The role of sex pheromones in a sexually cannibalistic praying mantid, *Pseudomantis albofimbriata*: identification and behavioural responses

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Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

July 2019

Dedication

To the departed soul of my beloved father

Table of contents

Abstract

Praying mantids are a group of charismatic predatory insects that use visual cues for mate location at short-distances, whereas long-distance communication mainly relies on chemical cues, i.e., air-borne pheromones. Pheromones have been assumed to be important components of the mantid mating system. This thesis examines the pheromone biology in the false garden mantid, *Pseudomantis albofimbriata* Stål which is a sit-and-wait predator, sexually cannibalistic and size dimorphic, mainly found in eastern Australia. Various aspects of mating system and reproductive biology have previously been investigated in *P. albofimbriata*, however direct evidence of female-emitted pheromones and their chemical identification is lacking. Before starting the chemical identification, I investigated the female calling behaviour and male responses. This behavioural study described female calling behaviour in this species for the first time and provided evidence that female calling behaviour and pheromone emission are intimately linked. Furthermore, the observations indicated the age and time window in which volatile pheromone collection would be most successful for *P. albofimbriata.* In addition, I made an attempt to identify the pheromone emission site in females and proposed that sex pheromone glands could be present on the intertergal membrane between the abdominal segments 3/4, 4/5, 5/6 and 6/7 on the female, however glandular tissue has not been found. The distribution and abundance of olfactory sensilla in males using scanning electron microscopy (SEM), which responsible for volatile chemical signal detection has also described. Then, the collection of volatiles from false garden mantids was conducted using three different methods - air trapping, solid-phase microextraction (SPME) and solvent extraction - to identify the suitable volatile collection method. The samples collected analysed through the gas chromatography linked to mass spectrometry (GC-MS). The results indicated that solvent extraction and air trapping methods identified a series of hydrocarbons, mainly alkanes, in praying mantid volatiles that is present in both male and female samples, suggesting these

methods were unable to identify female-specific compounds that might be candidates for pheromones. However, the SPME method successfully identified three acidic compounds such as tetradecanoic acid (C_{14}), pentadecanoic acid (C_{15}), *n*-hexadecanoic acid (C_{16}), that were female-specific. Then, I repeated the SPME method with slight modifications to identify the pheromone from female and similar results obtained indicate these compounds probable pheromone candidates in *P. albofimbriata*. I subsequently conducted behavioural assay to test male responses to these chemicals. Males were not significantly attracted to the synthetic samples of C_{14}, C_{15} and C_{16} acid compounds, demonstrating these compounds were unlikely to be sex pheromone components for this species, requiring further work on identifying additional chemicals in the female profiles. On the whole, this thesis makes a contribution to understanding the chemical ecology in praying mantid mating systems.

Declaration

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Md Mahmudunnabi July 2019

Acknowledgements

All praises to Allah (Almighty God), the supreme ruler of the universe allowed me to accomplish the work required for the fulfilment of the degree.

Most of all, I would like to express my heartfelt thanks to my supervisors Professor Marie E. Herberstein and Dr Katherine L. Barry for their exemplary support, valuable guidance, adequate motivation and outstanding positive encouragement throughout my candidature.

In addition to Mariella and Kate, the entire behavioural ecology lab group provided a very supportive and healthy environment for research. Thank you to Anu Jayaweera, Giselle Muschett, Patricio Lagos, Michael Strack and Nicole O'Donnell who welcome me to the lab and for sharing their office. I would also like to thank Mohammad Ameri, Kawsar Khan, and Darshana Rathnayake for their great company throughout my PhD journey. Thank you to all new members of the lab Georgina Bin, Alfonso Aceves and Michael Kelly for their presence makes the lab more vivid and congenial to me. My thanks are extended to senior members of lab group -Dr Matthew Bulbert, Dr Lizzy Lowe, Dr Jonas Wolf and Dr Chris Reid- for sharing their experience as a researcher to enhance my interactive knowledge and skills. Sincere appreciation goes to Jim McClean for his valuable time for proofreading some of my thesis chapter. Thank you to all volunteers for supporting my research- Barbara Dean, Alexia Dalley and Zoe Wild.

My special thanks go to Dr Soo Jean Park from the Department of Biomolecular Sciences, Macquarie University for her patience in teaching me gas chromatography linked to mass spectrometry (GC-MS) instrument to run my research project smoothly. Thank you, Dr Ian Jamie, Donald Cameron and Ben Hanssen for being welcoming during my days spent at their lab.

I would like to express my gratitude to Professor Simon George from the Department of Earth and Planetary Sciences for his valuable suggestions during designing and conceiving the chemical analysis of volatiles.

A large number of people have given me technical assistance over the period of entire PhD candidature. Specifically, I must thank Sue Lindsay, Nadia Suarez-Bosche, Nicole Vella and Naila Evans at Microscopy unit, Macquarie University for their generous support in operating fluorescence- and scanning electron microscope. My sincere gratitude to all the members of admin staff from the Department of Biological Sciences, Higher Degree Research Office and Campus Wellbeing who provided support and sophisticated administration solutions.

My earnest appreciation to my home institute, Bangladesh Agricultural Research Institute (BARI), Ministry of Agriculture, People Republic of Bangladesh for granting me study leave to conduct my PhD. I would also like to express my gratefulness to the 'Bangladesh-Sweden Trust Fund' for providing funds for a one way (Dhaka, Bangladesh to Sydney, Australia) air travel fare. Thanks are extended to Macquarie University for granting me a Macquarie University Research Excellence Scholarship (MQRES) and funding my entire research.

Thank you so much to my mother, father (who passed away during my PhD), sisters, brother and other members of my family for their unconditional love, continuous support and encouragement throughout my candidature.

Last but not least, my wife Jakia Sultana deserves special mention for her loving support and companionship over the past few years. It was nearly impossible to accomplish this research project without her wholehearted assistance. My heartiest loves to my son Zabir - who always inspires me to stay focus by showing his never-ending liveliness and to my daughter Madeeha - who joined our lives during the critical time of my PhD candidature as a precious gift.

INTRODUCTION

Insect communication

Communication is the process of information exchange between organisms, where a message is transmitted from one individual (emitter) to another individual that receives and processes that message. Communication plays important roles for insects such as finding mates, foraging for food and oviposition sites, as well as escaping predation. In insects, communication may occur in various modalities such as touch (tactile), sound (acoustic), vision (light or colour perception) and through chemicals (olfaction). Tactile communication can be used for recognising nest mates, such as in mole crickets, and other insects living under low light conditions (Alexander, 1967). Acoustic communication occurs by producing sound to attract individuals of the same species for mate assessment or to respond to another species as predator deterrence in crickets, cicadas, grasshoppers and katydids (Sanborn, 2008). Insect visual communication involves the production of light or colour. For example, fireflies and beetles use different frequencies of light flashes for species specific mate location (Forrest and Eubanks, 1995, Lewis and Cratsley, 2008). The species specific colour patterns on butterfly function for visual communication during courtship (Chapman, 1998). While the above examples demonstrate that insects communicate across many modalities (tactile, acoustic, visual and chemical), chemical communication is probably the most comprehensive and widespread modality (Greenfield, 2002).

Chemical communication is observed in many insect species (Lewis, 1984, Hansson & Wicher, 2016), which often have in complex structures for the production and perception of chemicals (Greenfield, 2002). Chemical communication is associated with kin and predator recognition, alarm, defence, aggregation, attraction, trail following, host marking of oviposition site, orientation, recruitment, and reproduction (Cardé and Millar, 2009, Yew and

Chung, 2015, Snellings et al., 2018). Effective communication over long distances is facilitated by chemical signals but it may not be sufficient for ensuring successful mating. Some insect may still require further close range (visual and mechanical) cues to initiate (Maxwell et al., 2010a). Chemical communication can offer several advantages over alternative modalities including its specificity, the potential for transmission of chemical signals across substantial distance, the ability to regulate the emission of chemicals, and the ability to detect and distinguish between chemical compounds and blends of compounds with a high level of sensitivity (Greenfield, 2002). Disadvantages are that chemical signals cannot be sent rapidly and an emitter's control over the direction in which the chemicals travel is being influenced by airflow and other environmental factors. In addition, it may be challenging for a receiver to localize the source of a distant chemical signal (Greenfield, 2002).

The term semiochemicals (Greek *semeion*, a mark or signal) describes the chemical substances that mediate interactions between organisms (Law and Regnier, 1971). Semiochemicals are divided into two major groups, allelochemicals and pheromones, depending on whether the interactions are interspecific or intraspecific, respectively. The term allelochemical was first proposed by Whittaker (1970) as "a chemical that is significant to organisms of a species different from its source for reasons other than food as such." Allelochemicals can be further classified into allomones, kairomones, synomones and apneumones, based on the advantage of the interaction (Nordlund and Lewis, 1976, Ruther et al. 2002, Hansson & Wicher, 2016). The term pheromones (Greek *pherein*, to carry or transfer and *horman*, to excite or stimulate) was introduced and defined as "substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behaviour or developmental process" (Karlson and Lüscher, 1959). The pheromones are usually beneficial for both emitter and receiver. According to their mode of influence, pheromones are broadly categorised as primer

(produce a lengthy physiological change in the receiver) and releaser (stimulate some immediate behavioural responses to the receiver) pheromones (Karlson and Lüscher, 1959, Wyatt, 2014, Hansson & Wicher, 2016). Releaser pheromones are often named after their function as sex pheromones, aggregation, dispersal, alarm, and trail pheromones (Wyatt, 2014). The best known are sex pheromones, which are highly species-specific chemicals that are involved in mate-finding or attraction and in initiating courtship (Wyatt, 2014).

The first sex pheromone was identified as 'bombykol' from the silk worm moth, *Bombyx mori* in 1959 (Butendandt et al.,1959). Since then, sex pheromones have been identified in many other species (El-Sayed, 2019 for details http://www.pherobase.com**)**. Sex pheromones are produced by one sex [usually females (Leal, 2005) but there are exceptions in males (Soldi et al., 2012)] and attract the opposite sex. Utilization of sex pheromones as the major means of bringing individuals together for reproduction is observed in many insects (Ali and Morgan, 1990). Sex pheromones are most often species specific and released when they are formed at defined periods of the day or night (Ali and Morgan, 1990). Typically, there is a synchronization between the time of release from females and the response from conspecific males (Ali and Morgan, 1990). They are produced in specific areas on the body such as pheromone glands in one sex and detected by the opposite sex through specific sensory receptors often on the antennae (Sakurai et al., 2014).

Isolation and Identification processes of pheromones

There are several methodologies that have been designed for the collection of volatiles from insects (Baker et al., 1981, Tumlinson et al., 1982, Golub et al., 1983, Shani and Lacey, 1984, Golub and Weatherston, 1984). These include two main methods: solvent extraction (direct extraction of the body parts/glands for residue analysis) and effluvial collection (trapping of volatiles on specific filters through air entrainment or solid–phase microextraction).

Once a sample is collected, identification of insect pheromone involves four essential components: analytical chemical methods, bioassays, organic synthesis and formulation (Tumlinson, 1990). The main analytical method used for the detection and structural elucidation of insect pheromones is chromatographic separation combined with mass spectrometry (MS) (Yew and Chung, 2015). Gas chromatography (GC) is the most widely used method for detection, quantification, and structural characterization of volatile pheromones (Sparkman et al., 2011a, Yew and Chung, 2015). GC separates a mixture of volatile compounds into its components by passage in a stream of inert gas though a heated column containing a stationary adsorbent so that different compounds pass through the column at different rates (Sparkman et al., 2011b). Mass spectrometry (MS) is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules (Sparkman et al., 2011c). Ionisation can be achieved by bombarding the molecule with electrons (electron impact, EI) or electron transfer from ionised molecules of a reagent gas (chemical ionisation, CI). The fragmentation pattern is characteristic of the molecular structure and may be used to deduce the structure empirically or by computer matching with a library of spectra of standard compounds. GC and MS make an effective combination for chemical analysis (Fig. 1). GC-MS is a highly sensitive instrument capable of detecting a nano-gram or less of a compound both in electron impact (EI) and chemical ionisation (CI) modes (Sparkman et al., 2011a).

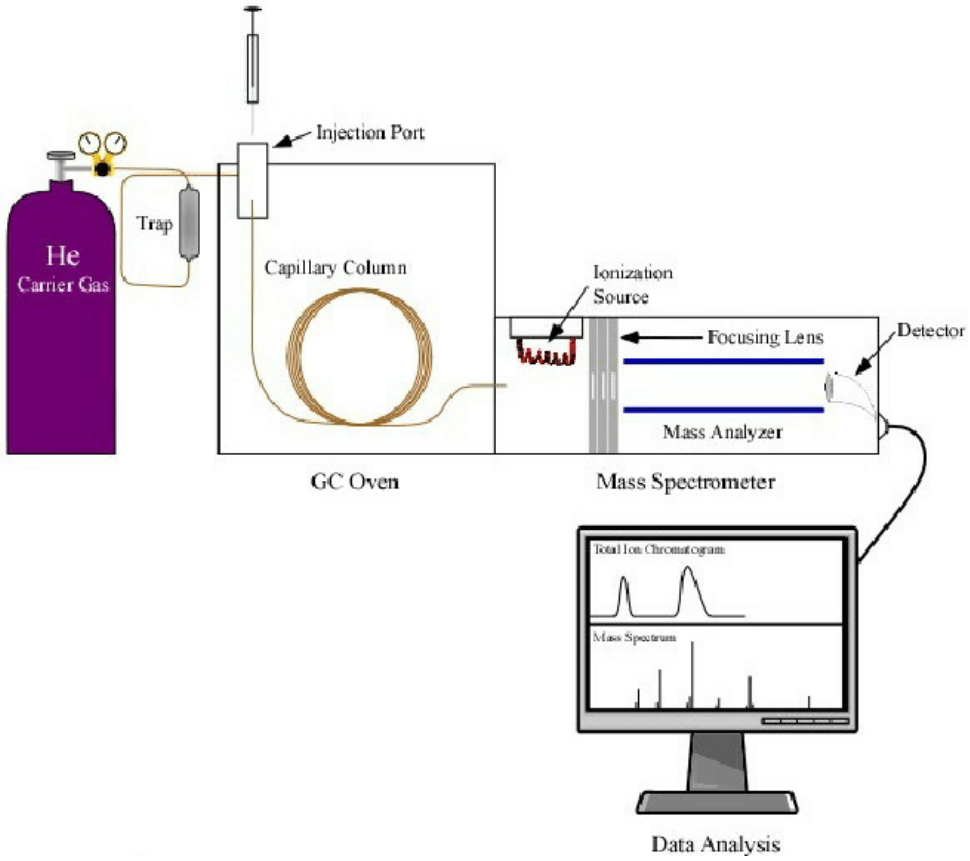

Figure 1. Schematic of a GC-MS system (Wu et al., 2012)

Bioassays are designed to assess the male or female response to the chemicals identified from the chemical analysis. These experiments are critical to differentiate between active components and inactive ones. The main types of bioassay used are behavioural bioassays or electrophysiological bioassays. Behavioural bioassays measure a change in behaviours in response to the test material in three experimental set ups: static cages/arenas, olfactometers or wind tunnels (Baker and Cardé, 1984). In static cages/arenas the insects are able to detect concentration gradients and respond accordingly. A typical example is the pitfall bioassay where two or more odour sources are placed in separate "pits" in the arena and the numbers of insects in the different pits are compared (Baker and Cardé, 1984). This type of experiment is normally used for walking insects. Various types of olfactometers have been used (Baker and Cardé, 1984), which usually involve sealed chambers connected to a pump that moves air through the system. The test insect is placed in the middle of the chamber and then has the

option of moving or flying to one or more of the test components in one of the inlets of the enclosed system. Wind tunnel bioassay (Baker and Linn, 1984) is one of the most common methods to test behavioural responses of flying insects to isolated compounds. Test insects are placed in the downward end and the test compound or stimuli, loaded into a dispenser, is placed at the upwind end of the tunnel. A fan either blows or sucks air through the system at a set rate. Test insects are then released in the tunnel and their behavioural responses to these stimuli can be directly noted by recording their upwind flight towards the source (Knudsen et al., 2018). This method has the advantage of permitting observations and measurements of the flight behaviour of the insect responses to odours and other sensory stimuli. Orientation to odour sources is a common reaction among many insects, which is often simply termed as 'attraction' a term that fails to reveal the complexity of this process and variety of underlying mechanisms (Cardé and Willis, 2008).

Electrophysiological assays that test fractions for biological activity and involves the use of the electrontennogram (EAG) were developed by Schneider (1957) and measure electrical depolarisation across the antenna of an insect. The depolarisation arises as a result of stimulation by the pheromone components passing over the antenna. This instrument is either used on its own or coupled with GC (Struble and Arn, 1984). EAG responses from male antennae to GC effluent was first described by Moorhouse et al. (1969). Components eluted from GC are separated into two fractions - one fraction goes into the GC detector, which is normally a flame ionization detector (FID) and the other is puffed over the antennae of the insect in the EAG. The responses of the antennal receptors are then measured and registered by using electrodes and amplifiers. Finally, the responses are correlated with the chromatogram obtained from the FID to indicate which compounds elicited a response by the antennae. Compounds that simulate the antennae, as reflected in the EAG recording, are matched to a

particular peak with same retention time on the GC. This peak is then identified by MS or other means and further analysed (Gouinguené et al., 2005).

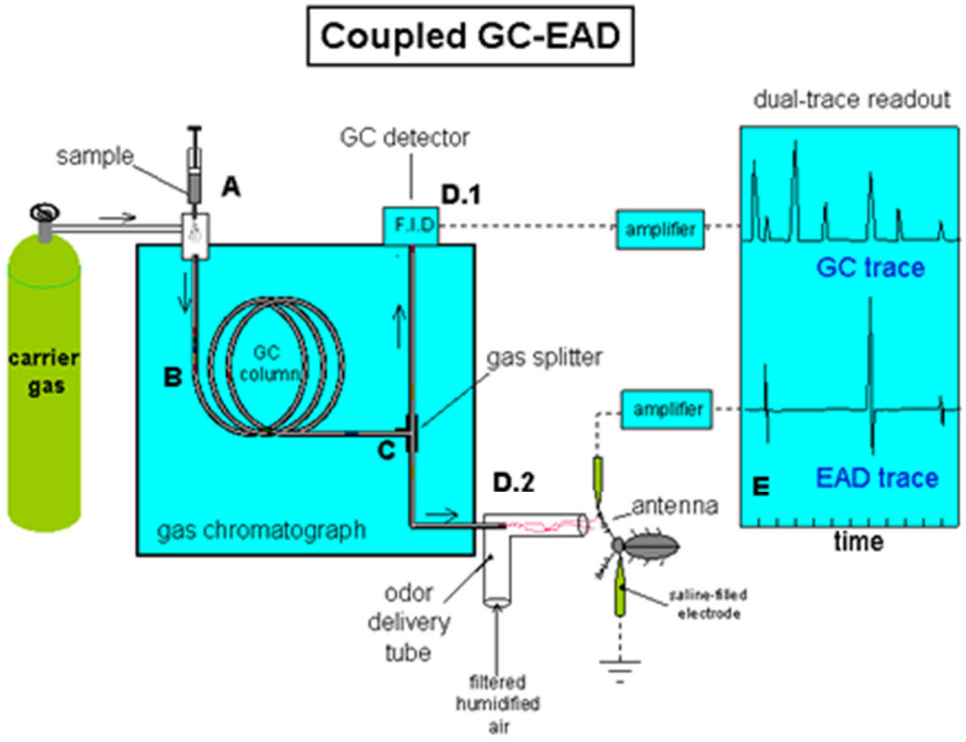

Figure 2. Schematic of a coupled gas chromatograph- electroantennographic detector (GC-EAD) instrumentation (https://www.srs.fs.usda.gov/idip/tools/gc-ead/)

Finally, an organic synthesis step is required in order to use the pheromone on a practical basis and synthesise the compound to a high degree of purity. It involves the construction of specific molecules in exactly the same configuration as the natural material (Tumlinson, 1990). The final stages of the identification involve the formulation of the blends at a slow release rate of pheromone over time. According to Tumlinson (1990), this final step is very challenging. It has been achieved by loading pheromone into rubber septa, plastic matrices or through the use of semi-permeable membrane through which the pheromone passes at a desired rate (Kuenen and Siegel, 2015).

