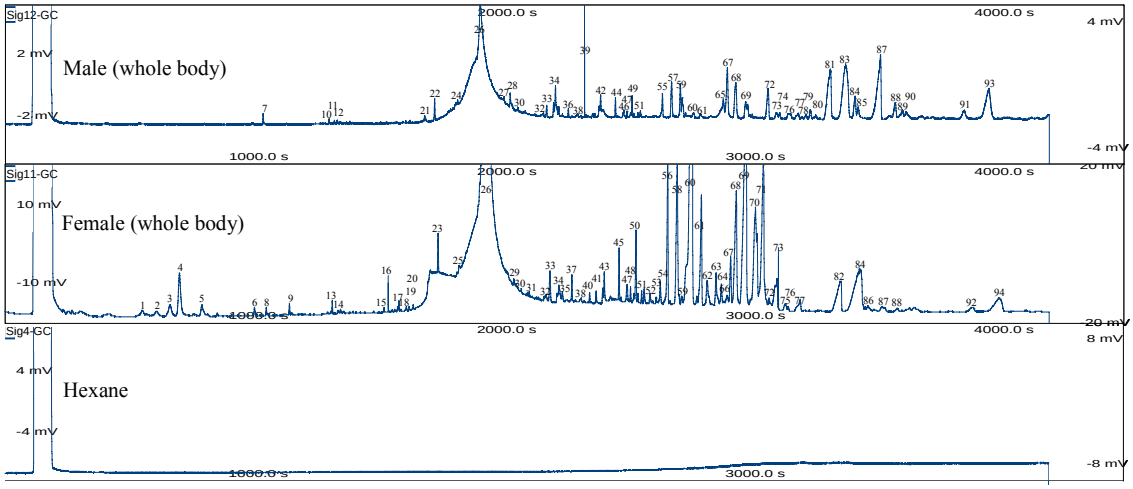


Figure 4 Gas chromatographs of whole body extracts of male and female *P. cerealellae*. Numbers on the peaks represent the various recognizable peaks (compounds) as analyzed by the GC. Peaks from male or female extracts having similar number were identified as being the same based on retention times (RT).



CHAPTER 8: ELECTROANTENNOGRAM AND BEHAVIORAL RESPONSES OF *PTEROMALUS CEREALELLAE* TO ODOR STIMULI ASSOCIATED WITH ITS HOST, *CALLOSOBRUCHUS MACULATUS*

INTRODUCTION

Research on the cues used by parasitoids to locate their hosts has received a major attention in the last three decades (Lewis et al., 1982; Vinson, 1984; 1991; Noldus, 1988; Noldus et al., 1991; Vet et al., 1991; Godfray, 1994; Agelopoulos et al., 1995; Sullivan *et al*., 2000; Mbata et al., 2004). Most of these studies have implicated semiochemical-mediated host location strategies for several species of parasitoids (Noldus and van Lenteren, 1985; Vinson, 1991; van Huis et al., 1994; Agelopoulos et al., 1995; Steidle et al., 2003; Mbata et al., 2004). The semiochemicals that mediate host location by parasitoids could originate from host insects (i.e. host-specific semiochemicals), hosts' habitat (environment), or produced from an interaction between host insects and their habitats (Turlings et al., 1991; Godfray, 1994; Quicke, 1997; Mbata, 2004).

Host-specific semiochemicals (kairomones) including host pheromones, host body chemicals (e.g., cuticular hydrocarbons) and volatile stimuli from host feces/or frass

may represent reliable host location cues for parasitoids of herbivorous insects (Lewis et al., 1982; Noldus and van Lenteren, 1985; Schöller and Prozell, 2002; Mbata et al., 2004, Lecomte and Thibout, 1993; Cortesero et al., 1993; Steinberg et al., 1993; Turlings et al., 1991; Agelopoulos and Keller, 1994a, b). However, use of host-specific semiochemicals for host location may be particularly challenging for parasitoids, which attack concealed hosts or whose hosts live endophytically within grains (Hawkins, 1994; Vet et al., 1995). Because they may not be directly exposed to host-specific semiochemicals at the initial stages of their host location behavior, due to the likelihood of chemical masking, parasitoids of concealed hosts may have to increase the number of information sources available to them in order to locate their hosts (Lewis et al., 1982; van Huis et al., 1994; Hawkins, 1994; Vet et al., 1995). Although, semiochemicals associated with the host habitat are non host-specific and less reliable, they are more easily detectable to parasitoids of concealed hosts and may signal host presence (Norlund et al., 1988; Vet et al., 1991; Vet & Dicke, 1992; Sullivan et al., 2000; Kalule and Wright, 2004). Indeed, studies have shown that many parasitoids of concealed hosts use non host-specific kairomonal cues, such as semiochemicals from the host habitat, for host location (Lewis et al., 1982; Vet et al., 1995; van Huis et al., 1994; Phillips, 1997). Parasitoids of concealed hosts may also use non-chemical cues such as host vibration signals to locate their hosts (Smirnov and Polejaeff, 1937; Kaschef, 1964).

Pteromalus cerealellae (Ashmead) (Hymenoptera: Pteromalidae) is a generalist ectoparasitoid of mature larvae and prepupae of several insect pests of stored products including *Sitotroga cerealella* Olivier, *Lasioderma serricorne* (Fab.), *Prostephanus truncatus* (Horn), *Sitophilus* spp., and the cowpea bruchid, *Callosobruchus maculatus*

(Fab.) (Ashmead, 1902; Brower, 1991; Wen et al., 1995; Mbata et al., 2005; Onagbola et al., 2007). Females of *P. cerealellae* lay eggs in these hosts, all of which develop inside seeds of cereal grains (plant hosts). Little is known about the cues used by female *P. cerealellae* to locate its concealed hosts. In a previous study, we observed that female *P. cerealellae* were capable of detecting and parasitizing dead (freeze-killed) and live *C. maculatus* larvae alike and could not differentiate between both host types (unpublished data), suggesting that larval vibrations may not be the principal host location cue used by this species. Being a generalist parasitoid, *P. cerealellae* presumably uses as host location cues volatile chemicals from different non-related plant-host complexes, as reported for *Lariophagus distinguendus* Först (Hymenoptera: Pteromalidae) (Steidle et al*.,* 2001).

In the first published report on semiochemical-meditated host location mechanisms in *P. cerealellae*, Mbata et al. (2004) reported significant orientation of mated females to various *C. maculatus* (bruchid) host-related odor stimuli including live and body extracts of virgin female *C. maculatus* and extracts of its oviposition marking pheromone, as well as uninfested and bruchid-infested cowpea seeds. The authors further reported that whole body extracts of virgin female bruchids elicited the strongest response in female *P. cerealellae.* These results suggest the use of host-related semiochemicals for host location by *P. cerealellae*.

The present study was carried out to further investigate the role of host-related semiochemicals in mediating host location by *P. cerealellae* and to determine the relative importance of semiochemicals from first (cowpea seeds) and second (bruchid-specific odors) trophic levels in host location. Based on the results of a preliminary experiment showing that female *P. cerealellae* could not differentiate between uninfested and

bruchid-infested cowpea seeds (unpublished data), we hypothesized that chemicals from the host habitat (cowpea seeds) are likely to play a relatively more important role in host location. In this paper, we evaluated the electroantennogram (EAG) and behavioral responses of female *P. cerealellae* to a variety of odor stimuli associated with its host, the cowpea bruchid, *C. maculatus*. Host-related stimuli evaluated include uninfested and bruchid-infested cowpea seeds (host habitat), and host-specific odors such as larval frass and whole body extracts of bruchid larvae, adult female bruchids and adult male bruchids. Additional tests were conducted in a 4-choice olfactometer to determine odor preference. Finally, chemical profiles of the various stimuli were characterized by gas chromatography to detect possible quantitative or qualitative differences in their chemical compositions.

MATERIALS AND METHODS

Insects

Pteromalus cerealellae was reared in our laboratory on the larvae of the cowpea bruchid, *Callosobruchus maculatus* (host insect). 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 5 d by placing ~25 pairs of 3-day-old mated *C. maculatus* in a glass jar containing ~100 g of cowpea seeds held at 30 ± 1ºC, 70 ± 5% r.h., and a photoperiod of L12: D12 (Mbata et al., 2005; Onagbola et al., 2007). Beetles were allowed to lay eggs on the seeds for 24 h after which they were removed with an aspirator. Infested seeds were incubated at the conditions specified above until the larvae had reached the fourth instar, which were then provided to *P. cerealellae* for

parasitization. The parasitoid was maintained by transferring ~30 adult pairs onto 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 d after infestation of cowpea seeds under our rearing conditions). Jars were held at the environmental conditions stated above for *C. maculatus*. Adult *P. cerealellae* were removed from the jars after 5 d of oviposition. Parasitized host larvae were incubated in a growth chamber at the above environmental conditions until the adult parasitoids started to emerge (~11 d after parasitization at the above conditions). Parasitized hosts (still contained within infested seeds) which bear the wasps' pupae were immediately transferred into 100 x 15 mm disposable Petri dishes to collect adult parasitoids immediately upon emergence.

Solvent extraction of chemical stimuli

Extraction of chemical stimuli from *C. maculatus* adults and larvae and from cowpea seeds was conducted using a method similar to that described by Shu et al. (1996, 1999) and Mbata et al. (2004). Three hundred, virgin adult females or males (2-3 days old) or fourth instars of *C. maculatus* were soaked in 6 mL laboratory grade hexane in a 10-mL glass vial for 2 h. To obtain fourth instars of *C. maculatus* for extraction, infested cowpea seeds containing fourth instar larvae (Mbata et al., 2005; Onagbola et al., 2007) of *C. maculatus* were carefully cracked opened with a fine knife to extract the bruchids (host)' larvae. The extraction process was repeated ten times for a total of 3000 adult females, males or larvae. The extracts were stockpiled in glass vials and concentrated under Nitrogen to obtain a concentration of ~1 insect equivalent per 0.2 μL (5 insects per μL of extract). Similar procedures were used to extract uninfested and bruchid-infested

cowpea seeds and larval frass. Approximately 5 g of uninfested insecticide-free cowpea seeds obtained from a local grocery store were extracted in 6 ml hexane in a 10 ml glass vial for 2 h. Cowpea seeds infested by *C. maculatus* were first cracked opened with a fine knife (at ~17 d after oviposition) to remove developing larvae (usually fourth instar) and frass and then extracted (with the larvae and frass removed) as described for uninfested seeds. Larval frass obtained from bruchid-infested cowpea seeds were removed into clean Petri dishes with fine a brush and stored in a freezer at -20°C until there was enough quantity for extraction. Approximately 4 g of frass were extracted in 5 ml hexane in a 10-mL glass vial for 2 h. Cowpea seeds and frass were weighed on a AR 2140 Adventurer™ balance (OHAUS Corp., Pine Brook, NJ). Extracts of cowpea seeds and frass were concentrated to 5 mg per µl. Each extract was made in three replicates, dehydrated in ~2 g of anhydrous Sodium Sulfate for ~12 h and stored in a freezer at -20°C until used.

Electroantennogram (EAG) recordings

The EAG response of 2-d old mated and unmated female *P. cerealellae* was tested to the following 7 stimuli treatments: (i) hexane extracts of uninfested cowpea seeds, (ii) bruchid-infested cowpea seeds, (iii) bruchid larvae (whole body, WB), (iv) larval frass, (v) adult female bruchids (WB), (vi) adult male bruchids (WB), and (vii) hexane (control). 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 an 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. Ten-μL aliquot of each solution (stimulus treatment) 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 μL aliquot of hexane. The tip of the pipette was placed ~3 mm into a small hole in the wall of a metal tube (13 cm long, 8 mm diameter) which orient towards 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 was allowed between successive stimulations for antennal recovery. A test series consisting of the above listed stimuli (treatments) was applied to an antennal preparation starting with the hexane control and following a random order thereafter. Recordings were obtained from at least 16 female *P. cerealellae* of different mating status.