Introduction to the study insect

Praying mantids are a group of charismatic predatory insects, comprised of over 2400 species in about 434 genera in 15 families (Otte, et al. 2019 for details http://Mantodea.SpeciesFile.org). They are well known for their sexually cannibalistic behaviour in which females kill and consume their mates before, during, or immediately after copulation (Roeder, 1935, Edmunds, 1988, Birkhead et al., 1988, Maxwell, 1999, Lelito and Brown, 2006, Lelito and Brown, 2008, Barry et al., 2008, Maxwell et al., 2010b, Brown et al., 2012, Jayaweera et al., 2015, Brown and Barry, 2016). They use both chemical and visual cues in their communication system. Chemical cues are used for long-distance communication and visual cues at a shorter range locate mates for copulation (Maxwell, 1999, Maxwell et al., 2010a, Barry et al., 2015). The use of air-borne sex pheromones for mate attraction has been documented in many mantid species including *Acanthops falcata* (Robinson and Robinson, 1979), *Sphodromantis lineola* (Hurd et al. 2004), *Hierodula patellifera* (Perez 2005), *Mantis religiosa*, *Empusa pennata* (Gemeno et al. 2005), *Stagmomantis limbata* (Maxwell et al., 2010a, Maxwell et al., 2010b), *Tenodera aridifolia sinensis* (Lelito and Brown, 2008, Maxwell et al., 2010a), *Hierodula majuscula* (Allen et al. 2012) and *Pseudomantis albofimbriata* (Holwell et al., 2007, Barry et al., 2010, Barry et al., 2011, Barry, 2013, Barry, 2015). All of these studies used indirect evidence such as observations of male arrival behaviour at the receptive females to infer the presence of pheromones. Nonetheless, some of these studies examined different aspects of mantid pheromone biology such as female calling behaviour in *Acanthops falcata* (Robinson and Robinson, 1979), *Hierodula patellifera* (Perez 2005), *Mantis religiosa*, *Empusa pennata* (Gemeno et al. 2005) and *Hierodula majuscula* (Allen et al. 2012) or antennal morphology in *Tenodera aridifolia sinensis* (Carle et al., 2014), *Tenodera angustipennis* (Slifer, 1968), *Sphodromantis lineola* (Hurd et al. 2004) and *Pseudomantis albofimbriata* (Holwell et al., 2007, Jayaweera and Barry, 2017). Despite this wealth of indirect

evidence, the isolation and identification of a putative sex pheromone from receptive females by chemical analysis has only been achieved in *Sphodromantis lineola* and the chemicals likely to function as pheromones are aldehydes, with the main chemical composition being a 1:3 ratio of tetradecanal to pentadecanal (Hurd et al., 2004).

This thesis aims to investigate aspects of pheromone biology in false garden mantid, *Pseudomantis albofimbriata* Stål, 1860. This is a common mantid species with a wide range of habitats, found amongst the foliage of trees, shrubs, and grasses throughout eastern Australia (Holwell et al., 2007). It is a sit-and-wait predator, sexually cannibalistic, sedentary in nature (Barry et al. 2008) and size dimorphic: females are larger than males and are unable to fly (Allen et al., 2014). The females move slowly due to their large abdomen and they continue to gain weight throughout their life. Males are smaller in body length (but weigh less) and they possess wings that are long enough to cover the whole abdomen, giving them the ability to fly (Allen et al., 2014). With only one generation per year, adults can only be found in the summer months from December to March. The species comes in two colour morphs- green and brown present in both male and female (Fig. 3).

Figure 3. Australian false garden mantids, *P. albofimbrita* A. Female & B. Male (Photo by Kate Barry)

Chapter outline

In the first thesis chapter, I describe the female calling behaviour and male responses in the false garden mantid *Pseudomantis albofimbriata*. This study provides evidence that female calling behaviour, pheromone emission and male responses are intimately linked. I identify the age and time window in which volatile pheromone collection is most successful for *P. albofimbriata.* This chapter is published in the **Journal of Natural History** and co-authored by Katherine L. Barry, who provided help with the experimental design, with conducting the experiment and providing feedback and suggestions during manuscript preparation. Marie E. Herberstein provided helpful feedback and suggestions on the manuscript.

In the second thesis chapter, I investigate the site of the pheromone emission in female abdomen and examined male antennae with scanning electron microscope to confirm the presence of olfactory receptors (sensilla) that are responsible for volatile chemical signals with their distribution pattern along the antennae. This chapter proposes that sex pheromone glands could be present on the intertergal membrane between abdominal segments 3/4, 4/5, 5/6 and 6/7, based on the presence of small pores. I also quantify the olfactory receptors (sensilla) on the male antennae, which are responsible for volatile chemical signal detection. Marie E. Herberstein helped with designing and conducting the study and also provided helpful comments and suggestions on the manuscript. Katherine L. Barry provided helpful feedback and suggestions on the manuscript.

In the third chapter, I develop a standard methodology for collecting and extracting volatiles from the false garden mantid to identify the pheromones. I evaluate collection of volatiles from false garden mantid using three different methods: air trapping, solid-phase microextraction (SPME) and solvent extraction. The collected samples are analysed through the gas chromatography linked to mass spectrometry (GC-MS). I show that the SPME

extraction is the most appropriate method for the collection and identification of insect volatiles in praying mantids. This chapter is co-authored by Soo Jean Park and Katherine L. Barry, who provided helpful suggestions during the design and conduct of the study and also provided helpful comments and suggestions on the manuscript. Marie E. Herberstein provided helpful suggestions on the manuscript preparation and continued guidance and support throughout the experiment.

My fourth chapter focuses on the isolation, identification and bioassay of possible component of sex pheromone based on the methods developed in chapter 3. To identify the female sex pheromones of *P. albofimbriata*, I collect volatiles by solid phase micro extraction (SPME) and analyse the samples by gas chromatography coupled with mass spectrometry (GC/MS). Several compounds are identified as potential pheromones and bioassay are carried out in the laboratory. Soo Jean Park and Katherine L. Barry provided helpful support in designing and conducting the experiment and also provided useful feedback and suggestions on the manuscript. Marie E. Herberstein provided helpful comments on manuscript preparation and continued guidance and support throughout the experiment.

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Chapter 1

Mantid sex pheromones: female calling behaviour and male responses in the Australian false garden mantid, *Pseudomantis albofimbriata* (Mantodea : Mantidae)

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Published in the Journal of Natural History

Mahmudunnabi, Md., & Barry, K. L. (2019). Mantid sex pheromones: female calling behaviour and male responses in the Australian false garden mantid, Pseudomantis albofimbriata (Dictyoptera: Mantidae). *Journal of Natural History*, 53(3-4), p. 171-183.

DOI: 10.1080/00222933.2019.1567857

Chapter 2

Morphological studies of praying mantids: potential sex pheromone emission sites in females and olfactory sensilla in males

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Abstract

The external morphology of the female abdomen and male antennae of Australian false garden mantids (*Pseudomantis albofimbriata*) was studied using stereomicroscopy and scanning electron microscopy to locate potential sites of pheromone emission in the female and to quantify the olfactory sensilla presence on the male antennae. We propose that sex pheromone glands could be present on the intertergal membrane between abdominal segments 3/4, 4/5, 5/6 and 6/7, based on the presence of small pores, however glandular tissue has not been found. The distribution pattern of three olfactory sensilla—basiconic, trichoid and grooved peg—varied along the longitudinal axis of the male antennae. Grooved peg sensilla were the most abundant type of sensilla, found on almost every flagellomere. Basiconic sensilla were found on the proximal and medial part of the antennae. Trichoid sensilla were further classified into 3 subtypes depending on their length and/or location, as long (type 1), medium (type 2) and short (type 3). Long trichoid sensilla were mainly found on the distal part of each flagellomere and the medial part of some flagellomere, while medium and short trichoid sensilla were found on every part of antennal articles. Different sizes of trichoid sensilla in mantids may suggest different functions in olfactory processing in praying mantids.

Keywords: Sex pheromone glands, intertergal membrane, basiconic sensilla, trichoid sensilla and grooved peg sensilla

Introduction

Many insects utilize sex pheromones as the main means of bringing males and females together (Ali and Morgan, 1990). Sex pheromones are species-specific, produced during defined temporal periods and released as they are formed, meaning they are not stored (Ali and Morgan, 1990). Sex pheromones are produced by glandular modifications of epidermal cells in the

integument of insects (Percy-Cunningham and Macdonald, 1987). The site of sex pheromone production varies widely between species. For example, pheromone glands are located on the front leg in the male red flour beetle, *Tribolium castaneum* (Faustini et al., 1981), the antennae of the male ant-loving beetle, *Palearctic Batrisinae* (De Marzo and Vit 1983) and the thorax in female wattle bagworm, *Kotochalia junodi* (Bosman et al., 1971) and the female bagworm moth, *Thyridopteryx ephemeraeformis* (Leonardt et al., 1983). However, pheromone glands are most commonly localized to the abdominal region in females (Schal et al., 1992, Liang and Schal, 1993, Ma and Roelofs, 2002).

Sex pheromones are received by olfactory receptors in insects. Olfactory receptors are most commonly found on the antennae (Dahanukar et al., 2005, Wilson, 2013, Li and Liberles, 2015), but may also be present on mouthparts (Dahanukar et al., 2005, Li and Liberles, 2015) or ovipositors (Yadav and Borges, 2017). In insects, the antenna consists of articles called antennomeres, and antennomere organisation is similar across all insect species, with a scape, pedicel and flagellum (Hansson, 1999). The flagellum is composed of articles known as flagellomeres, whose number and shape varies among species. Antennae bear numerous sensory organs (microstructures) called sensilla, and these microstructures vary in size and shape depending on their function (Hansson, 1999). Sensilla contain one or more bipolar olfactory receptor neurons. These sensory neurons transduce the chemical signal into an electrical response and bring the olfactory information from the periphery to the antennal lobes, which are the first relay station in the brain (Hildebrand, 1996, Mustaparta, 1996, Hansson, 1999). As with other insects, praying mantid antennae consist of antennomers organised with a scape, pedicel and flagellum (Carle et al., 2014, Jayaweera and Barry, 2017). Praying mantid antennae possess six different types of sensilla: chaetic (mechanoreception and contact chemoreception), campaniform (mechanoreception), coelocapitular (hygro- and thermoreception), basiconic, trichoid, and grooved peg sensilla (olfaction) (Carle et al., 2014,

Jayaweera and Barry, 2017). Although the sensilla arrangement and morphology has been investigated previously in praying mantid antennae, in this chapter, we focus on the distribution of olfactory sensilla, specifically the basiconic, trichoid and grooved peg sensilla, which play a role in sex pheromone detection.

In the present study, we used the false garden mantid, *Pseudomantis albofimbriata*, to locate the sex pheromone emission sites or glands in females and to investigate antennal morphology, especially the distribution of antennal sensilla responsible for chemoreception or olfaction in males. Previous studies on false garden mantids provide indirect evidence that females emit pheromones to attract males (Holwell et al., 2007, Barry et al., 2008, Barry et al., 2009, Barry, 2010, Barry et al., 2010, Barry and Kokko, 2010, Barry and Wilder, 2013, Barry, 2015, Jayaweera et al., 2015, Jayaweera and Barry, 2017). Our recent study confirmed that females of *P. albofimbriata* adopt a characteristic pheromone calling posture including the rhythmic pumping movement of the abdomen (Mahmudunnabi and Barry, 2019). This behaviour has been described for several mantid species, including *Acanthops falcata* (Robinson and Robinson 1979) and *Hierodula patellifera* (Perez 2005), as well as in several species of cockroach (Schal and Bell 1985; Liang and Schal 1993; Gemeno et al. 2003). As the abdominal pumping movement in *P. albofimbriata* exposes the intersegmental membranes, it is likely that these membranes are the site of pheromone production and release. However, the site of pheromone production in mantids has not been properly identified. Edmunds (1975) anticipated that sex pheromone glands may be present in the dorsal abdomen of *Trachodes afzelli* based on visual observations. Very similar observations were made for *A. falcata* (Robinson and Robinson 1979) and *H. patellifera* (Perez, 2005). No microscopic study to date has located the sex pheromone glands of praying mantids. Herein, we examined the potential site of pheromone emission in *P. albofimbriata*. In addition, we examined male antennae with

a scanning electron microscope to confirm and quantify the presence of olfactory receptors (sensilla), which are responsible for volatile chemical signal detection.

Methods

Insects

Pseudomantis albofimbriata juveniles were collected from Yamble Reserve, Quarry Road, Ryde NSW 2112 (33.8010° S, 151.1081° E), Australia during December 2016 to January 2017. The juveniles were raised to adulthood by methods previously described (Barry et al., 2008, Barry, 2010). Adult male and female mantids were housed separately in 425 ml inverted transparent cups with mesh for ventilation. Adult mantids were provided a diet of two small crickets, *Acheta domestica* (average body mass = 0.062 ± 0.003g, N = 20) three times a week (Barry et al. 2008; Barry 2010; Jayaweera et al. 2015) and were maintained under laboratory conditions at 14 L:10 D, temperature 25 ± 1°C and humidity 55 ± 5%.

Specimen preparation for microscopy

The animals were killed by freezing for 5 min (-18°C) then the legs, wings, thorax and head were separated from the abdomen using stainless steel scissors. The adult female abdomen was used for the study of abdominal glands, and adult male mantid antennae were detached from the point of attachment to the head capsule for antennal sensilla study.

Stereomicroscopy

Before using the scanning electron microscope, the female abdomen was observed using an Olympus SZX16 stereomicroscope equipped with CellSens imaging software. The abdomen was placed in a Petri dish for viewing the surface contours of the specimen.

Scanning electron microscopy (SEM)

For identifying abdominal glands, adult mantid abdomens were placed into a 10% solution of NaOH to dissolve tissue, then heated at 95°C in a water bath for 30 min to clear them (Sasso

Porto et al., 2016). The abdomens were next fixed overnight in 3% glutaraldehyde in 0.1 M phosphate buffer. After fixation, all specimens were washed three times with 0.1 M phosphate buffer for 20 min and again kept in 0.1 M phosphate buffer overnight in the fridge. The specimens were dehydrated through a series of ethanol baths (from 50% to 100% (v/v)) for 20 min in each and finally placed in 100% ethanol for 20 min twice over. Next, the samples were critical point dried with the Emitech K850 critical point drier. After drying, the specimens were mounted on 10 mm stub using carbon-adhesive tabs and sputter-coated with 20 nm thick gold using the Emitech K550 for imaging. Finally, images were taken with a JeolJSM 6480LA analytical scanning microscope.

For imaging antennal sensilla, antennae were fixed overnight in 3% glutaraldehyde in 0.1 M phosphate buffer. After fixation, all specimens were washed with 0.1 M phosphate buffer for 20 min three times and again kept in 0.1 M phosphate buffer overnight in the fridge. The specimens were dehydrated through a series of ethanol baths (from 50% to 100% (v/v)) for 20 min in each and finally placed in 100% ethanol for 20 min twice over. Then, samples were washed with 1:1 100% ethanol and hexamethyldisilazane (HMDS) for 20 min and finally washed with 100% HMDS for 20 min thrice over. After washing, the antennae were allowed to air dry overnight in a fume hood (Jayaweera and Barry, 2017). Following the drying, the antennae were mounted on a 10 mm stub using carbon-adhesive tabs and sputter-coated with 20 nm thick gold using the Emitech K550 for imaging. Finally, images were taken with a JEOLJSM 6480LA analytical scanning electron microscope. Additional images were taken with a PHENOM XL benchtop scanning electron microscope.

Sensilla identification and distribution of antennal flagellum

Previously, six types of morphologically distinct sensilla: chaetic sensilla, coelocapitula sensilla, campaniform sensilla, basiconic sensilla, trichoid sensilla, and grooved pegs have been found on the antennae of male *Pseudomantis albofimbriata* (Jayaweera and Barry, 2017) and

Tenodera aridifolia (Carle et al., 2014). Of these, three types of sensilla (basiconic sensilla, trichoid sensilla and grooved pegs) have been associated with olfaction (Holwell et al., 2007, Carle et al., 2014, Jayaweera and Barry, 2017), including in *P. albofimbriata*. In order to standardize the nomenclature of sensilla with that of other insect species, identification and classification of the sensilla types and terminology used in this work is based on the methodology described by Hansson (1999). Except for grooved peg sensilla which were previously termed as 'grooved basiconic sensilla' in *P. albofimbriata* (Holwell et al., 2007). However, following recent studies on antennal sensilla of *Tenodera aridifolia* (Carle et al., 2014) and *P. albofimbriata* (Jayaweera and Barry, 2017) with reference to Hansson (1999) we used the term 'grooved peg sensilla' in the current study.

Mantid antennae offered various senillar distribution along the antennal longitudinal axis and have many flagellomeres. To entitle the specific antennal regions, we divided the flagellum into 3 distinct parts from the base to the tip of the antenna. This division allows us to pinpoint the differences of the sensilla precisely on the entire antennae. We refer to the parts as proximal (flagellomeres #1 to # 30), medial (flagellomeres #31 to # 60), and distal (flagellomeres #61 to the tip), respectively.

Data analysis

Sensilla numbers for each flagellomere were counted directly from the computer screen. The length and diameter of sensilla were measured from digital images using ImageJ software (Schindelin et al., 2012). Six male antennae were investigated and the length of sensilla (6 of each type from each part of antenna) was measured to calculate averages per antennal article. The distribution and abundance of different types of sensilla on male antennae was analysed using Microsoft Excel software 2016 for Windows 10. All reported values are mean ± SD unless otherwise stated.