Data were first analyzed by using the standard least-squares fit model method (SAS Institute, 2003) to determine the effects of stimuli (treatments), mating, and interactions of both factors on absolute EAGs. Further analysis of the data was by using one-way ANOVA followed by Tukey's HSD comparison test to compare EAG responses of mated or unmated females to the different stimuli ($P < 0.05$; SAS, 2003). The effect of

mating on EAG response to each stimulus (treatment) was then compared by using the Students' t - test ($P < 0.05$; SAS, 2003).

Y-tube olfactometer bioassays

A Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) was used to test the attraction of mated female *P. cerealellae* (2-d old) to the stimuli treatments tested in the EAG experiment. 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 were separately connected to an extending glass tube (14.5 cm long, 19 mm diam.). There was a sieve inlayed 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 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 vertically hanging an office lamp (20 W, 250 lux) above (~50 cm high) the olfactometer tube.

The experiment was conducted to test the attractiveness of the following host-related stimuli treatments to mated female *P. cerealellae*: hexane extracts of uninfested cowpea seeds, bruchid-infested cowpea seeds, bruchid larvae (WB), larval frass, adult female bruchids (WB), and adult male bruchids (WB). Only mated females were tested in this experiment based on the results of a preliminary experiment, which showed no

significant effect of mating on the behavioral response of female *P. cerealellae* to the tested host-related stimuli (unpublished data). Each stimulus was delivered as a 25-µL sample placed on No. 1 filter paper strips (7 × 40 mm, Whatman® no. 1). This resulted in 125 larvae or adult equivalent or 125 mg of uninfested, infested or frass per loading (25 µL of each stimulus was used in the behavioral bioassays based on our preliminary data showing minimal response of female parasitoids to 10 µL dose of each stimulus). 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 25-µL aliquot of hexane (laboratory grade) was inserted into the second arm (solvent control). 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 treatment and the solvent control. After three individual parasitoids had been tested, a fresh odor stimulus was used and the olfactometer arms were reversed (180º). After each subset of 6 parasitoids had been tested, the olfactometer apparatus was rinsed with soap water and acetone, and then air-dried. Forty females were tested per choice test, and parasitoids were used only once. Bioassays were conducted at 25 ± 1˚C and 60 ± 5% r.h.

Data obtained on the percentage responses of mated female parasitoids to each stimulus versus control was analyzed by the use of a chi-square (χ^2) test to determine significant deviation ($P < 0.05$, SAS, 2003) from an expected 1:1 response.

Four-way olfactometer bioassays

The 4 most attractive odor stimuli treatments from the Y-tube bioasssays were further evaluated in a 4-way olfactometer (Analytical Research Systems, Gainesville, FL) to determine odor preference of mated female *P. cerealellae.* The 4-way olfactometer system used in this study was similar to the system described by Pettersson (1970) and Kalule and Wright (2004). It consists of a central chamber with orifices at the four corners through which purified and humidified air was drawn in, creating 4 potential odor fields, and a central orifice where mixing of the airflow from the arms occurred. Two of the orifices at the corners were designated for odor stimuli treatments and the other 2 for controls (i.e. only 2 stimuli treatments were compared at the same time). The 2 controls were hexane (control 1) and charcoal-filtered, humidified laboratory air (control 2). A constant airflow of 0.25 L/min was maintained through each of the 4 orifices at the corners of the olfactometer. Mixtures of air from the control arms and volatile odors from the treatment arms were suctioned-out from the olfactometer with a constant airflow of 1.5 L/min, through the central orifice. Glass tubes (1 by 5 cm) containing the test stimuli or controls were attached with teflon connectors to each of the 4 arms of the olfactometer. Volatile odors were delivered as a 25-μL sample (resulting in 125 larvae equivalent or 125 mg of uninfested, infested or frass per loading) placed on Whatman No. 1 filter paper strips (7×40 mm). After allowing for solvent evaporation (~15 s), the filter paper strip was inserted into a glass tube, which was connected to an arm of the four-way olfactometer.

The following four attractive host-related hexane extracts were tested in a pair-wise fashion (binary test) for a total of six paired treatments (Table 1): uninfested cowpea

seeds, bruchid-infested cowpea seeds, bruchid larvae (WB), and larval frass. A mated female *P. cerealellae* (2-d old) was introduced singly into a glass tube (1 × 5 cm), which was connected to the central orifice of the olfactometer to expose females to the volatile odors/air mixtures in a relatively limited area. Once in the chamber, a parasitoid was given 5 min to make a choice among the 4 air fields (i.e. 2 treatments and 2 control fields). If the parasitoid had not made a choice within this duration, it 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 for that stimulus. After five individual parasitoids had been tested, a fresh odor stimulus was used and the olfactometer was cleaned as previously described. In order to remove any directional bias in the chamber, the olfactometer was rotated after 10 parasitoids had been tested. Each binary test (consisting of 2 treatments and 2 controls) was replicated 4 times (i.e. 4 rotations of the olfactometer arms) with each replicate consisting of 10 females, resulting in a total of 40 females per binary test. All observations were made at ~25 ± 1ºC, 60 ± 5% r.h. under ambient, incandescent light of ~250 lux.

For each binary comparison, the number of female parasitoids which chose the two control arms was minimal (< 20 %) and not included in the statistical analyses. Data obtained from all 4 replicates were pooled and analyzed with chi square (χ^2) to test for significant deviation ($P < 0.05$, SAS, 2003) from an expected 1:1 response of female parasitoids to any 2 paired treatments.