Results

Female abdominal morphology

Generally, female praying mantid abdomens consisted of 11 segments plus a non-segmental apical telson (Fig.1A). The segments preceding the genitalia are known as pregenital segments. Mantids bore an undivided ventral sclerotized plate, sternite and a large undivided dorsal plate, the tergites - in segment from 1 to 6. On the dorsal and ventral sides, the posterior part of a segment formed a transverse fold that covered part of the following segment: this is often known as an 'intersegmental membrane' or by the more recently introduced term: 'intertergal membrane' (dorsally) (Brannoch et al., 2017). Segments 7-9 were morphologically modified for genital function and constitute the genital segments. Segment 7 followed their preceding segments. Tergite 8 and tergite 9 were both short and vaguely separated from the dorsal main parts. For the post genital segments, segment 10 was a transverse plate resembling the preceding segments but its median part was strongly expanded to the posterior. There was a pair of segmented cerci at the end of segment 11 (Fig. 1B). The cerci consisted of a series of sclerotized sections, known as cercomere, and were covered with long and short trichoid sensilla (Fig. 1C).

Pheromone emission site

Scanning electron micrographs of female abdomen showed pores on the intertergal membranes between abdominal segments 3/4, 4/5, 5/6 and 6/7 (Fig. 2). The dorsal view of the intertergal membrane showed a well-defined area containing a number of pores that may be the pheromone gland openings. Similar types of pores have been found in many cockroaches but in the tergal region (Schal et al., 1992, Liang and Schal, 1993, Sreng, 2006). In contrast, no pores were observed in intertergal membranes of male abdominal segments (Fig. 3).

Male antennal morphology

Antennae of the male false garden mantid, *Pseudomantis albofimbriata*, consisted of a scape, pedicel and flagellum (filiform in shape) which was divided into numerous flagellomeres (98.17 ± 2.61; n = 6). Mean antennal length was 27.73 ± 0.83 mm (n = 6) and the first flagellomere was longer (425 µm) than other flagellomeres, while their length (120 to 375 µm) varied from base to tip of the antenna. Basal flagellomeres were wider (175 µm) than other flagellomeres but were progressively thinner towards the tip (45 µm) of the antenna.

Sensilla distribution on the antenna

We found that the distribution pattern of the three olfactory sensillae (basiconic sensilla, trichoid sensilla and grooved pegs) varied along the longitudinal axis of the mantid antennae. The numerical distributions of olfactory sensilla across the parts of the flagellum are presented in Fig. 4 (Supplementary data provided as appendices in Tables 1–3) and described as follows:

Proximal part (flagellomeres #1 to # 30): All three types of olfactory sensilla were found on the proximal part of the antenna (Fig. 5). Grooved peg sensilla and sensilla trichodea first appear on the 6th flagellomere and 8th flagellomere, respectively. Sensilla basiconica first appear on 15th flagellomere and were present on the remaining flagellomeres.

Medial part (flagellomeres #31 to # 60): As with the proximal part, every type of olfactory sensilla was found on this part of the antenna (Fig. 6). However, the sensilla on the medial part were morphologically stout and more easily visible than on the proximal part.

Distal part (flagellomeres #61 to the tip): Only grooved peg sensilla and sensilla trichodea were found on this part of the antennae (Fig. 7). The length of sensilla of all types increase towards the tip of the antenna (for example, grooved peg sensilla are 5.61 ± 0.59 µm, 10.93 ± 0.95 µm and 13.83 ± 0.94 µm in length for proximal, medial and distal parts, respectively).

The grooved peg sensilla

The grooved peg sensilla were the most abundant type of sensilla. This type of sensilla was short and their length did not vary systematically along the antennae, ranging from 4.9 to 15.1 µm (average length 10.12 ± 3.51 µm, n = 18). The grooved peg sensillae were usually located in shallow depressions on the flagomeres (Fig. 5 B, 6 B & 7 B, C).

The basiconic sensilla

Basiconic sensilla occurred on the proximal and medial parts of the antenna. This type of sensilla was very similar to the grooved peg sensilla except for a slightly extended tip while they were also positioned in a depression (Fig. 5 C & 6 C). They were rod-shaped, hair-like structures and ranged from 14.8 to 29.8 µm in length (average length 24.27 ± 1.70 µm, n = 18).

The trichoid sensilla

The trichoid sensilla were more abundant on the distal than the basal part of the antenna. This type of sensillum was hair-like. They varied greatly in length (9.1 to 49.3 µm), with their length increasing towards the tip of the antenna. We subclassified this type of sensillum into three subtypes depending on their length and/or location. A similar subclassification has been applied to the moth *Helicoverpa armigera* (Diongue et al., 2013), the cockroach *Peripalaneta americana* (Watanabe et al., 2012) and the mantid species, *Tenodera aridifolia* (Carle et al., 2014). Based on length and position, the sensilla were classified as long (type 1, average length 47.57 ± 1.70 µm, n=12), medium (type 2, average length 34.45 ± 4.89 µm, n=18) and short (type 3, average length 16.21 ± 4.38 µm, n=18). Long trichoid sensilla were mainly found on the distal part of each flagellomere and the medial part of some flagellomere, while medium and short trichoid sensilla were found on all antennal segment parts (Fig. 5 D, E; 6 D-F & 7 D-F).

Discussion

In this study we aimed to locate potential pheromone emission sites of female false garden mantids based on the external morphology of the abdomen. We identified 1-2 pores per 10 µm on the abdominal intertergal membranes of segments 3/4, 4/5, 5/6 and 6/7. The abdominal morphology of *Pseudomantis* is quite similar to that of *Sphodromantis* (Brannoch et al., 2017). As we have seen in our previous studies, females perform an abdominal pumping movement that exposes the intertergal membranes during calling behaviour, which is likely linked to pheromone emission in false garden mantids (Mahmudunnabi and Barry, 2019). Hence, we are proposing this could be the potential pheromone emission site in the female false garden mantid. While the absence of similar pores on the male abdomen, supports our interpretation, we were unable to identify glandular tissue.

The location of praying mantid pheromone glands has been identified in *Trachodes afzelli* between abdominal tergites 5 and 6 (Edmunds, 1975), and on the anterior margin of abdominal tergite 7 in *Acanthops falcata* (Robinson and Robinson, 1979). Pheromone glands are located on the intersegmental membranes in many insect species including the mantid, *Hierodula patellifera* (Perez, 2005) and several species of cockroach (Schal and Bell, 1985). Further studies at the ultrastructure level with a histological approach using transmission electron microscopy are required to identify the specific location of these intertergal glands in *P. albofimbriata*.

The external morphology of male false garden mantid antennae, along with the distribution pattern of the sensilla responsible for olfaction, are described in the current study. The antennae of *Pseudomantis albofimbriata* are very similar to the antennae of other praying mantids described to date, including *Sphodromantis lineola* (Hurd et al., 2004), *Hierodula majusculata* (Allen et al., 2012) and *Tenodera aridifolia* (Carle et al., 2014). The results of these studies, along with previous studies on *P. albofimbriata* (Holwell et al., 2007, Jayaweera

and Barry, 2017), revealed that male antennae possess several types of olfactory sensilla. It has previously been established that the grooved peg sensilla of mantids occur in different shapes (Slifer, 1968). Accordingly, our study found that in *P. albofimbriata*, the length and number of grooved peg sensilla vary, and some sensilla contained a closed terminal tip whilst others contained an irregular pore at the tip. Some of them were entirely closed and others partially sealed. This level of variation is also observed in this type of sensilla in other insects (Bowen, 1995, Diehl et al., 2003, Watanabe et al., 2012). Olfactory sensory neurons were observed on groove peg sensilla in cockroaches (Fujimura et al., 1991), confirming their role in the olfactory function.

The basiconic sensilla contained structures similar to those in other insect species where they function as olfactory receptors (Toh, 1977, Shanbhag et al., 1999, Shanbhag et al., 2000). This type of sensillum helps to detect odour molecules of plants reported in *Yponomeuta* moths (Van der Pers, 1981) and terpenes and alcohols in fruit flies (de Bruyne et al., 2001), and banana odours in mantid species (Carle et al., 2014). The odorant molecules of alcohols and terpenes are main components of plants especially flowers and fruits. Therefore, the function of basiconic sensilla is considered as plant odour detectors. We found this type of sensilla sparsely distributed on the antenna similar to *Tenodera aridifolia* (Carle et al., 2014). While at first gland it seems illogical to expect plant odour detectors on carnivorous insects, they might be involved in locating good sites for hunting for the praying mantids. We observed the juvenile mantids on the flowers of *Lomandra longifolia* thus for heavily-scented nectar attract a lot of herbivorous insects, and therefore, mantids locate these as preferred hunting sites.

The olfactory structures of trichoid sensilla have been well described in insect species from fruit flies to cockroaches (Toh, 1977, Shanbhag et al., 1999, Shanbhag et al., 2000). They are recognised as primary sex pheromone detectors in many insect species including mantids (Carle et al., 2014). In our study, we identified three subtypes of trichoid sensilla, based on

their length and/or location. We identified long (type 1), medium (type 2) and short (type 3) trichoid sensilla. The different lengths of these sensilla may indicate that they perform different functions. For example, in cockroaches, two sensory neurons were present in long trichoid sensilla, which respond antagonistically (in an 'on' and 'off' fashion) to olfactory stimuli (Hinterwirth et al., 2004, Burgstaller and Tichy, 2011), whereas neurons in the other two subtypes (short and medium) did not respond in this manner. Furthermore, the axons of sensory neurons retained in long and short trichoid sensilla terminate different position in the antennal lobe, which is the primary olfactory centre in insects (Watanabe et al., 2012). These findings strongly suggest that different sizes of trichoid sensilla in mantids perform different functions in olfactory processing. For example, they could detect different molecules of the female pheromone blends.

In most insect species, single-walled sensilla such as trichoid and basiconic sensilla facilitate signal encoding for pheromone reception (Hallberg et al., 1994, Kim et al., 2000, Brockmann and Brückner, 2001, Nakanishi et al., 2009, Syed et al., 2010). Though the grooved peg sensilla are doubled-walled in nature and the involvement of this type of sensilla in sex-pheromone processing is not well documented, we anticipate that the grooved peg sensilla have some involvement in sex-pheromone reception in *P. albofimbriata*, as suggested for other mantid species such as *T. aridifolia* (Carle et al., 2014) and *H. majusculata* (Allen et al., 2012). Further confirmation with electrophysiological recordings would be of great interest.

Our report is the first attempt to localize a potential pheromone emission site in the female abdomen of praying mantids and to quantify the presence of olfactory sensilla that are responsible for chemical detection in male garden mantid antennae using scanning electron microscopy. We are currently elucidating the chemical components of the *P. albofimbriata* sex pheromone, which will allow us to the identification of pheromones using analytical procedures.

Acknowledgements

Many thanks to Sue Lindsay, Nadia Suarez-Bosche, Nicole Vella and Naila Evans at Microscopy unit, Macquarie University for their generous support in operating fluorescence- and scanning electron microscope. Thanks are also extended to Macquarie University for funding this research.

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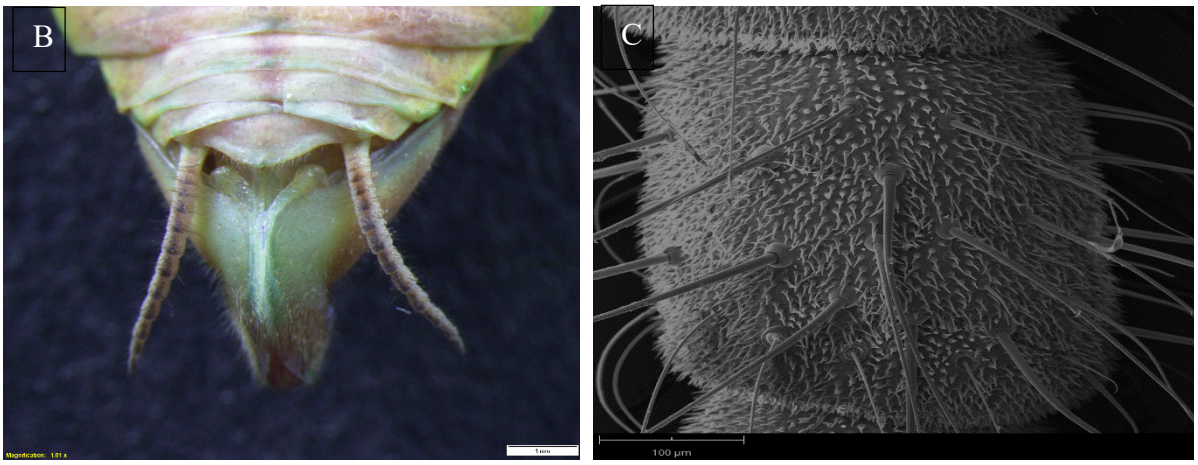

Figure 1. A. Micrograph of a female *P. albofimbriata* showing entire abdominal segments. B. Micrograph of a female *P. albofimbriata* showing the tip abdominal segments, including cerci. C. Scanning electron micrograph showing a cercomere.

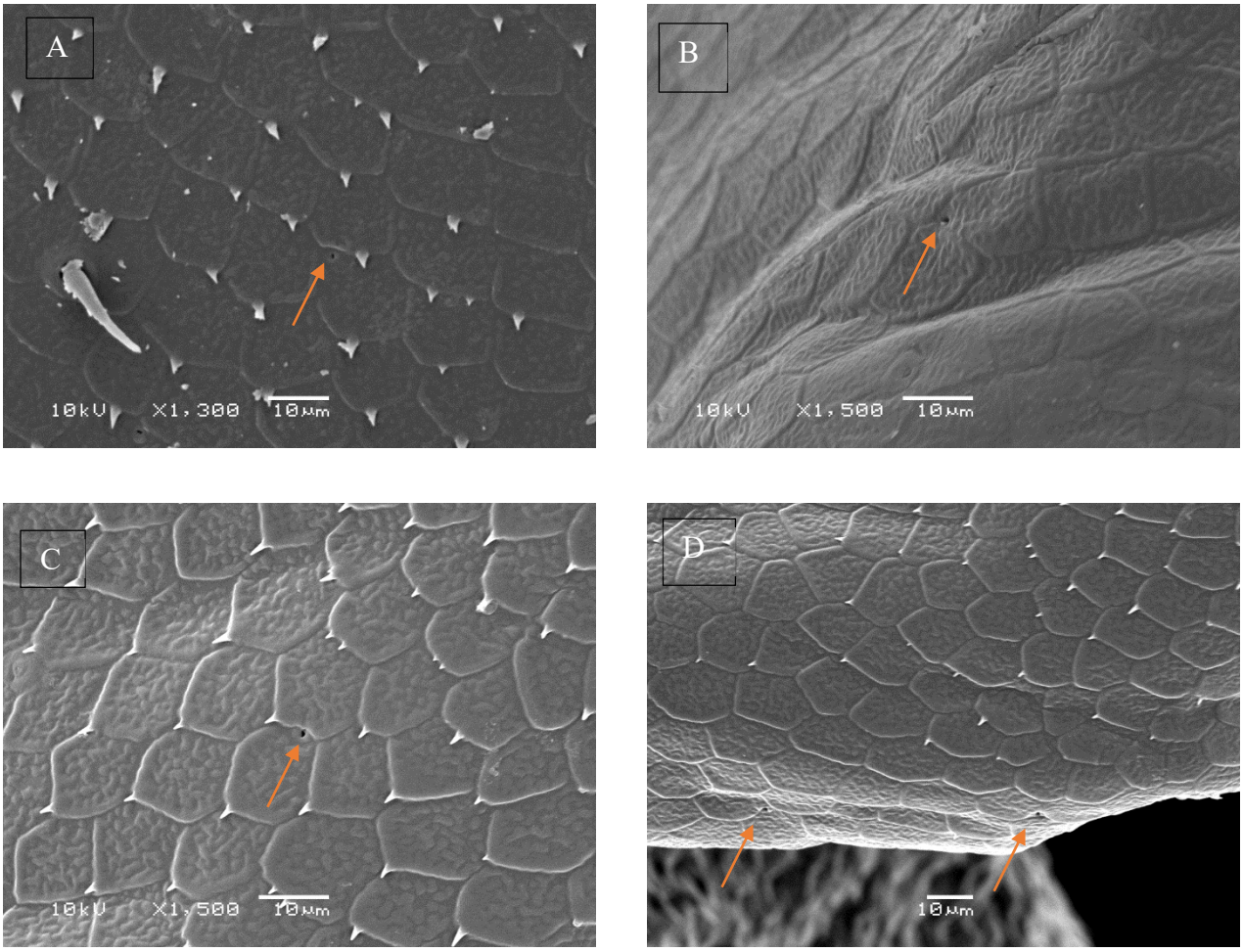

Figure 2. Scanning electron micrograph showing the dorsal view of the intertergal membrane 3 & 4 (A), 4 & 5 (B), 5 & 6 (C) and 6 & 7 (D) in female; arrows indicate pore like structures.