GC analyses

Hexane extracts of each of the tested stimulus treatment (uninfested cowpea seeds, bruchid-infested cowpea seeds, bruchid larvae (WB), larval frass, adult female bruchids (WB), adult male bruchids (WB)) were analyzed in a gas chromatograph (Shimadzu GC 17A, Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector (FID) set at 320ºC. The GC column used was a non-polar Restek® Rtx-IMS capillary column (30 m × 0.25 mm ID; 0.25-µm film thickness). The temperature conditions were 2 min at 80ºC, increased at a rate of 5ºC/min to 280ºC, and held at this temperature for 18 min. The injector temperature was set at 275ºC and operated in splitless mode throughout the analyses. The carrier gas was Helium (purity 99.96%) with a flow rate of 1.5 mL/min and at a constant pressure of 100 KPa. Approximately 2 µL of each extract was injected into the GC for analysis. The retention time (RT) of the recognizable peaks (compounds) was determined and compared among the treatments.

RESULTS

Electroantennogram (EAG) responses

Standard least squares modeling revealed significant effects of stimuli treatments ($F = 49.70$, $df = 6$, $P < 0.0001$), mating ($F = 15.94$, $df = 1$, $P < 0.0001$), and treatment × mating interaction ($F = 3.88$, $df = 6$, $P < 0.0001$) on EAG response of female *P. cerealellae*. Based on the recorded significant mating effect, the effect of stimuli on EAG response was compared separately for unmated and mated females. All of the stimuli elicited significantly greater EAG response in mated females compared to the hexane

control (Fig. 1). However, extracts of uninfested seeds, infested seeds, bruchid larvae, and larval frass elicited significantly greater EAG responses than extracts of adult female bruchids and adult male bruchids. Similar results were obtained for unmated females (Fig. 1). In general, mated females showed greater EAG response than unmated females but this was not always significant. Students' *t* - test showed that extracts of infested seeds ($t = 2.31$, $df = 1$, $P = 0.028$) and bruchid larvae ($t = 3.93$, $df = 1$, $P = 0.0005$) elicited significantly greater EAG response in mated than in unmated females (Fig. 1). In contrast, whole body extract of adult male bruchids elicited a marginally significantly greater EAG response in unmated than in mated females ($t = -2.10$, $df = 1$, $P = 0.044$). No significant differences were observed in the responses of mated and unmated female parasitoids to extracts of uninfested seeds ($t = 1.15$, $df = 1$, $P = 0.259$), larval frass ($t = 1.78$, $df = 1$, $P = 0.084$) and adult female bruchids ($t = 0.22$, $df = 1$, $P = 0.826$).

Y-tube olfactometer bioassays

Chi square analyses showed significant olfactometer response of mated female *P. cerealellae* to extracts of uninfested seeds (90%; $\chi^2 = 12.8$, $df = 1$, $P = 0.0003$), bruchid-infested seeds (85%; $\chi^2 = 9.8$, $df = 1$, $P = 0.0017$), larval frass (89.5%; $\chi^2 = 11.8$, $df = 1$, $P = 0.0006$), bruchid larvae (84.2%; $\chi^2 = 8.9$, $df = 1$, $P = 0.0029$), and adult female bruchids (79%; $\chi^2 = 0.64$, $df = 1$, $P = 0.0116$). However, whole body extract of adult male bruchids did not elicit significant behavioral response in female parasitoids (65%; $\chi^2 = 1.8$, $df = 1$, $P = 0.179$) (Fig. 2).

Four-way olfactometer bioassays

The results of the pair-wise comparisons (binary test) of the 4 most attractive stimuli in a 4-way olfactometer bioassay are summarized in Table 1. Mated female *P. cerealellae* did not show significant preference between extracts of uninfested seeds vs. infested seeds ($\chi^2 = 0.03$, $df = 1$, $P = 0.8527$), uninfested seeds vs. bruchid larvae ($\chi^2 = 0.53$, $df = 1$, $P = 0.4652$), infested seeds vs. bruchid larvae ($\chi^2 = 0.81$, $df = 1$, $P = 0.3692$), infested seeds vs. larval frass ($\chi^2 = 1.13$, $df = 1$, $P = 0.2888$), and bruchid larvae vs. larval frass ($\chi^2 = 0.57$, $df = 1$, $P = 0.4497$). However, when extract of uninfested seeds was paired with extract of larval frass, female parasitoids showed significant preference for uninfested seeds (53%) over larval frass (23%) ($\chi^2 = 4.8$, $df = 1$, $P = 0.0285$) (Table 1). Furthermore, there was a consistent trend for a preference for cowpea seeds (uninfested or infested) over bruchid-specific stimuli (bruchid larvae or larval frass), although this was not always significant (Table 1).

GC analyses

GC analyses revealed major qualitative and quantitative differences in the chemical profiles of the different host-related extracts. The total number of recognizable peaks obtained from extracts of uninfested seeds, bruchid-infested seeds, bruchid larvae, larval frass, adult female bruchids, and adult male bruchids were 18, 21, 50, 18, 69 and 72, respectively (Fig. 3; Table 2). These peaks could be categorized into 3 broad groups based on their likely sources: i) cowpea seed-based peaks (**x**, $n = 10$), ii) bruchid larvae-based peaks (***x***, $n = 29$), and iii) adult female bruchid-based peaks (x, $n = 3$) (Table 2). As

expected, some of the peaks that occurred in extracts of bruchid-infested seeds were present in extracts of uninfested seeds (n = 4), bruchid larvae (n = 12), and adult female bruchids (n = 3). Larval frass extract had 18 recognizable peaks, which consisted of 3 cowpea seeds-based peaks and 11 bruchid larvae-based peaks, but no frass-specific peaks (Fig. 3; Table 2). In addition, all of the 18 larval frass peaks were also recorded in bruchid-infested seed extract, confirming that larval frass is a product of infested cowpea seeds, which, in turn, is derived from uninfested cowpea seeds and bruchid larvae. Extracts of adult female and male bruchids contained 69 and 72 recognizable peaks, respectively, with 64 peaks in common (Fig. 3; Table 2), and shared 4 (peak numbers 28, 29, 48, 56) peaks with uninfested cowpea seeds, suggesting these peaks to be cowpea seed-based.

Given the results of the behavioral bioassays, which implicated cowpea seeds as the likely source of compounds that elicited significant response in female *P. cerealellae* (Fig. 2), attention was paid mainly to the cowpea seed-based peaks that may explain the significant results recorded in the behavioral bioassays. Based on the results of the Y-tube olfactometer bioassays demonstrating significant attraction of female *P. cerealellae* to all tested extracts, except for whole body extract of adult male bruchids (Fig. 2), we hypothesized that: i) uninfested cowpea seeds is the primary source of the attractive compounds (peaks) in extracts of uninfested seeds, infested seeds, bruchid larvae and frass, ii) the attractive compounds are likely to be present either in relatively higher amounts or in optimal ratios in uninfested cowpea seeds, and this quantitative differences may explain the observed relatively (but not always significant) greater response of female parasitoids to uninfested cowpea seeds extract, iii) the attractiveness of whole

body extract of adult female bruchids is mediated by adult female bruchid-specific compounds, which may not be present in cowpea seeds extracts or in whole body extracts of adult male bruchids.

Several of the cowpea seed-based peaks were present in the attractive extracts of uninfested cowpea seeds, infested cowpea seeds, whole body larvae and larval frass (Table 2). Extracts of uninfested cowpea seeds and larval frass had three peaks (peak numbers 48, 67, 84) in common (Fig. 3; Table 2). All three peaks were expressed in similar amounts in both extracts, suggesting that the observed preference of the parasitoid for uninfested cowpea seeds over larval frass is not due to quantitative differences in the expression of these peaks, but, instead, may be due to the peaks that occured in uninfested cowpea seeds but not present in larval frass (qualitative differences in chemical profile), including peak numbers 1, 4, 19, 27, 28, 29, 56, 76, 98, 101, 103, 104, 105, 109 and 110 (Fig. 3; Table 2). Similarly, qualitative differences in the chemical profiles of whole body extracts of adult female and male bruchids (Fig. 3; Table 2), in particular the two adult female bruchid-specific peaks (peak numbers 2 and 26, represented by F in Table 2), may possibly explain why extract of the later failed to elicit significant behavioral response in female *P. cerealellae* as did extract of the former.

DISCUSSION

The results of this study demonstrated the electroantennogram and behavioral responses of female *P. cerealellae* to various host-related odor stimuli, as have been reported for several other parasitoid species (Elzen et al., 1983; Turlings et al., 1991; Steinberg et al., 1993; van Huis et al., 1994; Röse et al., 1997; Steidle and Schöller, 1997;

Pettersson et al., 2001; Kalule and Wright, 2004; Mbata et al., 2004). Specifically, results from Y-tube bioassays showed significant attraction of female *P. cerealellae* to all tested stimuli, with the exception of whole body extract of adult male bruchids. Further evaluation in a 4-way olfactometer demonstrated preference of female *P. cerealellae* for extract of uninfested cowpea seeds compared to other stimuli, although a significant difference was recorded only between uninfested cowpea seeds and larval frass. Overall, these results suggest that semiochemicals from the 1st trophic level (cowpea seeds) are relatively more important than host bruchid-specific semiochemicals in mediating host location by female *P. cerealellae*.

Females of various parasitoid species, in particular parasitoids of concealed hosts, are known to use volatiles from the habitat (food) of their hosts rather than host-specific semiochemicals for host location (Elzen et al., 1983; Norlund et al., 1988; Martin et al., 1990; Tumlinson et al., 1992; Wickremasinghe and van Emden, 1992; Souissi, 1999; Kalule and Wright, 2004). For instance, the solitary larval and pupal ectoparasitoid of bruchid beetles, *Eupelmus vuilleti* (CRW) (Hymenoptera: Eupelmidae) (Cortesero et al., 1993), an egg parasitoid of *C. maculatus*, *Uscana lariophaga* Steffan (Hymenoptera: Pteromalidae) (van Huis et al., 1994) and the generalist parasitoid, *L. distinguendus* (Steidle et al., 2001) have been reported to be attracted to volatiles emanating from uninfested cowpea seeds.

Several aspects of our results on semiochemical-mediated host location behavior of *P. cerealellae* are in agreement with an earlier report on the same species by Mbata et al. (2004), including observed attraction of female parasitoids to extracts of uninfested and bruchid infested cowpea seeds and to whole body extract of adult female bruchids.

Our results showing greater attraction of female *P. cerealellae* to cowpea seed odor than to host bruchid-specific odor are, however, disagree with those of Mbata et al. (2004), which indicated that host bruchid-specific stimuli (e.g., extracts of female beetles and infested seeds containing larval frass) were more attractive than extract of uninfested cowpea seeds. Findings similar to those of Mbata et al. (2004) are also common in the literature on parasitoid host location behavior (e.g. Noldus and van Lenteren, 1985; Steinberg et al., 1993; Turlings et al., 1993; Colazza et al., 1997; Steidle and Schöller, 1997; Reddy et al., 2002; Sullivan et al., 2000). For example, females of *L. distinguendus* were shown to prefer rice and wheat grains infested by stored grain beetles over uninfested grains (Steidle et al*.,* 2001). Similarly, females of *U. lariophaga* (van Huis et al., 1994), *Roptrocerus xylophagorum* Ratzeburg (Hymenoptera: Pteromalidae) (Sullivan et al., 2000) and *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) (Eben et al., 2000) were reported to prefer odors of infested over uninfested hosts' foods. The disparity between some of our results and those reported by Mbata et al. (2004) may be due to differences in experimental design and protocols. For example, Mbata et al. (2004) made use of head space volatile collection techniques to obtain odors from infested and uninfested cowpea seeds, whereas hexane extracts of infested and uninfested cowpea seeds were tested in the present study.