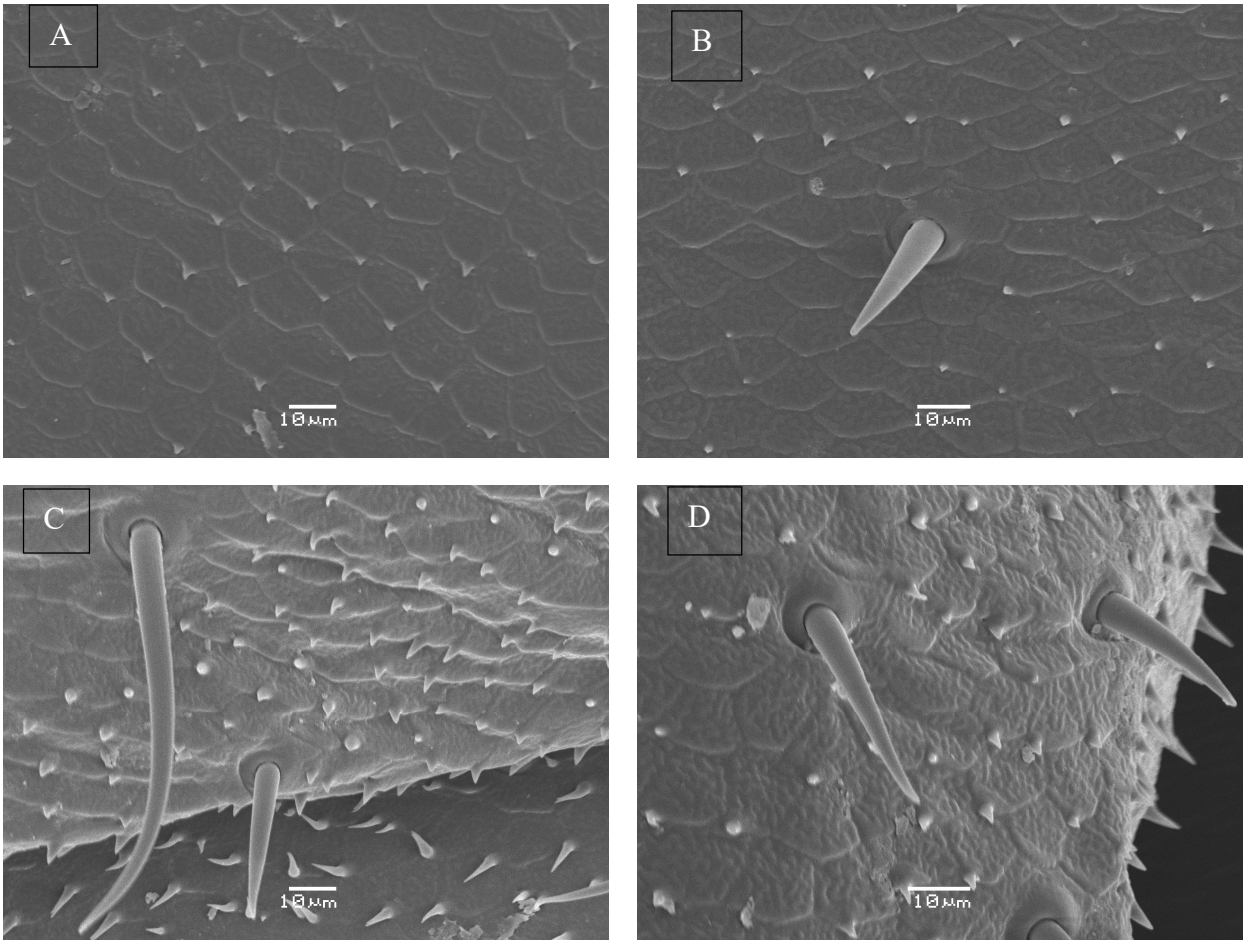

Figure 3. Scanning electron micrograph showing the dorsal view of the intertergal membrane 3 & 4 (A), 4 & 5 (B), 5 & 6 (C) and 6 & 7 (D) in male; without pore like structures.

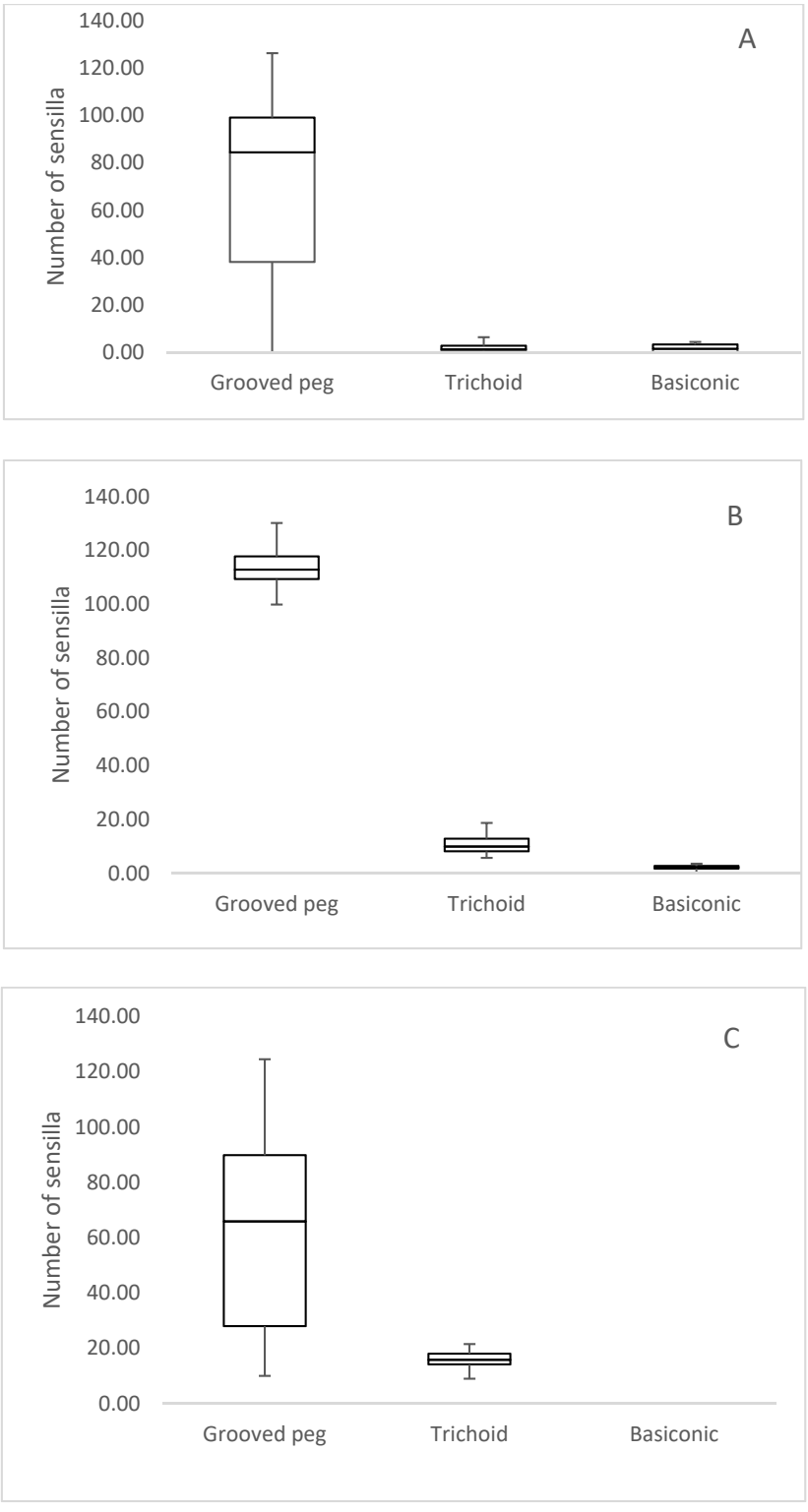

Figure 4. Box plots of the olfactory sensilla per flagellomere on different parts [proximal (A), medial (B) & distal (C)] of male *P. albofimbriata* antennae.

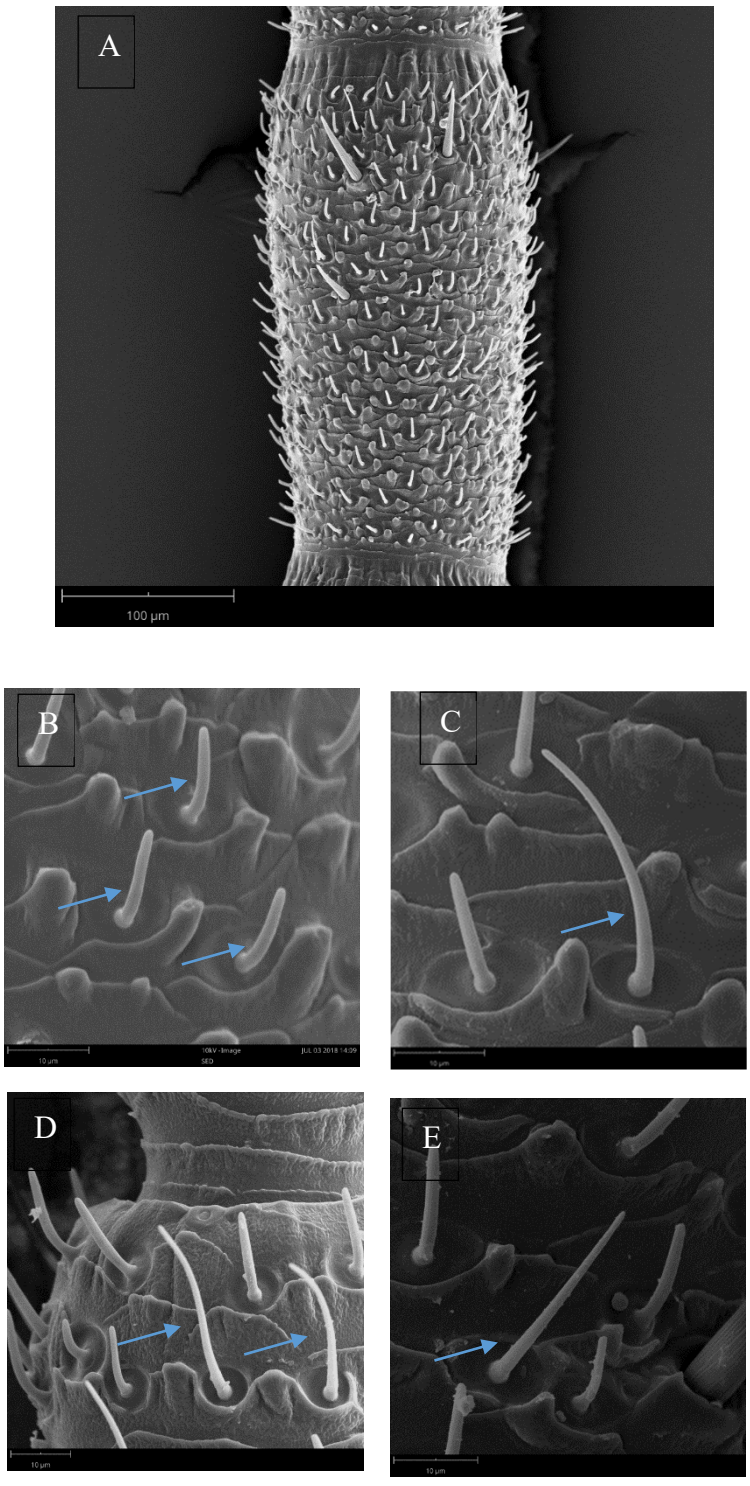

Figure 5. Scanning electron micrographs of a flagellomere (A) of *P. albofibriata* on the proximal part of the male antenna. Three different types of olfactory sensilla were identified: Grooved peg (B), basiconic (C) and trichoid sensilla (D, E). Two types of trichoid sensilla were distinguished depending on their position and length: medium trichoid sensilla (type 2, D) and short trichoid sensilla (type 3, E).

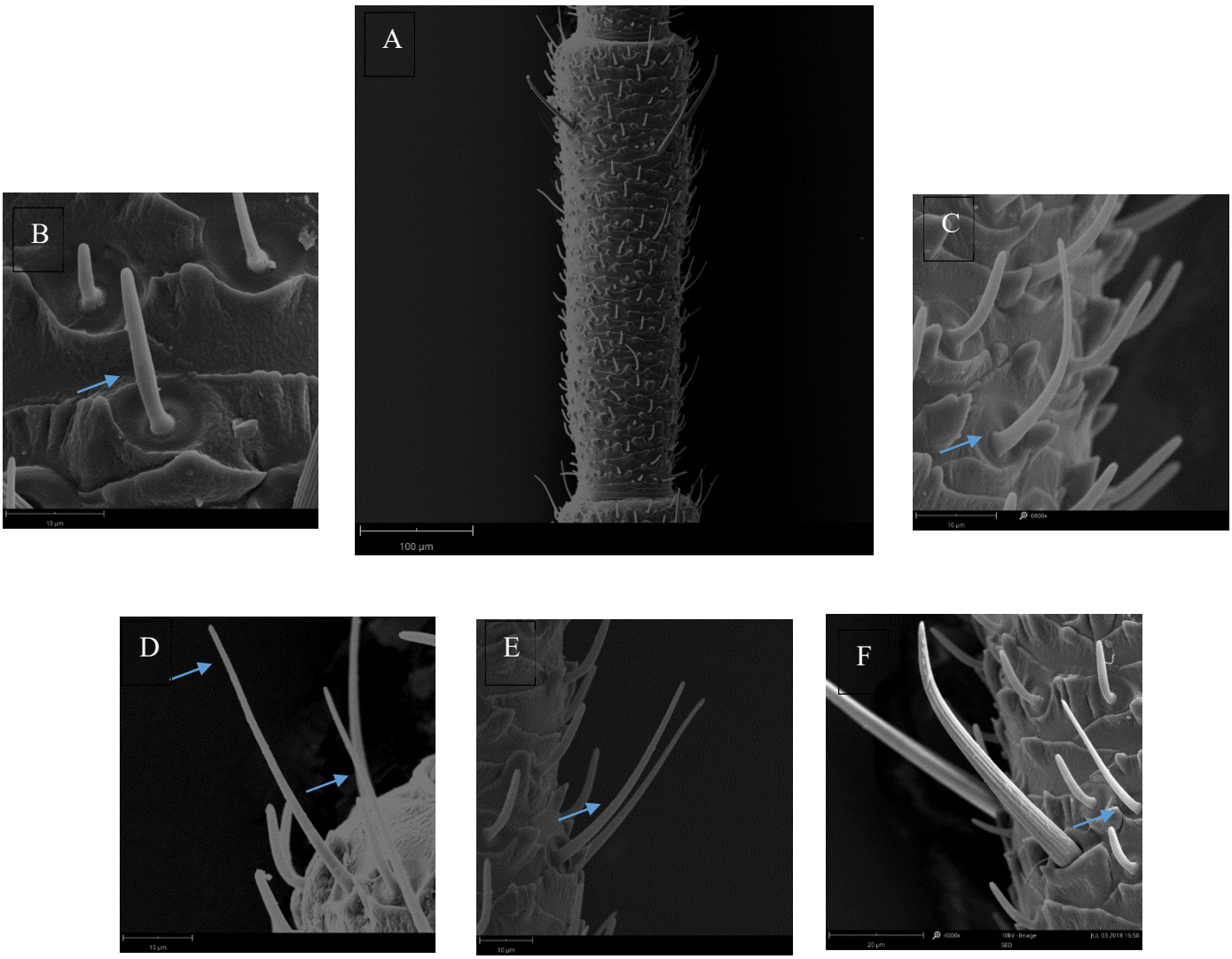

Figure 6. Scanning electron micrographs of a flagellomere (A) of *P. albofibriata* on the medial part of a male antenna. Three different types of olfactory sensilla were identified: Grooved peg (B), basiconic (C) and trichoid sensilla (D-F). Three types of trichoid sensilla were distinguished depending on their position and their length: long trichoid sensilla (type 1, D) medium trichoid sensilla (type 2, E) and short trichoid sensilla (type 3, F).

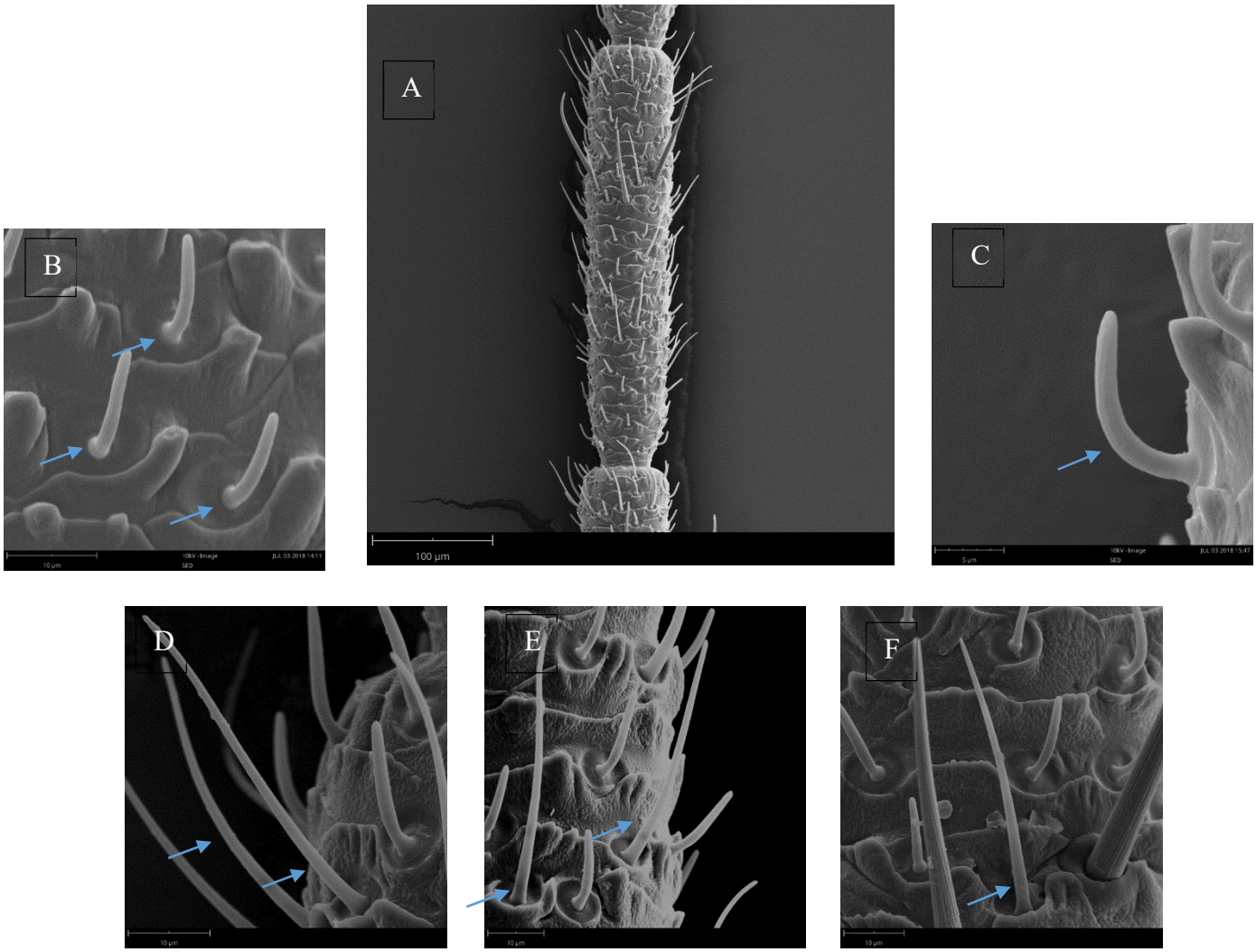

Figure 7. Scanning electron micrographs of a flagellomere (A) of *P. albofibriata* on the distal part of a male antenna. Two types of olfactory sensilla were identified: Grooved peg (B & C) and trichoid sensilla (D-F). Three types of trichoid sensilla were distinguished depending on their position and their length: long trichoid sensilla (type 1, D) medium trichoid sensilla (type 2, E) and short trichoid sensilla (type 3, F).