Attraction of female *P. cerealellae* to whole body extracts of adult female bruchids, as recorded in this study and also by Mbata et al. (2004), is likely mediated by female bruchid sex pheromones (Shu et al., 1996; Phillips et al., 1996). Many parasitoid species, in particular egg parasitoids, are known to use host sex pheromones as host-location cues (i.e. as kairomones) (e.g. Lewis et al., 1982; Noldus and van Lenteren,

1985; Colazza et al., 1997; Reddy et al., 2002, Mbata et al., 2004). For example, Reddy et al. (2002) demonstrated attraction of the egg parasitoid, *Trichogramma chilonis* Ishii (Hymenoptera: Trichogrammatidae) and the larval parasitoid, *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae), to the sex pheromones of their host, *Plutella xylostella* (Lepidoptera: Plutellidae). Host location in the egg parasitoid, *U. lariophaga* was also reported to be mediated by the host bruchid's sex pheromones (van Huis et al., 1994). Attraction of female *P. cerealellae* to adult female bruchid (*C. maculatus*) may appear illogical (Mbata et al., 2004), given that the mature larvae needed for parasitization may not occur until several weeks after the short lived adult female bruchids had died. The results of our GC analyses suggest that this attraction is probably mediated by the adult female bruchid-specific peaks (peak numbers 2 and 26). Both peaks have low retention times indicating high volatility and, thus, may constitute the unindentified sex pheromone components of adult female bruchids (Shu et al., 1996; Mbata et al., 2004). In the same vein, the non-attractiveness of whole body extract of adult male bruchids, which was reported by Mbata et al. (2004), may be explained by absence of these two adult female-specific peaks in extracts of adult male bruchids.

Contrary to the results of Mbata et al. (2004), which showed greater attraction of female *P. cerealellae* to infested cowpea seed than to uninfested cowpea seeds, we did not record any significant difference in the response of female parasitoids to both stimuli. Findings similar to ours were reported for female *L. distinguendus*, which also showed no significant preference for bruchid-infested cowpea seeds over uninfested cowpea seeds (Steidle et al., 2001). The results of our GC analyses revealed about 10 cowpea-seed based peaks occuring in extract of uninfested cowpea seeds as well as in extracts of

infested cowpea seeds, whole body larvae and larval frass and, possibly, may explain the attractiveness of these extracts. As a generalist parasitoid, *P. cerealellae* presumably uses general plant odor cues for host location (Godfray, 1994; Steidle et al., 2001). Thus, it is likely that the cowpea-seed based peaks recorded in the current study will occur in other grains. Indeed, chemical analyses of different grains (e.g. rice, wheat and cowpea) have revealed the presence of common general plant volatile compounds including methanol, ethanol, hexanal, hexanol, methylbutanal, pentanal, pentanone, phenylacetaldehyde, and naphtalene (Bullard & Holguin, 1977; Maga, 1978; Legendre et al., 1978; Fisher et al., 1979; Ram et al., 1999). We hypothesize that attraction of generalist parasitoids such as *P. cerealellae* and *L. distinguendus* to stored grain pests may be mediated by some of these common general grain volatile compounds.

Based on their results, Steidle et al. (2001) proposed a 2-step process for host location by *L. distinguendus*, which may also be applicable also to other generalist parasitoids of concealed hosts. First, female parasitoids use general plant (grain) odor cues (host food) to locate their concealed hosts or host habitat, regardless of the plant-host complex. Second, female parasitoids must differentiate between healthy (uninfested) and infested grain seeds. This second step may be accomplished by the use of chemical cues associated with host feces/frass in infested seeds, as demonstrated for *L. distinguendus* on some plant-host complexes (Steidle and Schöller, 1997; Steidle et al., 2001). Our results support the first step regarding the use odors of general plant odor as host location cues by generalist parasitoids. However, we could not demonstrate the ability of *P. cerealellae* females to differentiate between bruchid-infested and uninfested cowpea seeds using chemical cues. Steidle et al. (2001) also reported that while *L.*

distinguendus females showed preference for infested seeds from the complexes rice-*Sitophilus granarius* and wheat-*Rhyzopertha dominica* over uninfested seeds, the parasitoid could not differentiate between bruchid-infested and uninfested cowpea seeds. The authors proposed that their results may be due to the possible masking of host feces odor in cowpea, compared to rice or wheat. In any case, the inability of *P. cerealellae* (this study) and *L. distinguendus* (Steidle et al*.,* 2001) to distinguish between bruchid-infested and uninfested cowpea seeds may suggest that females of both species are primarily attracted to bruchid hosts using cowpea volatiles. Once in the host habitat (a mixture of uninfested and infested cowpea seeds), females may search randomly for infested cowpea seeds (Steidle et al*.,* 2001) or use visual or tactile cues such as those associated with bruchid egg plugs to differentiate between infested and uninfested cowpea seeds. Indeed, we have observed that *P. cerealellae* females drum the tip of their antennae on the surface of cowpea seeds prior to oviposition (unpublished data), possibly as a mechanism to differentiate between infested and uninfested cowpea seeds. It is unlikely that larval vibrations are used as cues for this purpose, given a previous study, which showed that female *P. cerealellae* were capable of detecting and parasitizing dead (freeze-killed) and live *C. maculatus* larvae alike and could not differentiate between both host types (unpublished data).

These results showed that *P. cerealellae* locates its bruchid host primarily by using innate volatile cues from cowpea seeds, but may use volatile cues associated with adult female bruchids. Further studies including gas chromatography coupled with electroantennogram detection (GC-EAD) and gas chromatography coupled with mass spectrometer (GC-MS) are necessary to identify the compounds (peaks) mediating the

attraction of female *P. cerealellae* to uninfested cowpea seeds and adult female bruchids. Similarly, further research is needed to determine the mechanisms (cues) used by female *P. cerealellae* to distinguish between infested and uninfested cowpea seeds.

ACKNOWLEDGEMENTS

This research was supported in part by the Alabama Agricultural Experiment Station and an Auburn University Competitive Research grant to HYF. I would like to thank Dr. Li Chen for technical assistance.

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Table 1 Response of mated female *P. cerealellae* to attractive host-related stimuli in a four-choice olfactometer.

Stimuli source	% reacting to		χ^2 (1 df)	*P*
	T1	T2		
Uninfested seeds (T1) vs. Bruchids infested seeds (T2)	37.5	35.0	0.03	0.8527
Uninfested seeds (T1) vs. Bruchid larvae (T2)	42.5	32.5	0.53	0.4652
Uninfested seeds (T1) vs. Larval frass (T2)	52.5	22.5	4.80	0.0285*
Bruchids infested seeds (T1) vs. Bruchid larvae (T2)	45.0	32.5	0.81	0.3692
Bruchids infested seeds (T1) vs. Larval frass (T2)	47.5	32.5	1.13	0.2888
Bruchid larvae (T1) vs. Larval frass (T2)	40.0	30.0	0.57	0.4497

Table shows percentage choice of female *P. cerealellae* between two stimuli (T1 and T2) sources. * Indicate significant difference in percentage females' choice (χ^2, $P < 0.05$).

Table 2 Peaks (compounds) present in the various host-related extracts and their retention times.

Peak No.	Retention time (s)	Tested stimuli (Treatments)					
		Uninfested seeds	Infested seeds	Bruchid larvae	Larval frass	Adult ♀ bruchid	Adult ♂ bruchid
1	1123.6	C					
2	1314.5					F	
3	1481.8			***x***		***x***	
4	1524.5	C					
5	1289.1			L			
6	1323.6					x	x
7	1410.9					x	x
8	1527.3					x	x
9	1834.5					x	x
10	1840.0					x	x
11	1856.4			L			
12	1876.4					x	x
13	1911.8					x	x
14	1914.5					x	x
15	1919.1					x	x
16	1927.3					x	x
17	1954.5					x	x
18	2003.6			***x***			***x***
19	2013.6	**x**				**x**	
20	2054.5			***x***			***x***
21	2055.5			***x***		***x***	***x***
22	2118.2			***x***			***x***
23	2143.6						x
24	2178.2			***x***			***x***
25	2190.9			***x***		***x***	***x***
26	2196.4					F	
27	2233.6	**x**		**x**			**x**
28	2244.5	**x**		**x**		**x**	**x**
29	2270.9	**x**		**x**		**x**	**x**
30	2279.1					x	x
31	2295.5			***x***		***x***	***x***
32	2330.0					x	x
33	2336.4					x	x
34	2357.3			L			
35	2347.7			***x***		***x***	***x***
36	2387.3			***x***		***x***	***x***
37	2419.9			***x***		***x***	***x***
38	2430.7					x	x
39	2438.0					x	x

40	2443.4			***x***		***x***	***x***
41	2438.6		***x***	***x***	***x***		
42	2458.7					x	x
43	2466.9					x	x
44	2485.8			***x***		***x***	***x***
45	2487.7		*x*		*x*	*x*	
46	2501.2		x				
47	2515.7			L			
48	2521.1	**x**	**x**	**x**	**x**	**x**	**x**
49	2528.3			L			
50	2531.0					x	x
51	2546.4		***x***	***x***	***x***		
52	2550.0			L			
53	2558.1		***x***	***x***	***x***	***x***	***x***
54	2573.5			L			
55	2582.5			***x***		***x***	***x***
56	2587.0	**x**				**x**	**x**
57	2606.0			L			
58	2614.2					x	x
59	2624.1			***x***		***x***	***x***
60	2638.6					x	x
61	2645.8					x	x
62	2649.4		***x***	***x***	***x***	***x***	***x***
63	2660.2					x	x
64	2665.7					x	x
65	2681.9		***x***	***x***	***x***	***x***	***x***
66	2696.4		x		x		
67	2709.0	**x**	**x**	**x**	**x**		
68	2727.1		*x*		*x*	*x*	*x*
69	2733.4		***x***	***x***	***x***		
70	2747.0		***x***	***x***	***x***	***x***	***x***
71	2761.4		***x***	***x***	***x***	***x***	***x***
72	2770.5			L			
73	2792.2		***x***	***x***	***x***		
74	2799.4			***x***		***x***	***x***
75	2817.5					x	x
76	2839.2	**x**		**x**			
77	2858.1			***x***		***x***	***x***
78	2860.8					x	x
79	2871.7						x
80	2877.1		***x***	***x***	***x***		
81	2889.8					x	x
82	2900.6					x	x
83	2919.6					x	x
84	2929.5	**x**	**x**	**x**	**x**		
85	2933.1					x	x

86	2940.4			L			
87	2954.8					x	x
88	2886.1		***x***	***x***	***x***		
89	2989.2		*x*		*x*	*x*	*x*
90	3027.1			L			
91	3034.3			L			
92	3047.0					x	x
93	3060.5					x	x
94	3072.3					x	x
95	3077.7					x	x
96	3091.3					x	x
97	3113.9					x	x
98	3121.1	**x**	**x**				
99	3147.3			L			
100	3157.2					x	x
101	3180.7	C					
102	3206.0					x	x
103	3240.4	C					
104	3296.4	C					
105	3328.9	C					
106	3362.3		***x***	***x***		***x***	***x***
107	3421.1			L			
108	3431.9					x	x
109	3463.6	C					
110	3583.7	C					
111	3668.7						x

Table shows uninfested cowpea seed-based peaks (**x**), bruchid larval-based peaks (***x***), adult female bruchid-based peaks (*x*), uninfested cowpea seed-specific peaks (C), bruchid larval-specific peaks (L), and adult female bruchid-specific peaks (F).

Figure 1 Electroantennogram (EAG) responses of mated and unmated female *P. cerealellae* to various host-related stimuli treatments. Figure shows mean (-mV ± SE) absolute EAG response of mated (white bars) and unmated (grey bars) female parasitoids to extracts of uninfested cowpea seeds, bruchid (*C. maculatus*)-infested cowpea seeds, and larval frass, and whole body extracts of bruchid larvae, adult female bruchid, and adult male bruchid, as well as the solvent (hexane) control. For each mating group (mated or unmated), means followed by similar letters are not significantly different ($P < 0.05$, Tukey's HSD test). White or grey bars followed asterisks (*) indicate significant difference between responses of mated and unmated parasitoids to the tested stimuli ($P < 0.05$, Students' *t* - test).