Chapter 3

Sex pheromones of the Australian false garden mantid, *Pseudomantis albofimbriata*: volatile collection and analysis

Md Mahmudunnabi, Soo Jean Park & Katherine L Barry

Abstract

In praying mantids, sex pheromones play a vital role in mate finding and sexual recognition. Various aspects of the mating system and reproductive biology have previously been investigated in the false garden mantid, *Pseudomantis albofimbriata*, however direct evidence of female-emitted pheromones and their chemical identification is lacking. In this chapter, we attempt to develop a standard methodology for collecting and extracting volatiles for chemical identification of pheromones in praying mantids. We collected volatiles from female praying mantids using three different methods: air trapping, solid-phase microextraction (SPME) and solvent extraction. The samples collected were analysed through gas chromatography linked to mass spectrometry (GC-MS). Solvent extraction and air trapping methods identified a series of hydrocarbons, mainly alkanes, in praying mantid volatiles. These volatiles were present in both male and female samples, suggesting these methods were unable to identify female-specific compounds that might be candidates for pheromones. However, the SPME method successfully identified compounds that were female-specific, such as tetradecanoic acid, pentadecanoic acid, *n*-hexadecanoic acid, making these compounds likely pheromone candidates in *P. albofimbriata*. The results indicate that SPME extraction is the most appropriate method for the collection of insect volatiles to identify the pheromones of praying mantids.

Keywords: Chemical identification, solvent extraction, air entrainment, SPME, GC-MS, hydrocarbon, chromatogram.

Introduction

In insects, sex pheromone communication systems involve the release of specific chemicals from the pheromone producer into the environment, with the chemo-signals then transmitted to the receiver through air (in rarer cases through water) and detected by chemoreceptive sensilla present on antenna, where the processing of signals occurs to mediate appropriate sexual behavioural responses in the receiver. The chemical identification of sex pheromones started in the 1950s and the first pheromone identified was that of the silk worm moth, *Bombyx mori*, which has become the classic example of sex pheromone, attracting male silk moths from great distances (Karlson and Butenandt, 1959). Pheromones come from diverse chemical classes such as hydrocarbons (linear or branched), esters, acids, alcohols, aldehydes, epoxides, ketones, isoprenoids, triglycerides (El-Sayed, 2019). These compounds can be found in different isomeric forms and it is recognised that the relative proportions of the respective linear isomers are critical to their function as insect sex pheromones (Leadbetter and Plimmer, 1979). The same component mixed in a different ratio might represent the pheromone of a different species (Kaissling, 2014). Naturally occurring insect sex pheromones are known to consists of common straight-chain unsaturated hydrocarbons with one or more double bonds and a terminal functional group such as an ethyl ester, alcohol or aldehyde (Morse and Meighen, 1986), while many insect pheromones are only hydrocarbons with high molecular weight but without functional group that show little variation in volatility (Ferveur, 2005; Howard and Blomquist, 2005; Wicker-Thomas, 2007). Pheromones that are highly volatile can be detected at a long distance from the source, while detection of less volatile compounds requires direct contact with a chemosensory organ (Touhara, 2008; Wicker-Thomas, 2007).

Many studied insect sex pheromones are produced by the female (Leal, 2005), but male-specific sex pheromones have also been identified recently, including 5,9,17-trimethylhenicosane from the true bug *Phthia picta* (Soldi et al., 2012) and 11-*cis*-vaccenyl

acetate, from *Drosophila* (Benton, 2007; Kurtovic et al., 2007), both of which inhibit male-male courtship. In *Drosophila melanogaster*, a combination of both male-specific and female-specific pheromones have been identified. For example, 7,11-heptacosadiene (7,11-HD) is produced by the female and acts as an aphrodisiac for males, whereas the male hydrocarbon, 7-tricosane (7-T), is an anti-aphrodisiac for other males (Ferveur, 2005, Farine et al., 2012).

Sex pheromones of herbivorous insects have received more research attention and recognition than those of predatory insects, mainly due to the economic importance of herbivores as pests (Landolt and Phillips, 1997). Nevertheless, predators can be considered as important pest control agents (Debach and Rosen, 1975), and praying mantids may prove to be effective in this regard (Hurd et al., 2004). Approximately 2400 praying mantid species have been described worldwide (https://mantodearesearch.com/) with some species using air-borne sex pheromones for mate attraction (Hurd et al., 2004, Holwell et al., 2007, Lelito and Brown, 2008, Barry et al., 2010, Maxwell et al., 2010a, Barry et al., 2011, Barry, 2015, Barry, 2013, Maxwell et al., 2010b). Interestingly, all of these studies lack direct evidence of female-emitted pheromones. Instead, they use indirect evidence to imply the presence of pheromones, such as observations of male behaviour towards the receptive females. As males are attracted from a distance in the absence of visual cues, it has been concluded that males locate females by means of chemical cues (Barry, 2010, Barry et al., 2010, Maxwell et al., 2010a). The only chemically identified praying mantid pheromone is from *Sphodromantis lineola.* Pheromone identification was achieved in *S. lineola* through volatile collection from individual adult female mantids using solid phase micro-extraction (SPME) for almost an entire day, then the collected volatiles were analysed through gas chromatography linked to mass spectrophotometry (GC/MS) analysis. The results revealed that the chemicals used as primary pheromones in *S. lineola* are aldehydes, tetradecanal and pentadecanal (Hurd et al., 2004).

Pheromone isolation can be carried out using different methodologies: solvent extraction, trapping of released volatiles on specific filters through air entrainment or solid-phase microextraction (SPME) (Baker et al., 1981, Tumlinson et al., 1982, Golub et al., 1983, Golub and Weatherston, 1984, Shani and Lacey, 1984). Solvent extraction methods provide information on the amount of pheromonal components present at the time of extraction. This method can be performed in two ways: whole body extraction (Leoncini et al., 2004) or on the excised gland from the appropriate part of the insect, such as the abdominal tip of a moth where pheromone glands are usually located (Golub et al., 1983). Extracts are then injected into a GC or GC-MS system for analysis. This method can be time consuming along with hundreds of insects are needed for the extraction of the pheromone (Haverty et al., 1996, Hung et al., 2001). Furthermore, this procedure may result in the extraction of unwanted contaminants that originate from the insects or the glands.

Effluvial collections or air entrainment as called "dynamic sampling" (Agelopoulos and Pickett, 1984) methods involve the trapping of volatile components produced by the insect over a period of time, either in a cold trap (Sower et al., 1971) or on a solid absorbent such as activated charcoal, Tenax or Porapak Q (Tumlinson et al., 1982). The adsorption method involves first trapping the volatile components onto an absorbent material and then eluting the trapped organic components with a solvent (Rai et al., 1997, Hinkens et al., 2001). Another technique, Solid-Phase Micro Extraction (SPME) is a viable alternative to solvent extraction and a convenient sampling method that involves adsorption of volatiles onto a polymer-coated, fused-silica fibre (Martos and Pawliszyn, 1997). The application of SPME for insect studies was initiated by Malosse et al. (1995) who analysed the air-borne volatile pheromones of the sugarcane weevil, *Metamasius hemipterus*. Numerous studies followed to identify pheromones from different insects using the SPME method (Frérot et al., 1997, Auger et al., 1998, Jones and Oldham, 1999, Clarke et al., 1999, Sledge et al., 2000, Rochat et al., 2000, Bland et al.,

2001, Peppuy et al., 2001, Augusto and Luiz Pires Valente, 2002, Tullio et al., 2003, Djozan et al., 2005, Lievers and Groot, 2016). After collecting volatiles from insects, the SPME fibre can then be inserted into an analytical instrument such as a GC or GC-MS for separation and quantification without first requiring the application of a solvent.

In this study, we attempt to develop a standard methodology for collecting and extracting volatiles from the Australian false garden mantid, *Pseudomantis albofimbriata* to identify the pheromones. Previous studies have inferred that males use long-distance air-borne pheromones to locate females in false garden mantid (Barry 2010; Barry et al. 2010), however, the pheromone and its components have not yet been identified. Therefore, we conducted an evaluation of volatile collection from false garden mantid using three different methods: air trapping, solid-phase microextraction (SPME) and solvent extraction. Then, the collected samples were analysed through the gas chromatography linked to mass spectrometry (GC-MS) to establish the most appropriate method for the collection and identification of insect volatiles in praying mantids.

Materials and Methods

Study species, collection and housing

The false garden mantid, *Pseudomantis albofimbriata*, is a sit-and-wait predator. These mantids occur in a wide range of habitats, found mostly amongst the foliage of trees, shrubs, and grasses throughout eastern Australia (Holwell et al., 2007). They are sexually cannibalistic, sedentary in nature (Barry et al. 2008) and size dimorphic: females are larger than males and are unable to fly (Allen et al., 2014).

Juvenile mantids were collected in December 2016-February 2017 from Yamble reserve, Quarry Road, Ryde NSW 2112 (33.8010° S, 151.1081° E), Australia, and the juveniles were raised to adulthood by methods described previously (Barry et al., 2008). The sex of *P.*

albofimbriata individuals was determined by differences in the adult abdomen and wing morphology. Adult mantids were housed individually in 425 ml inverted transparent cups with mesh for ventilation, under laboratory conditions with a temperature of 25 ± 1°C, humidity 55 ± 5% and with a diurnal period of 14 light hours per day. Adult male and female mantids were used in this study.

Collection of volatiles

We trialled a number of different volatile collection methods to identify a suitable collection method, including solvent extraction of body parts, trapping of released volatiles on specific filters through air entrainment, and solid-phase microextraction.

Solvent extraction

Abdominal extracts were obtained from unmated sexually mature male (n = 6) and female (n = 6) praying mantids. Previous studies suggest that praying mantids become sexually mature approximately two to three weeks after post adult emergence (Roeder, 1935, Liske and Davis, 1987, Birkhead et al., 1988, Lawrence, 1992, Kynaston et al., 1994, Mahmudunnabi and Barry, 2019). Hence, individuals at 22 ± 2 days post adult emergence were chosen for this experiment. The animals were killed by freezing them for 5 min (-18°C), and subsequently the abdomens were separated from the thorax using stainless steel scissors. The abdomen was placed in a 5 mL plastic vial and rinsed with three aliquots of 2 mL hexane, with each aliquot rinse lasting 2 min. The three extracts were combined and then the solvent was evaporated under a gentle stream of nitrogen to reduce the volume to 1 mL of extract. The extract was transferred into a small vial sealed with a Teflon-lined cap and stored at -30°C.

Air entrainment/trapping

Volatiles were collected by an entrainment method for which a custom-made trapping system was developed (Fig. 1). Volatiles were collected from unmated sexually mature males and females were utilized as a batch collection of volatiles. Volatiles were collected from 11 batches of six females each. Different females were used in each batch. Volatiles were collected from six batches of six males, again using different males each time. In a single run, six mantids were placed individually in containers (500 mL). Air was drawn in through the container at 2 L/min by a vacuum pump (John Morris Scientific, Germany), through an active charcoal filter (20 cm, 2 cm, 6-18 mesh) and out through a collection filter containing Tenax GR (60-80 mesh; 6.35mm x 3.5 mm). Collections were carried out for 8-10 hrs. Entrained volatiles were eluted from the Tenax GR filters with hexane (1 mL). Volatiles collected were stored in a small vial sealed and stored at –30°C.

Solid phase micro extraction (SPME)

SPME was used for collecting compounds emitted from mantids (unmated females, n = 6. and males, n = 6). Before the collection, SPME fibres (coated 100 μm polydimethylsiloxane, Supelco, Bellefonte, PA) were conditioned at 220 °C for 15 min in a GC injector, using the splitless mode. Then the tip of the fibre was placed in a 200 mL round bottle containing an unmated female/male mantid. The lid of the bottle was sealed with a septum and kept for a duration of 10–12 hr during the entire dark period. The sorption was conducted at 25 °C for a duration of 10–15 min under low ambient lighting. When finished, the SPME fibre with the adsorbed volatiles was directly injected into the GC linked to MS (injector at 270 °C; splitless for 1 min) for chemical analysis.

Gas chromatography-mass spectrometry (GC-MS) parameters

Gas chromatography-mass spectrometry (GC-MS) data were acquired using a Shimadzu GCMS QP2010 instrument with an Rtx-5 (Restek, 30 m × 0.25 mm id × 0.250 μm) capillary column and helium as carrier gas (1 mL/min). The sample injection was operated in splitless mode and the temperature of the injector was set at 270°C. The oven temperature was programmed from 60°C for 3 min, increased to 250°C at a rate of 10°C/min, and held at this temperature for 30 min (Hurd et al. 2004). The ionisation method was electron impact with a voltage of 70 eV over a mass range m/z 47 – 600. The transfer line and the trap were held at 290°C and 200°C, respectively. The chemical structure of each compound was elucidated by comparison with data in the mass spectral library (Wiley9, NIST107 and NIST21) of the software present in GCMS solution. Observed mass spectra from expected chromatographic peaks were identified by comparison of the mass spectra of the authentic standards. Authentic synthetic standards were obtained from Sigma-Aldrich Pty Limited. All chemicals were > 95% pure.

GC-MS chromatograms evaluation

The chromatograms obtained from separately sampled males and females by the solvent extraction method, the air entrainment/trapping method and the SPME method were overlayed, and sex specific peaks were searched for. Male chromatograms were compared with other male chromatograms and female chromatograms were compared with other female chromatograms. We expected that female-only peaks would be prime candidates for sex pheromones.

Results

In the case of air trapping, the chromatograms showed no obvious differences in the peaks between individual male and female samples and they followed very similar patterns (Fig. 2). This method produced 10 identifiable peaks, which were mainly cuticular hydrocarbons (*n*-

alkane), as well as eight unidentified peaks (methyl branched hydrocarbons), all of which were similar in both male and female samples. However, some differences were observed in the peaks between male and female samples generated from solvent extraction (Fig. 3). Solvent extraction produced 10 identifiable peaks and nine unidentified peaks, but they did not follow similar patterns, i.e. not eluting from same retention time for male and female samples. Comparing the solvent extraction with the air trapping method showed that both methods produced very similar results (Table 1). The praying mantid volatiles found by these two methods contained a series of hydrocarbons, mainly alkanes.

The chromatograms obtained from the extraction by SPME generated different peaks between male and female samples, with sex specific peaks. This method produces dissimilar results between individual male and female samples including three female-specific peaks (Fig. 4). Male samples produced fewer peaks (9) than females (13). SPME samples contained some compounds that were eluted from the fibre itself (labelled with Arabic numerals 1, 2 and 3 in Fig. 4, in both male and female samples), acid compounds (labelled with Roman numerals) only in female samples and unidentified compounds (denoted by asterisks) in both male and female samples (Table 2).

Discussion

In our study, solvent extraction and air trapping methods identified a series of cuticular hydrocarbons, mainly alkanes, in the praying mantid volatiles present in both male and female samples. The cuticular hydrocarbons of mantids also identified from cuticular extracts of other mantids species but function has not been tested (Jones, et al., 1997). Cuticular hydrocarbons of many insects not only protect against desiccation but also play a major role in the chemical communication including in aggregation, species, nest or mate recognition and the signalling of reproductive status (Howard, 1982, Van oystaeyen et al., 2014 & Snellings et al., 2018).

Using the SPME method, we identified acidic compounds that were female-specific. These acidic compounds, tetradecanoic acid, pentadecanoic acid, *n*-hexadecanoic acid, are known to function as pheromones in other insects, such as the common green bottle fly, *Lucilia sericata* (Gołębiowski et al., 2012), the leaf cutter ant, *Acromyrmex octospinosus* (Ortius-Lechner et al., 2000) and the crazy ant, *Paratrechina longicornis* (Witte et al., 2007). All of these three compounds are biosynthetically closely related to the aldehydes; tetradecanal and pentadecanal that had been identified in another mantid species (Hurd, et al., 2004), therefore possible pheromone candidates in the false garden mantid.

Conventional methods of collecting volatiles from insects to analyse insect pheromones involve extraction by solvents. This method is time consuming and results may vary depending on time of extraction and type of solvent used (Bagnères and Morgan, 1990, Haverty et al., 1996). Another shortcoming is that unwanted components originating from the insects might also be extracted by this process. The air trapping method used here could also not avoid contamination during eluting the trapped compounds with a solvent system. Eventhough, we collected volatiles as a control without mantids to check that contamination. On the other hand, SPME is a suitable method for the extraction of volatiles to identify insect pheromones, generating sex specific peaks for *P. albofimbriata* and other insects (Frérot et al., 1997, Auger et al., 1998, Jones and Oldham, 1999, Clarke et al., 1999, Sledge et al., 2000, Rochat et al., 2000, Bland et al., 2001, Peppuy et al., 2001, Augusto and Luiz Pires Valente, 2002, Tullio et al., 2003, Djozan et al., 2005, Lievers and Groot, 2016). Our results demonstrate that SPME extraction is the most appropriate method for the collection of insect volatiles to identify the pheromones of praying mantids. Prior to this study, the only chemically identified pheromone in mantids was for *Sphodromantis lineola* and was also obtained by solid phase micro-extraction (SPME) (Hurd et al. 2004). This method may also prove to be very useful in volatile collection from live insects because sampling with a SPME fibre is non-destructive, solvent-

free, less time consuming and will allow volatile collection from the same individual (if required) over time. Therefore, SPME extraction is the most appropriate method for volatile collection to identify the pheromones of praying mantids.

Acknowledgements

Many thanks to Anu Jayaweera, Alexia Dalley and Barbara Dean for their help with collection and rearing of mantids. My special thanks goes to Dr Ian Jamie and Donald Cameron from the Department of Biomolecular Sciences at Macquarie University for allowing us to run the research project in their lab. Thanks to Mariella Herberstein for helpful comments on manuscript preparation and continued guidance and support throughout the experiment. Thanks are also extended to Macquarie University for funding this research.

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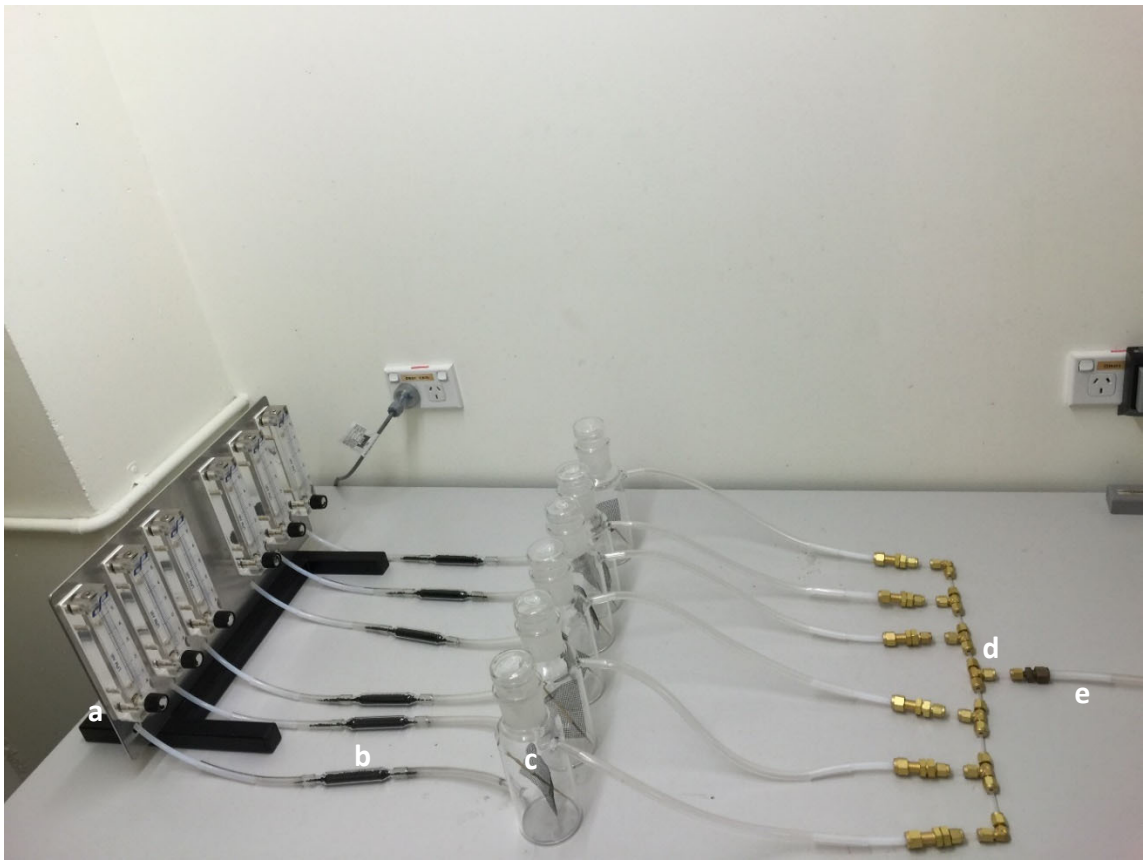

Figure 1. Setup of air trapping system for collecting volatiles from false garden mantids. Flowmeter (a), active charcoal filter (b), container for mantids (c), air merge (d) and tenax filters (e).

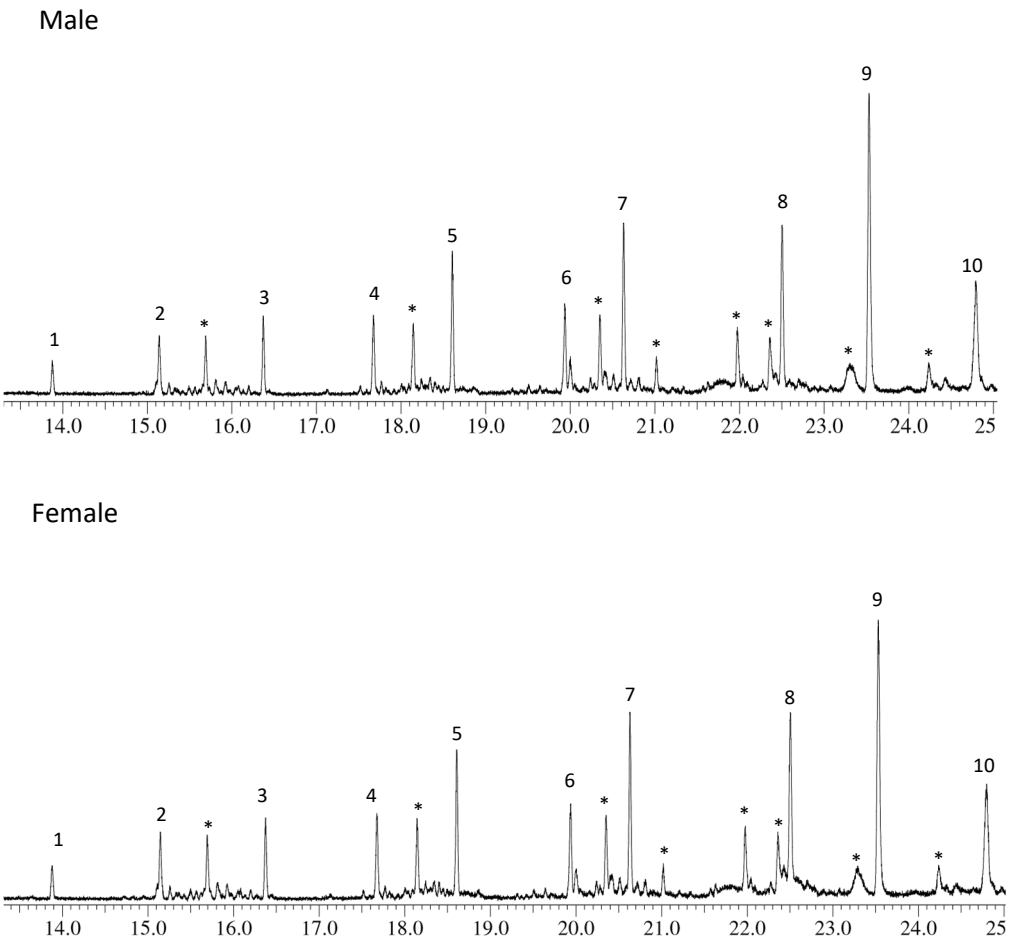

Figure 2. Total ion chromatograms of air trapping samples from male and female praying mantids *P. albofimbriata,* demonstrating the general patterns found in qualitative comparisons between the sexes. Labelled peaks correspond to those in Table 1. Only the most abundant compounds are labelled here.

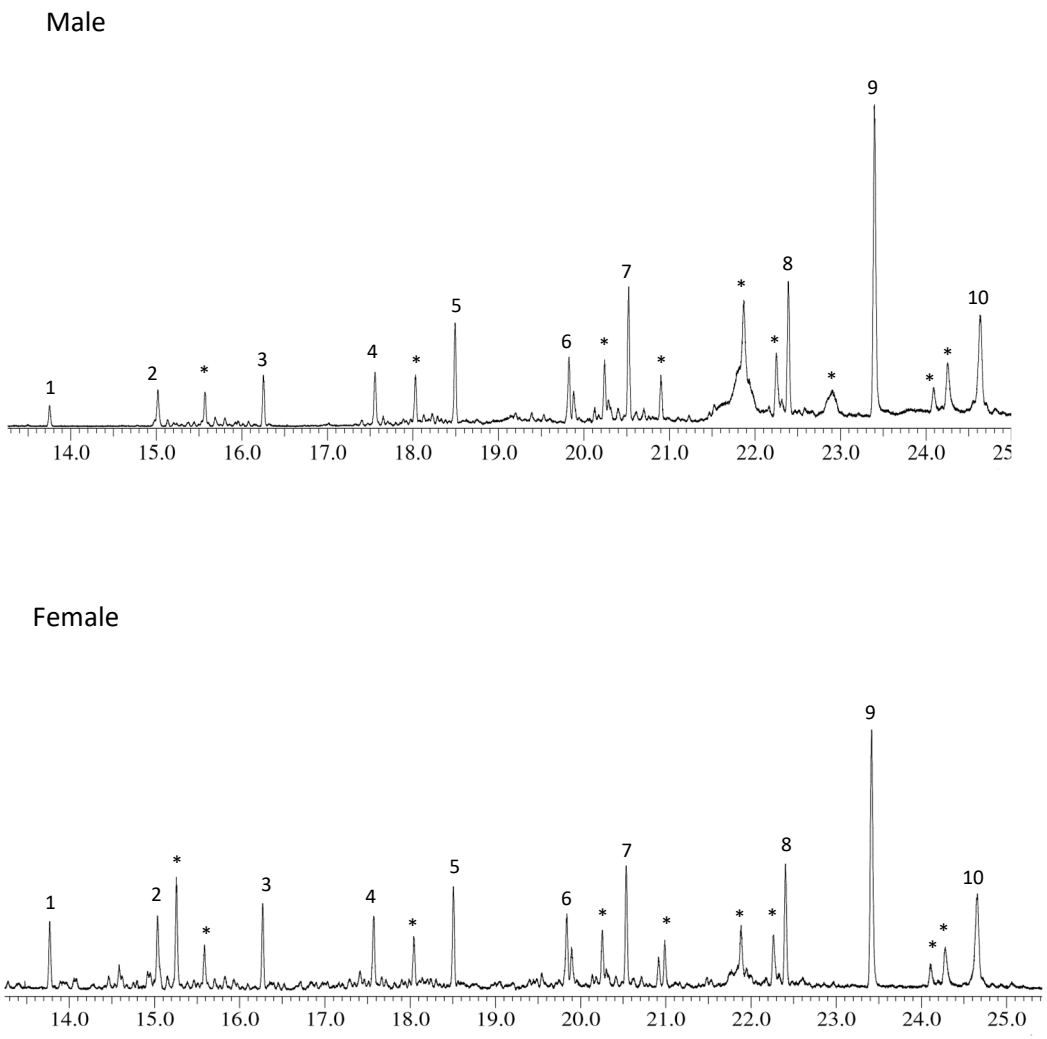

Figure 3. Total ion chromatograms of solvent extraction samples from male and female praying mantids *P. albofimbriata*, demonstrating the general patterns found in qualitative comparisons between the sexes. Labelled peaks correspond to those in Table 1. Only the most abundant compounds are labelled here.

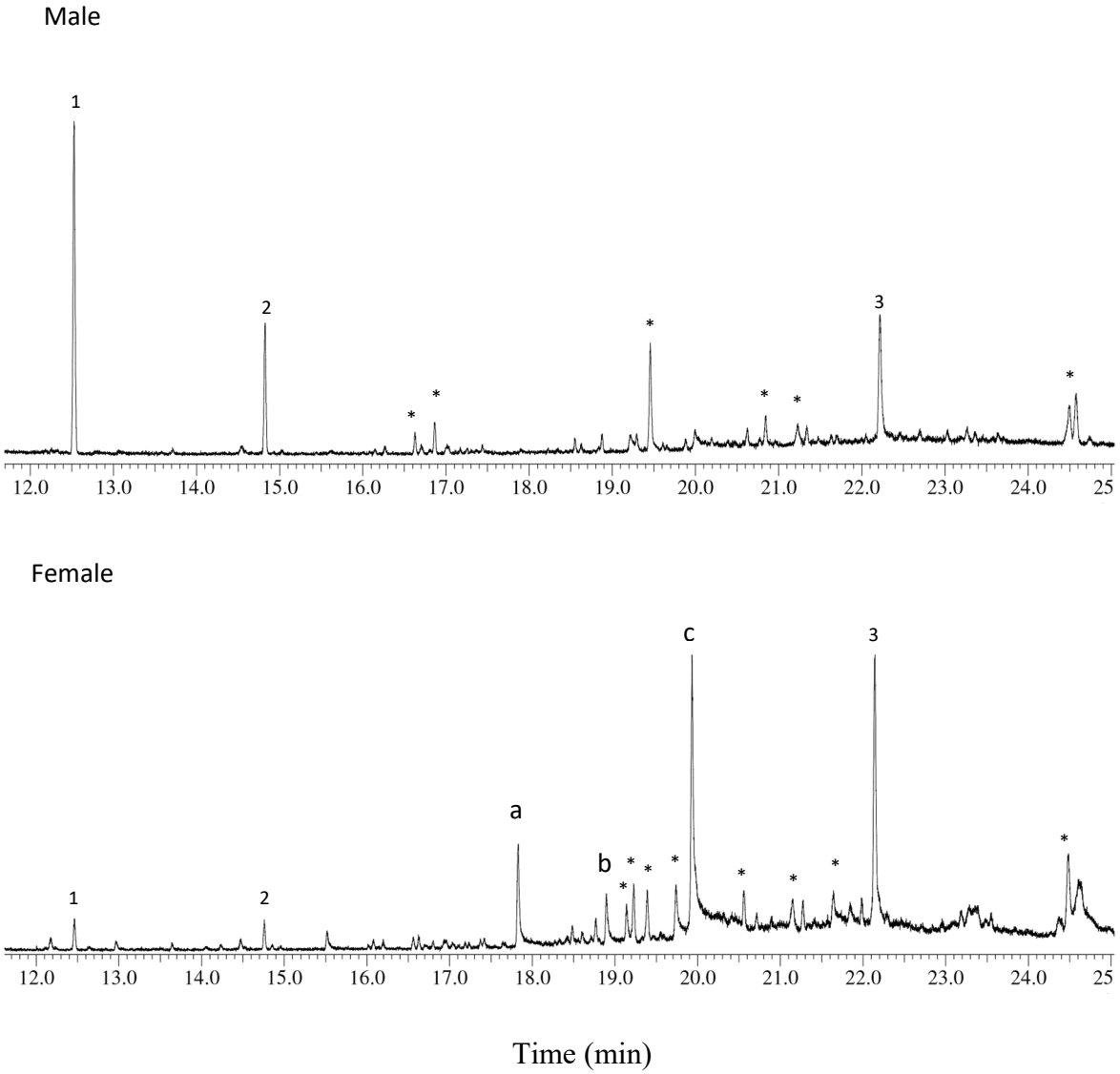

Figure 4. Total ion chromatograms of SPME samples from male and female praying mantids *P. albofimbriata*, demonstrating the general patterns found in qualitative comparisons between the sexes. Labelled peaks correspond to those in Table 2. Only the most abundant compounds are labelled here.

Table 1. Compounds identified by air trapping and solvent extraction samples from a male and female praying mantid *P. albofimbriata*. Numbers correspond to those presented in Fig. 2 & 3, only the most abundant compounds are listed.

Peak no.	Compound
1	tetradecane
2	pentadeane
3	hexadecane
4	heptadecane
5	octadecane
6	nonadecane
7	eicosane
8	heneicosane
9	2-propenoic acid, n-pentadecyl ester
10	tetracosane
*	unidentified (methyl branched hydrocarbons)

Table 2. Compounds identified by SPME samples from male and female praying mantids *P. albofimbriata*. Numbers & letters correspond to those presented in Fig. 4 (letters used for the specific peaks found in females only). Only the most abundant compounds are listed.

Peak no.	Compound
1	cyclohexasiloxane, dodecamethyl-
2	3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane
3	phenol
a	tetradecanoic acid
b	pentadecanoic acid
c	*n*-hexadecanoic acid
*	Unidentified

Chapter 4

Identification and bioassay of sex pheromone of the Australian false garden mantid, *Pseudomantis albofimbriata*

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Abstract

The false garden mantid, *Pseudomantis albofimbriata* is a sit-and-wait predator that is sexually cannibalistic, size dimorphic and mainly found in eastern Australia. Pheromones are an important component of their biology and play a vital role in mate finding and sexual recognition. The pheromone components of the false garden mantid have not yet been identified. Three compounds were detected during preliminary analyses of volatiles sampled by solid-phase microextraction (SPME) from female false garden mantids using gas chromatography linked to mass spectrometry (GC-MS). The compounds were identified as tetradecanoic acid (C_{14}), pentadecanoic acid (C_{15}) and *n*-hexadecanoic acid (C_{16}) by comparing their GC retention time and mass spectra with those of synthetic standards. In bioassays, males were not significantly attracted to the synthetic samples of C_{14}, C_{15} and C_{16} acid compounds, demonstrating these compounds were unlikely to be sex pheromone components for this species. Further work, including the use of electroantennograms as well as behavioural trials at modified concentrations, is discussed.

Keywords: SPME, GC-MS, Tetradecanoic acid, Pentadecanoic acid and *n*-Hexadecanoic acid.

Introduction

Insect pheromones play an important role in reproductive biology by facilitating mating behaviour, especially in terms of species recognition, sex differentiation and mating partner assessment. The pheromones that mediate the responses of one sex to the other and that bring individuals together for reproduction are referred to as sex pheromones (Ali and Morgan, 1990). Although there is a lot of variation in how sex pheromones facilitate mate finding in insects, females usually produce sex pheromones that are attractive to males. For instance, sex pheromones transmit species-specific messages over a distance to facilitate mate finding observed in short-lived insects (Greenfield, 1981; Lebreton et al., 2017). Sex pheromones play

a central role in mate finding and sexual recognition in many insects including praying mantids. Praying mantids are a group of diverse and charismatic predatory insects, and some species are known to use air-borne sex pheromones for mate attraction including *Acanthops falcata* (Robinson and Robinson, 1979), *Sphodromantis lineola* (Hurd et al., 2004), *Hierodula patellifera* (Perez, 2005), *Mantis religiosa*, *Empusa pennata* (Gemeno et al., 2005), *Stagmomantis limbata* (Maxwell et al., 2010a, Maxwell et al., 2010b), *Tenodera aridifolia sinensis* (Lelito and Brown, 2008, Maxwell et al., 2010a), *Hierodula majuscula* (Allen et al., 2012) and *Pseudomantis albofimbriata* (Holwell et al., 2007, Barry, 2010, Barry et al., 2010, Barry et al., 2011, Barry, 2013, Barry and Wilder, 2013, Barry, 2015, Mahmudunnabi and Barry, 2019).

In the present study, we used the false garden mantid *Pseudomantis albofimbriata* (Mantodea: Mantidae), which is a well-studied sexually cannibalistic Australian species used as a model for a wide variety of behavioural, physiological, and ecological studies over the last 12 years. Various studies have examined different aspects of the mating system and reproductive biology of *P. albofimbriata* including mate location and mate choice, female calling behaviour, male arrival behaviour and antennal morphology (Holwell et al., 2007, Barry et al., 2008, Barry et al., 2009, Barry, 2010, Barry et al., 2010, Barry and Kokko, 2010, Barry and Wilder, 2013, Barry, 2015, Jayaweera et al., 2015, Jayaweera and Barry, 2017, Mahmudunnabi and Barry, 2019). Collectively, these studies infer the presence of female-emitted pheromones through indirect evidence such as observations of male behaviour towards the receptive females. Males are attracted from a distance and move towards the female without using any visual cues. This strongly indicates that males utilise female chemical cues or air-borne pheromones to locate and assess potential mates (Barry, 2010, Barry et al., 2010). However, the chemical identity of this pheromone remains unknown. Therefore, our aim is to identify the chemical components of the female sex pheromones in *P. albofimbriata*. In our

previous study revealed that solid phase micro extraction (SPME) extraction is the most appropriate method for the collection of insect volatiles (Chapter 3). Using this method, during preliminary analyses of volatiles we found three female specific compounds: tetradecanoic acid, pentadecanoic acid and hexadecanoic acid, which were possible candidates for sex pheromones in the false garden mantid. To confirm this identification, we slightly modified the SPME sampling techniques (see in methods) and repeated the volatiles collection from unmated females and analysed by gas chromatography coupled with mass spectrometry (GC/MS). Behavioural assays were then conducted to confirm the potential pheromone components.

Bioassay methods were designed to allow assessment of the pheromone components, which encompass only a small portion of the total chemical constituents of the insect. Any chemical compounds considered as a pheromone which requires to demonstrate the behavioural activity towards the compounds that used in intraspecific communication (Baker and Cardé, 1984). Chemical compounds produced by female to be evidence of their function as sex pheromones, which requires a demonstration of male responses (Karlson and Lüscher, 1959). A change in behaviours in response to the test material measured in an experimental setup - behavioural bioassay - can be conducted in static cages/arenas, olfactometers or wind tunnels (Baker and Cardé, 1984). These generally involve sealed chambers connected to a pump that draw air through the system. The test insect is placed in the middle of the chamber and has the option of moving or flying to one or more of the test components in one of the inlets of the enclosed system. To assess how male false garden mantids respond to the identified chemicals by following similar methods employed by Hurd et al. (2004) in a slightly modified Y-maze has been used.