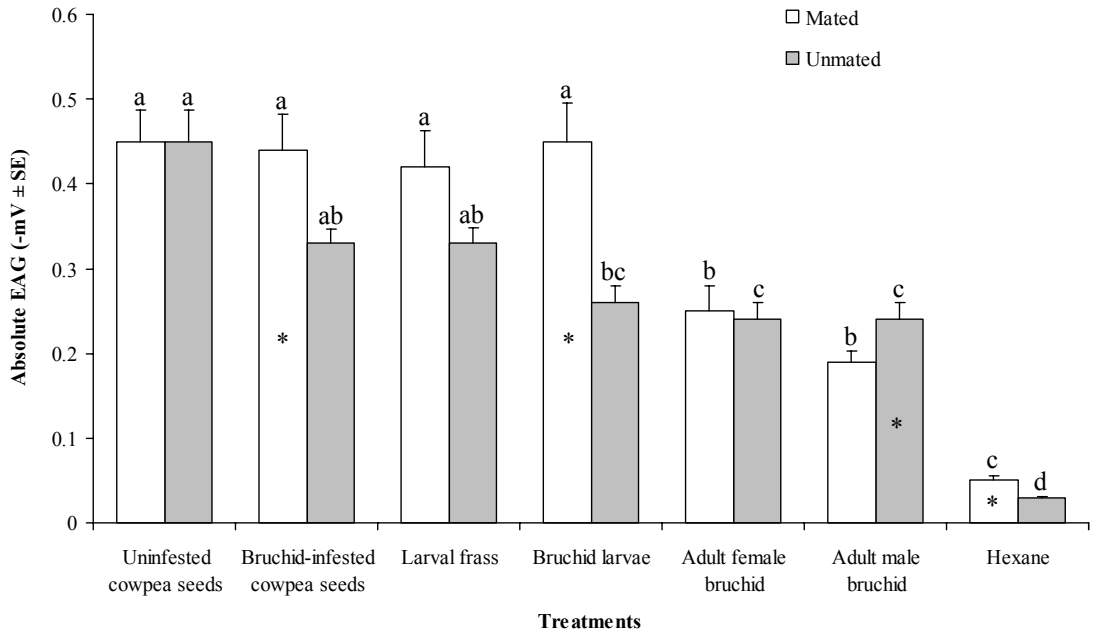

Figure 2 Responses of mated female *P. cerealellae* to various host-related stimuli treatments in a Y-tube olfactometer. Figure shows responses of mated female parasitoids when given a choice between hexane (control) and different host-related stimuli: extracts of uninfested cowpea seeds, bruchid (*C. maculatus*)-infested cowpea seeds, larval frass, bruchid larvae, adult female bruchid, and adult male bruchid. Grey bars indicate the percentage responses to the control; white bars indicate the percentage responses to the tested stimuli. N = 40 individuals per choice test. Asterisks (*) indicate significant differences within a choice test ($P < 0.05$, χ^2).

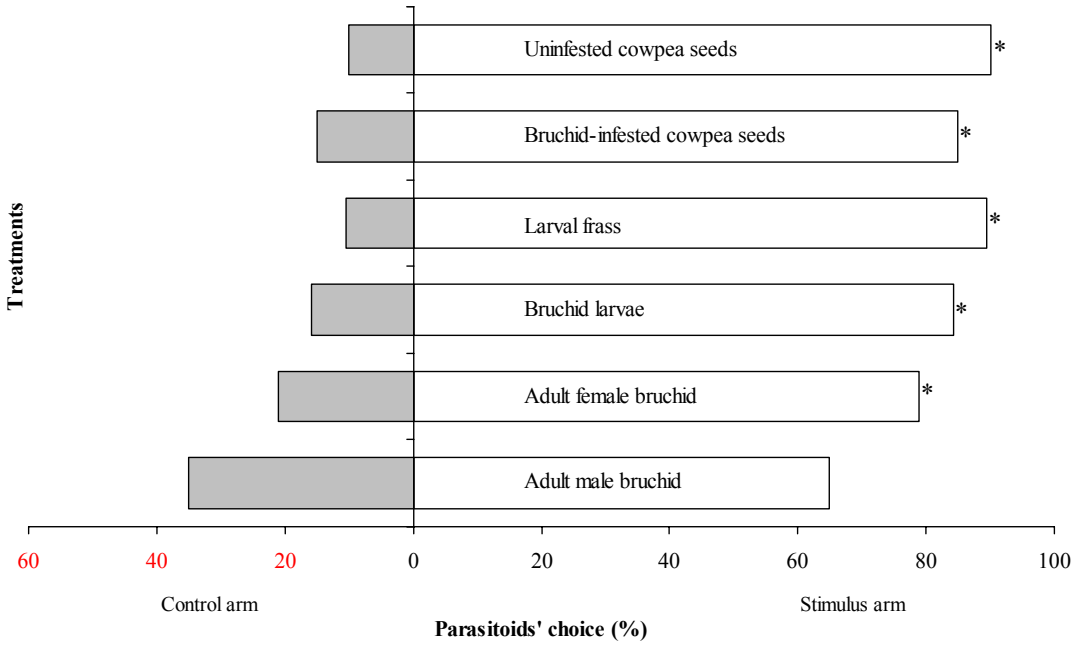

Figure 3 Gas chromatographs of the various extracts tested in electroantennogram and behavioral tests: uninfested cowpea seeds, bruchid (*C. maculatus*)-infested cowpea seeds, larval frass, bruchid larvae, adult female bruchid, and adult male bruchid. Numbers on the peaks represent the various recognizable peaks (compounds) as analyzed by the GC. Peaks from different extracts having the same number were identified as being the same based on retention times (RT).