Materials and Methods

Insects

Individuals of *P. albofimbriata* were collected in December 2017-February 2018 from Yamble Reserve, Quarry Road, Ryde NSW 2112 (33.8010° S, 151.1081° E), Australia and the juveniles were raised to adulthood by methods previously described (Barry et al., 2008, Barry, 2010). All juveniles were housed individually in 200ml inverted transparent cups of which the bottom end was replaced by mesh for ventilation. The animals were maintained on a diet of two extra-small crickets *Acheta domestica* (average body mass = 0.026 ± 0.001g, n = 20) three times a week until they reached adulthood, and sprayed with water daily. The sexes of *P. albofimbriata* adults were separated based on differences in the abdomen and wing morphology. Adult males and female mantids were housed separately in 425 ml inverted transparent cups with mesh for ventilation. Adult mantids were maintained on a diet of two small crickets, *Acheta domestica* (average body mass = 0.062 ± 0.003g, N = 20) three times a week, which is considered a normal feeding requirement for praying mantids of this species (Barry et al., 2008, Barry, 2010, Jayaweera et al., 2015). The laboratory conditions consisted of a temperature of 25 ± 1°C, humidity 55 ± 5%, with a diurnal period of 14 light hours per day, however the light period was reversed to make the observations easier for the researcher. Ten hours of dark was set from 0830hrs to 1830hrs (AEDT) (Mahmudunnabi and Barry, 2019). Unmated females (22 ± 1 day post emergence) were used for headspace sampling, whereas males (22–29 days post emergence) were used for behavioural studies.

Collection of volatiles

Female false garden mantids become mature and chemically attractive to males around 2-3 weeks after adult emergence (Mahmudunnabi and Barry, 2019). Calling behaviour of false garden mantids has been recorded throughout the night starting from early evening until morning with a peak calling activity around 11pm (Mahmudunnabi and Barry, 2019). This

information allowed us to determine the suitable volatile collection time for pheromone identification. We collected volatile emitted compounds from the headspace of individual adult female mantids using a solid phase microextraction (SPME) device containing a fibre coated with 100μm polydimethylsiloxane (Supelco, Bellefonte, PA). The SPME fibre was conditioned at 220°C for 15 minutes in a GC injector, splitless mode. For headspace sampling, we placed one mature female mantid into a round bottom flask (500ml). Then the flask was sealed with aluminium foil and the mantid was kept in the flask for 10 hours that span the entire dark period. The fibre was then exposed to the flask and the sorption was conducted at 25°C for a duration of 10–15min under low ambient lighting. When finished, the SPME fibre with the adsorbed volatiles was directly injected into the GC linked to MS (injector at 270°C; splitless for 1 min) for chemical analysis. This procedure was repeated for 15 individual unmated females.

Gas chromatography-mass spectrometry (GC-MS) parameters

Gas chromatography-mass spectrometry (GC-MS) data were acquired using a Shimadzu GCMS QP2010 instrument with an Rtx-5 (Restek, 30 m × 0.25 mm id × 0.250 μm) capillary column and helium as carrier gas (1 mL/min). The sample injection was operated in splitless mode and the temperature of the injector was set at 270°C. The oven temperature was programmed from 60°C for 3 min, increased to 250°C at a rate of 10°C/min, and held at this temperature for 30 min (Hurd et al. 2004). The ionisation method was electron impact with a voltage of 70 eV over a mass range m/z 47 – 600. The transfer line and the trap were held at 290°C and 200°C, respectively. The chemical structure of each compound was elucidated by comparison with data in the mass spectral library (Wiley9, NIST107 and NIST21) of the software present in GCMS solution. Observed mass spectra from expected chromatographic peaks were identified by comparison of the mass spectra of the authentic standards. Authentic synthetic standards obtained from Sigma-Aldrich Pty Limited. All chemicals were > 95% pure.

Behavioural assay

Behavioural assays were designed to assess how male false garden mantids respond to the chemicals identified from the GC-MS analysis. The Y-maze arena (60cm long × 60cm wide × 15cm high), side walls and floor were made of black plexiglass and the roof was covered with transparent plastic cling wrap (Fig. 1). A V-shaped interior partition was created by inserting cardboard that divided the arena into two 30 cm corridors at its apex, pointed toward the end where a male mantid was placed. An aquarium pump produced airflow of 2000 cc per min purified through an active charcoal filter (20 cm, 2 cm, 6-18 mesh) and split into two hoses entering through a hole in the centre of the far wall of each corridor. The behavioural assays were carried out in a dark room to eliminate visual cues, however, a red light was used to allow observations without disturbing the mantids as they cannot perceive red light (Briscoe and Chittka, 2001).

Synthetic chemical compounds of tetradecanoic acid (myristic acid), pentadecanoic acid and hexadecanoic acid (palmitic acid) were obtained from Sigma-Aldrich Pty Limited. The chemicals were diluted at 1/10 in *n*-hexane and 50 µL of each chemical and their blend (chemicals were mixed in a 1:1:1 ratio) was pipetted onto filter paper (Whatman filter paper 1, diameter 25 mm, thickness 180 µm and pore size 11 µm) to perform the behavioural essay.

At the start of the test, each of two strips of filter paper were soaked in either a solvent (*n*-hexane) as control or the solvent plus synthetic chemical compounds and air-dried (for 5 min). Each was then placed on top of the inverted cups sitting under the hole at each corridor. Male mantids were released individually into the arena and observed for 1 hr to record the response of males towards the stimuli (test chemicals or control). Each male (n = 45) was exposed to each chemical once in a random order. As in previous studies in this species demonstrates that males respond towards the calling females in a glass Y-maze, confirming that males make a simultaneous choice between two chemical stimuli (Barry et al., 2010).

Therefore, we not repeated calling females as a positive control in our current study. The natural proportions of three putative pheromone compounds have not identified. Instead, we applied used similar concentrations that have been successfully used in similar studies (Cini et al., 2011). All experiments were conducted at a temperature of 25 ± 1°C and relative humidity 55 ± 5%, and took place during the dark period because males search for females primarily during the night (Mahmudunnabi and Barry, 2019). The arena was thoroughly cleaned with soap solution and wiped with ethanol after each use, and left in the fresh air for several hours between each treatment run.

The response of males towards the stimuli (test chemicals or control) was recorded as either entering the arm of the maze, or as a non-response if the male did not enter the maze arm. Attractive index was then calculated using the following formula (Deb and Kumar, 2016):

$$\text{Attractive index} = \frac{\text{(No. of mantids responded to the test chemical} - \text{No. of mantids responded to the control)}}{\text{(No. of mantids released} - \text{No. of mantids responded to the control)}}$$

The number of males responding was analysed using Chi-square tests.

Results

Pheromone identification

Chromatographic profiles for all fifteen females were very similar, eight of them yielded identical results. Total ion chromatogram of a representative female is shown in Figure 2. Only eight peaks (found in all the samples) were identified in the chromatogram (Table 1). The mass spectra of peaks 4, 5 and 7 suggested that they were Compound I, Compound II and Compound III, respectively. The remaining peaks were products of degradation of the SPME coating.

To identify the three compounds that were most likely to be relevant for mantid pheromones, we analysed mass fragmentations (see supplementary data provided as appendices in Figures 1&2), which identified a molecular ion at m/z 228 corresponding to a molecular formula of $C_{14}H_{28}O_2$. The presence of γ-hydrogen rearrangement identified a product at m/z 60. These data and NIST library search suggested a C_{14} acid compound. The mass spectrum of the natural compound found in *P. albofimbriata* females was identical to that of the authentic standard sample of tetradecanoic acid. Similarly, the spectra of compound II showed a molecular ion at m/z 242 corresponding to a molecular formula of $C_{15}H_{30}O_2$ and a fragment ion at m/z 60. These data were consistent with a C_{15} acid compound. The mass spectrum of the natural compound found in *P. albofimbriata* females matched that of the authentic standard sample of pentadecanoic acid. Finally, the spectra of compound III found in *P. albofimbriata* females had a molecular ion at m/z 256 corresponding to a molecular formula of $C_{16}H_{32}O_2$. The peak exhibited similar fragmentation patterns to those of I and II, except their molecular ions had differences of 28 (C_2H_4) and 14 (CH_2), respectively, suggesting C_{16} acid, the same type of compounds but with a different carbon length. The mass spectrum of the natural compound found in *P. albofimbriata* females was identical to that of the authentic standard sample of hexadecanoic acid.

The retention time of these three peaks matched those of authentic standards of tetradecanoic acid, penatadecanoic acid and hexadecanoic acid samples. The mass spectra of peaks 4, 5 and 7 suggested that they were Compound I (tetradecanoic acid, C_{14}), Compound II (penatadecanoic acid, C_{15}) and Compound III (hexadecanoic acid, C_{16}), respectively.

Behavioural Assay

The presence of potential pheromone components was further investigated by behavioural assays. Overall, the mantids responded similarly to all three compounds and their mix (χ^2= 2.56, df = 6, P= 0.8615). The attractive index for all three compounds and their blend was very

low (Table 2) with no indication that the compounds were attractive to the males. This was further supported by the substantial number of males that were non-responders, that is, those that did not move away from the release area.

Discussion

We identified three acid compounds - tetradecanoic acid (myristic acid), pentadecanoic acid and hexadecanoic acid (palmitic acid) - as potential components of the pheromone from false garden mantid volatiles. These compounds are not novel and have been previously reported as used in the chemical communication systems of other arthropods including spiders, flies, ants, butterflies and bees (Table 3). These compounds that are often found in insect frass have been shown to act as aggregation kairomones (Keesey et al., 2016). Twenty eight identical compounds including tetradecanoic acid, pentadecanoic acid and hexadecanoic acid were identified both on spider silk and the cuticle of female European house spiders, *Tegenaria atrica* and were used as contact chemical signals in their mating system (Prouvost et al., 1999). Sixteen cuticular fatty acids ranging from C_6 to C_{20} were identified in males and females of the blowfly, *Lucilia sericata* (Gołębiowski et al., 2012). Tetradecanoic acid was found in cuticular extracts of male and pentadecanoic acid in female Asian citrus psyllid, *Diaphorina citri* (Mann et al., 2013).

In our bioassay, we tested the compounds that were identified from female garden mantids, however none of the synthetic chemical compounds tested elicited any response activity in male garden mantids. This suggests that these compounds do not function as sex pheromones of false garden mantids, which indicates that we need to re-run the assays with various potential changes. First, trialling lower concentrations of compounds since the original concentration of synthetic compounds has not yet been quantified. And second, investigating the smaller peaks from chromatograms as potential pheromone candidates. Third, use another method such as gas chromatography linked to mass spectrophotometry detection (GC-EAD)

or with single sensillum recording (GC-SSR) (Wibe, 2004), where by the end of the GC column splits into two parts; one part passes into the normal flame ionization detector (FID) and other part passes to an antenna or a single olfactory sensillum of the study insect. The responses of the antennal receptors are then measured and registered by using electrodes and ampflifiers. Finally, GC-EAD analyses of the headspace volatile emitted by females could identify the electrical responses from the antennae of male *P. albofimbrita* against chemicals in the headspace, which may function as pheromone components or aggregation kairomones for this species. It is also observed that the amount of males often reduces toward the end of the life cycle (Hurd et al., 1994) and female mantids often aggregate by producing the increased concentration of pheromone during the egg-laying portion of their life cycle, perhaps responding to each others' pheromones for attracting males (Hurd,1999). It indicates aggregation kairomones in mantids is highly possible, which required for further investigation.

Acknowledgements

Many thanks to Dr Ian Jamie and Donald Cameron from the Department of Biomolecular Sciences at Macquarie University for allowing us to run the research project in their lab. To Zoe wild for her help to run the behavioural assay. Thanks to Mariella Herberstein for helpful comments on manuscript preparation and continued guidance and support throughout the experiment. Thanks are also extended to Macquarie University for funding this research.

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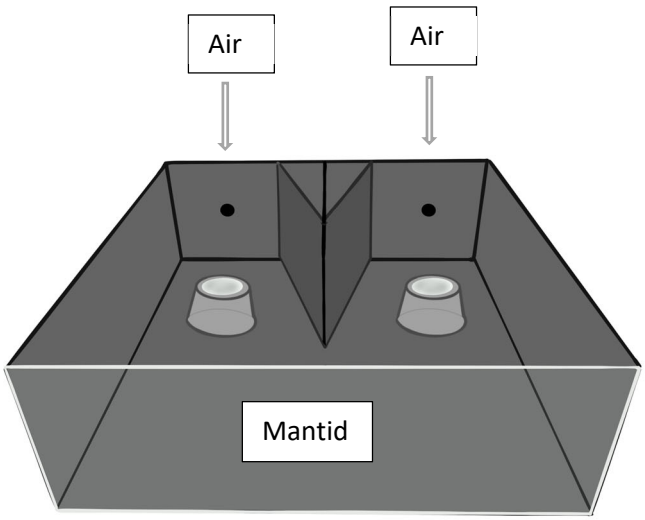

Figure 1. Schematic of the Y-maze used to test the responses of the male mantids towards the chemical compounds identified from female false garden mantids.

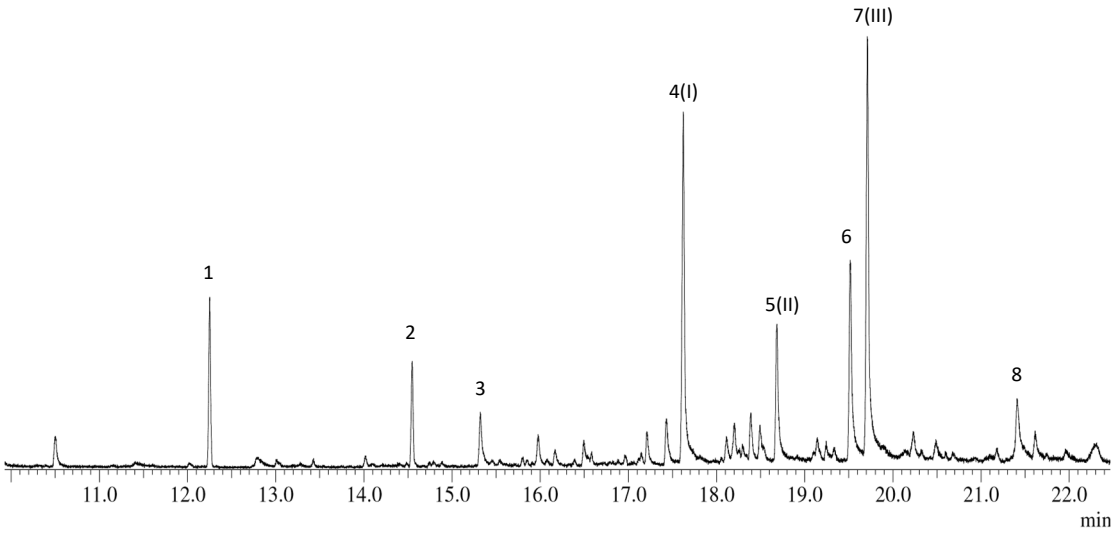

Figure 2. Total ion chromatograms of SPME sample from a female *P. albofimbriata*. Labelled peaks correspond to those in Table 1 and explained in the text. The x-axis scale gives retention time in minutes.

Table 1. Compounds identified by SPME samples from female praying mantids *P. albofimbriata*. Numbers correspond to those presented in Fig. 2. The compounds found in all the sampled females are listed.

Peak no.	Compound
1	cyclopentasiloxane, decamethyl-
2	cyclohexasiloxane, dodecamethyl-
3	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane
4	tetradecanoic acid
5	pentadecanoic acid
6	unidentified
7	*n*-hexadecanoic acid
8	phenol

Table 2. Behavioural study data of male false garden mantids showing their responses either towards the test compounds or control and non-responsive. Calculated attractive index and percent male responses also presented.

Test compound	No. of mantids released	No. of mantids responded	No. of nonresponsive mantids	Attractive index (% of males responding)
tetradecanoic acid (TA)	45	18	17	0.23 (22.86)
n-hexane (control)		10		
pentadecanoic acid (PA)	45	14	20	0.09 (8.82)
n-hexane (control)		11		
hexadecanoic acid (HA)	45	13	22	0.09 (8.57)
n-hexane (control)		10		
Blends of TA+PA+HA	21	7	11	0.22 (22.22)
n-hexane (control)		3		

Table 3. Evidence for arthropod species utilization of tetradecanoic acid (myristic acid), pentadecanoic acid and hexadecanoic acid (palmitic acid) in their chemical communication system

Species	Tetradecanoic acid	Pentadecanoic acid	Hexadecanoic acid	References
European house spider, *Tegenaria* spp.	Yes	Yes	Yes	(Prouvost et al., 1999, Trabalon et al., 1997)
Asian citrus psyllid, *Diaphorina citri*	Yes	Yes	-	(Mann et al., 2013)
Tobacco budworm, *Heliothis virescens*	Yes	-	-	(Teal and Tumlinson, 1989)
Common green bottle fly, *Lucilla sericata*	Yes	Yes	Yes	(Gołębiowski et al., 2012)
Leaf-cutter ant, *Acromyrmex octospinosus*	Yes	Yes	Yes	(Ortius-lechner et al., 2000)
African milkweed butterflies, *Amarius* sp	Yes	Yes	Yes	(Schulz et al., 1993)
Crazy ant, *Paratrechina longicornis*	Yes	-	Yes	(Witte et al., 2007)
Bumble bee, *Bombus* spp.	Yes	-	Yes	(Bertsch et al., 2004)
Grasshoppers, *Melanoplus* spp.	Yes	-	Yes	(Jackson, 1981)
The bull ant, *Myrmecia gulos*a	-	-	Yes	(Cavill et al., 1970)

Species	Tetradecanoic acid	Pentadecanoic acid	Hexadecanoic acid	References
Fire ant, *Solenopsis* spp.	-	-	Yes	(Cabrera et al., 2004)
Male african sugarcane borer, *Eldana saccharina*	-	-	Yes	(Burger et al., 1993)
Sulfur butterfly *Colias erate poliographus*	-	-	Yes	(Ômura et al., 2015)
Sawfly, *Nematus* spp.	-	-	Yes	(Boeve et al., 1992)
Red mason bee, *Osmia bicornis*	-	-	Yes	(Conrad et al., 2017)

Conclusions

This thesis examines the pheromone biology of sexually cannibalistic praying mantids. The primary focus of this research was to identify the pheromones of the Australian false garden mantid, *Pseudomantis albofimbriata* (Mantodea: Mantidae).

Based on indirect evidence, female false garden mantid emit sex pheromones that attract males from a long distance and males use both chemical and visual means to locate and assess female conspecifics at closer range for copulation (Maxwell, 1999, Holwell et al., 2007, Barry et al., 2010, Maxwell et al., 2010, Barry et al., 2015). Males are able to locate conspecific females when visual cues are obscured, which indicates air-borne pheromones play an important role in their reproductive biology (Holwell et al., 2007, Barry et al., 2010, Barry et al., 2011, Barry, 2013, Barry, 2015). It was predicted that female pheromone emission (calling) and male responses are intimately linked in false garden mantids (Barry et al., 2010), but no calling behaviour has been observed and described previously. My study provides experimental evidence that female false garden mantids adopt a calling posture comprising the rhythmic pumping movement of the abdomen during pheromone emission (Chapter 1). This behaviour has also been recorded in several other species of praying mantid (Robinson and Robinson, 1979, Perez, 2005, Gemeno et al., 2005). Based on these observations, I predicted that the potential site of pheromone emission on the body is likely to be exposed during the abdominal pumping movement. Therefore, I attempted to localize a potential pheromone emission site on the female abdomen using microscopy. I was able to locate pores on the intertergal membrane between abdominal segments 3/4, 4/5, 5/6 and 6/7 (Chapter 2). However, further studies at the ultrastructure level with a histological approach using transmission electron microscopy are required to identify the specific location of these intertergal glands in *P. albofimbriata*.

The female emitted pheromones require sensilla that can detect these pheromones, which in insects are typically located on the male antennae. The sensilla arrangement and

morphology of the male antennae of false garden mantids were investigated previously (Holwell et al., 2007, Jayaweera and Barry, 2017). However, the results of my research provide more detailed data on the olfactory sensillae distribution pattern specifically the basiconic, trichoid and grooved peg sensilla present on the male antenna (Chapter 2). I identify the presence of olfactory sensilla that have potential to play a role in sex pheromone detection on male antennae of false garden mantid. Here, I identified three subtypes of trichoid sensilla - long (type 1), medium (type 2) and short (type 3) - based on their length and/or location. There is some evidence that different sizes of trichoid sensilla in insects perform different functions in olfactory processing (Hinterwirth et al., 2004, Burgstaller and Tichy, 2011, Watanabe et al., 2012, Carle et al., 2014). The trichoid and basiconic sensilla are single-walled and have previously been shown to facilitate signal detection during pheromone reception in most insects (Hallberg et al., 1994, Kim et al., 2000, Brockmann and Brückner, 2001, Nakanishi et al., 2009, Syed et al., 2010). Hence, I anticipated that these sensilla, including the grooved peg sensilla which are doubled-walled, have some involvement in sex-pheromone reception in *P. albofimbriata*, as suggested for other mantid species such as *T. aridifolia* (Carle et al., 2014) and *H. majusculata* (Allen et al., 2012). Electrophysiological recordings with single sensillum recordings (GC-SSR) are yet to be carried out to confirm this claim.

Before isolation of the pheromone it was necessary to develop a standard methodology for collecting and extracting volatiles from the false garden mantid. There are several collection methods available for the collection of volatiles from insects (Baker et al., 1981, Tumlinson et al., 1982, Golub et al., 1983, Shani and Lacey, 1984, Golub and Weatherston, 1984), yet there is no universal method that can successfully collect volatiles from any insect. I trialled several methods for capturing potential chemicals that have been successfully applied recently - air trapping, solid-phase microextraction (SPME) and solvent extraction (Ho et al., 2014, Wang et al., 2015, Deb and Kumar, 2016, Lievers and Groot, 2016, Stanley et al., 2018, Visser et al.,

2018). Among the tested methods, SPME is a suitable method for the extraction of volatiles to identify pheromones in most insects (Frérot et al., 1997, Auger et al., 1998, Jones and Oldham, 1999, Clarke et al., 1999, Sledge et al., 2000, Rochat et al., 2000, Bland et al., 2001, Peppuy et al., 2001, Augusto and Luiz Pires Valente, 2002, Tullio et al., 2003, Djozan et al., 2005, Lievers and Groot, 2016), including the praying mantid *Sphodromantis lineola* (Hurd et al. 2004). In my study, the solvent extraction and air trapping methods identified a series of hydrocarbons, mainly alkanes, in the false garden mantid volatiles present in both male and female samples. However, I found that the SPME extraction identified compounds that were female-specific (Chapter 3).

I based the optimal volatile collection time on the pheromone release posture or calling behaviour of females. Therefore, I utilised the description of the calling behaviour of *P. albofimbriata* and the age and time window in which volatile pheromone collection with SPME method would be most successful for *P. albofimbriata* (Chapter 1). Using this method, I identified several female specific compounds: tetradecanoic acid (C_{14}), pentadecanoic acid (C_{15}) and hexadecanoic acid (C_{16}), which were not found in males, therefore, possible candidates for sex pheromones in the false garden mantid (Chapter 3 & 4). However, this is not sufficient evidence of their function as pheromones, which requires a demonstration of male responses to the possible pheromone (Karlson and Lüscher, 1959). Consequently, I designed a behavioural assay to assess how male false garden mantids respond to the identified chemicals by following similar methods employed by Hurd et al. (2004) in a slightly modified Y-maze. However, no consistent response from male *P. albofimbriata* was observed during these behavioural assays. This suggests that even though these compounds were female specific, they did not function as pheromone (Chapter 4). I considered only most abundant peaks, however, there were other much smaller peaks that were not identified or tested. Therefore, further studies are required to investigate the smaller peaks in the chromatograms

obtained by GC-MS as potential pheromone candidates. However, I cannot dismiss the possibility that these compounds are indeed pheromone components or aggregation kairomones for this species, because my experimental set up may not have allowed males to respond appropriately. For example, perhaps the concentration of the chemicals was unnaturally high or low, or the design of the Y-maze was inappropriate. Therefore, wind tunnel bioassays (Baker and Linn, 1984, Knudsen et al., 2018), a method to test behavioural responses of flying insects to isolated compounds, could be a possible alternative. Furthermore, varying the concentration of the compounds may be necessary to elicit an appropriate male response. This might require the quantification of the amount of each compound released from the female. Finally, using gas chromatography linked to an electroantennographic detection (GC-EAD) to analyse the headspace volatile emitted by females could identify the electrical responses from the antennae of male *P. albofimbrita* against chemicals (biologically active compounds) in the headspace, that may otherwise be overlooked.

Overall, my research highlights some approaches to isolation, spectrometry and behavioural assay to explore the existing chemical communication system in the false garden mantid and describes future areas for research to understand the chemical ecology of praying mantid mating systems.

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Appendices

Appendix A

Table 1. Number of olfactory sensilla on the proximal part (flagellomeres #1 to # 30) of male *P. albofimbriata* antennae.

No. of flagellomeres	Grooved peg sensilla (Mean±SD, n=6)	Sensilla trichodea (Mean±SD, n=6)	Sensilla basiconica (Mean±SD, n=6)
6	1.80 ± 1.47	0.00	0.00
7	4.00 ± 1.15	0.00	0.00
8	29.17 ± 2.54	1.00 ± 0.00	0.00
9	65.17 ± 2.54	1.00 ± 0.00	0.00
10	92.83 ± 13.92	1.00 ± 0.00	0.00
11	100.83 ± 13.92	1.00 ± 0.00	0.00
12	101.83 ± 13.92	1.50 ± 0.50	0.00
13	126.17 ± 23.25	1.20 ± 0.40	0.00
14	125.83 ± 27.94	2.00 ± 1.00	0.00
15	87.17 ± 2.67	1.50 ± 1.26	1.17 ± 0.90
16	80.83 ± 2.67	1.33 ± 1.11	2.00 ± 1.83
17	71.83 ± 2.79	1.50 ± 0.96	2.83 ± 1.46
18	70.83 ± 2.67	0.83 ± 0.37	3.67 ± 1.60
19	70.50 ± 2.99	1.83 ± 0.69	3.67 ± 1.80
20	76.50 ± 2.63	1.33 ± 0.47	4.17 ± 2.11
21	90.50 ± 4.99	1.33 ± 0.47	4.50 ± 1.71
22	99.17 ± 3.24	2.50 ± 1.12	3.83 ± 1.21
23	93.83 ± 2.67	3.17 ± 1.21	3.50 ± 1.61
24	81.50 ± 3.86	3.00 ± 1.00	2.83 ± 2.11
25	98.50 ± 2.99	4.33 ± 2.69	3.17 ± 0.90
26	97.50 ± 7.54	6.50 ± 1.38	4.00 ± 2.45
27	95.17 ± 2.67	4.00 ± 1.53	4.00 ± 1.83
28	116.50 ± 7.54	4.83 ± 1.95	3.00 ± 0.58
29	109.17 ± 2.67	5.83 ± 0.37	3.17 ± 0.90
30	111.50 ± 2.99	4.50 ± 1.50	3.17 ± 1.86

Table 2. Number of olfactory sensilla on the medial part (flagellomeres #31 to # 60) of male *P. albofimbriata* antennae.

No. of flagellomeres	Grooved peg sensilla (Mean±SD, n=6)	Sensilla trichodea (Mean±SD, n=6)	Sensilla basiconica (Mean±SD, n=6)
31	100.83 ± 2.67	7.00 ± 2.89	3.17 ± 2.11
32	110.50 ± 4.99	7.50 ± 2.50	2.00 ± 0.82
33	113.50 ± 4.99	8.83 ± 3.34	2.67 ± 1.70
34	108.83 ± 2.67	7.33 ± 4.53	1.33 ± 0.94
35	120.50 ± 4.99	5.67 ± 3.50	1.83 ± 0.90
36	118.50 ± 4.99	7.00 ± 3.00	1.83 ± 1.07
37	115.83 ± 2.67	6.67 ± 4.82	2.00 ± 0.82
38	113.50 ± 4.99	8.50 ± 1.38	2.17 ± 1.21
39	107.83 ± 2.67	8.00 ± 3.00	2.00 ± 1.15
40	99.83 ± 2.67	9.33 ± 3.68	3.33 ± 2.36
41	112.17 ± 9.33	9.00 ± 2.77	2.17 ± 1.07
42	110.50 ± 4.99	10.17 ± 1.21	2.50 ± 1.12
43	106.83 ± 2.67	9.00 ± 2.45	2.83 ± 1.57
44	116.50 ± 4.99	7.83 ± 3.08	3.00 ± 1.63
45	110.83 ± 3.24	9.00 ± 4.40	2.33 ± 1.25
46	113.83 ± 2.67	9.67 ± 1.25	2.83 ± 2.11
47	117.50 ± 2.99	10.50 ± 3.64	3.17 ± 1.86
48	120.50 ± 4.99	11.83 ± 3.80	1.67 ± 0.94
49	108.83 ± 2.67	10.50 ± 2.75	3.50 ± 0.96
50	117.83 ± 5.81	11.83 ± 0.69	2.67 ± 0.75
51	113.83 ± 2.67	13.00 ± 2.77	1.83 ± 0.69
52	109.17 ± 2.79	11.50 ± 3.69	2.17 ± 0.69
53	111.83 ± 2.67	13.67 ± 5.76	1.67 ± 0.47
54	124.50 ± 4.99	18.67 ± 3.50	2.67 ± 0.47
55	130.17 ± 9.33	14.00 ± 5.39	2.00 ± 0.82
56	122.50 ± 4.99	15.17 ± 2.79	0.67 ± 0.75
57	111.83 ± 2.67	16.17 ± 3.08	0.00
58	107.83 ± 2.67	12.83 ± 3.89	0.00
59	109.83 ± 2.67	12.83 ± 4.84	0.00
60	124.17 ± 6.67	13.67 ± 3.09	0.00

Table 3. Number of olfactory sensilla on the distal part (flagellomeres #61 to the tip) of male *P. albofimbriata* antennae.

No. of flagellomeres	Grooved peg sensilla (Mean±SD, n=6)	Sensilla trichodea (Mean±SD, n=6)
61	124.50 ± 2.67	13.33 ± 3.04
62	116.83 ± 4.99	14.33 ± 1.80
63	108.83± 2.67	13.67 ± 1.70
64	89.17 ± 9.33	15.83 ± 3.39
65	93.83 ± 2.67	16.67 ± 1.70
66	100.50 ± 4.99	15.33 ± 1.80
67	102.50 ± 4.99	19.00 ± 2.94
68	93.83 ± 2.67	18.00 ± 4.43
69	101.50 ± 4.99	15.50 ± 1.80
70	101.50 ± 2.99	16.50 ± 4.39
71	89.83 ± 2.67	13.67 ± 2.13
72	105.83 ± 3.53	18.00 ± 2.38
73	85.17 ± 2.79	15.00 ± 2.45
74	79.83 ± 2.67	16.17 ± 3.13
75	82.17 ± 2.79	18.00 ± 3.74
76	71.17 ± 5.81	18.50 ±2.69
77	80.17 ± 2.67	18.33 ± 2.21
78	89.50 ± 4.99	11.67 ± 8.28
79	73.83 ± 2.67	13.17 ± 10.02
80	71.83 ± 2.67	19.00 ± 3.46
81	64.17 ± 3.53	15.67 ± 7.67
82	65.83 ± 2.67	20.00 ±1.41
83	44.50 ± 2.63	17.33 ±2.43
84	43.83 ± 2.67	19.17 ±0.37
85	41.83 ± 2.67	21.50 ± 2.50
86	42.83 ± 2.67	19.17 ± 3.58
87	33.17 ± 3.53	18.50 ± 1.80
88	37.83 ± 2.67	18.67 ± 2.98
89	42.17 ± 2.67	17.00 ± 1.00
90	22.50 ± 3.86	16.83 ± 1.95
91	27.83 ± 2.67	15.67 ± 0.47
92	29.50 ± 2.63	15.33± 1.49
93	28.00 ± 3.51	14.17 ± 0.37
94	24.00 ± 3.85	15.20 ± 0.98
95	20.00 ± 3.85	16.00 ± 1.10
96	19.00 ± 3.85	15.00 ± 1.67
97	17.00 ± 3.85	13.60 ± 1.50
98	18.50 ± 1.50	13.00 ± 2.35
99	15.50 ± 2.50	13.25 ± 2.28
100	11.50 ± 0.50	11.00 ± 1.00
101	10.00 ± 0.00	9.00 ±0.00

Appendix B

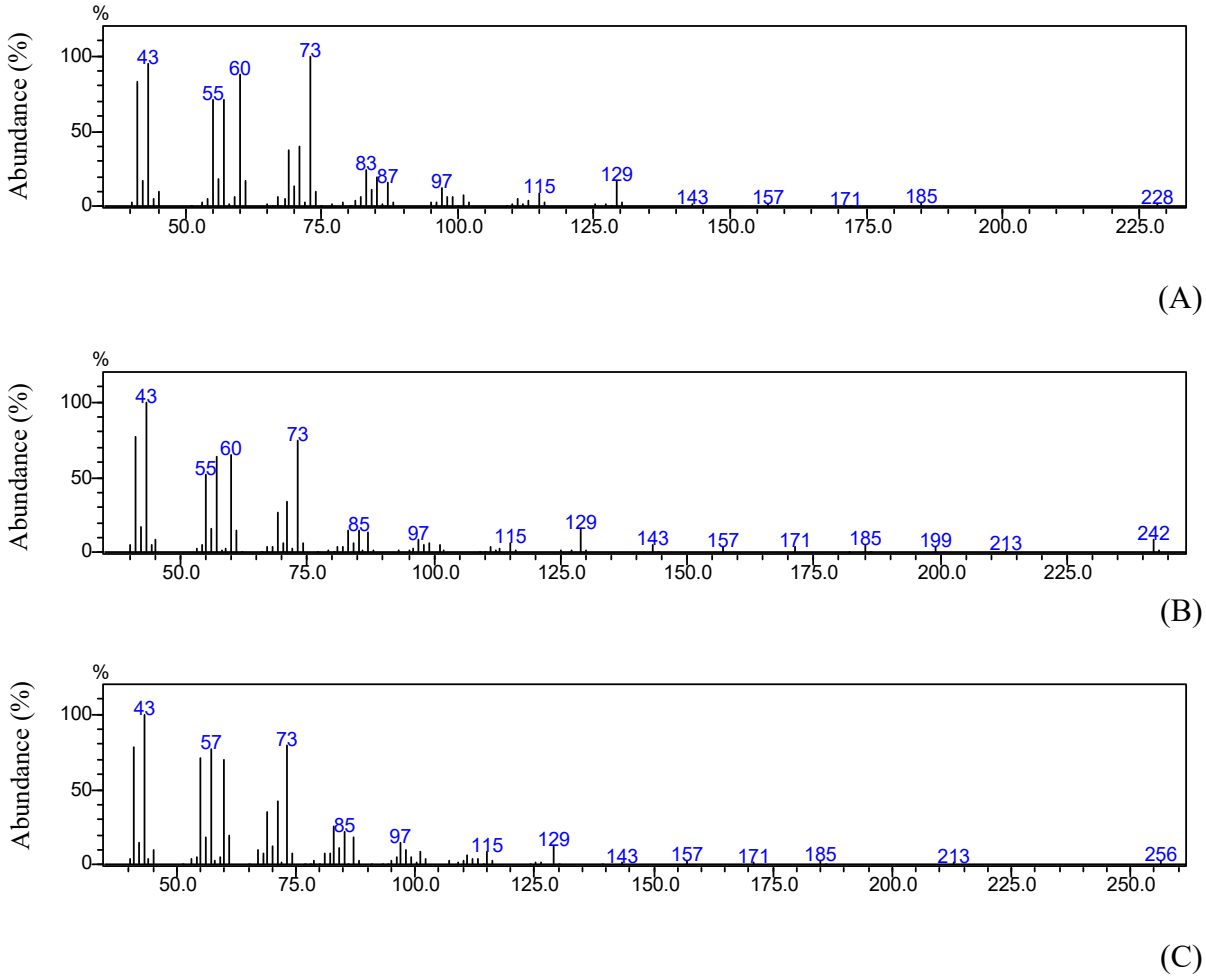
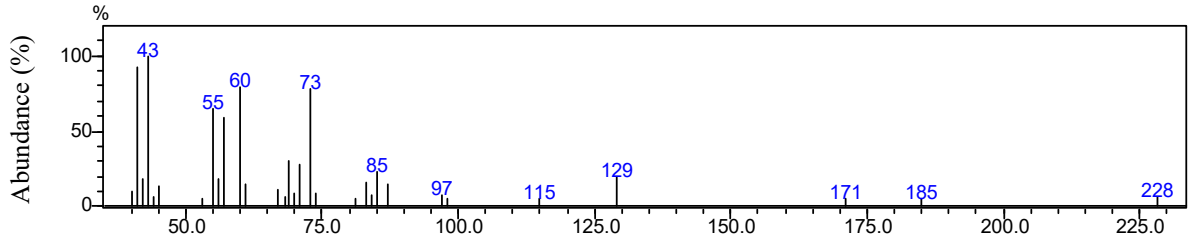
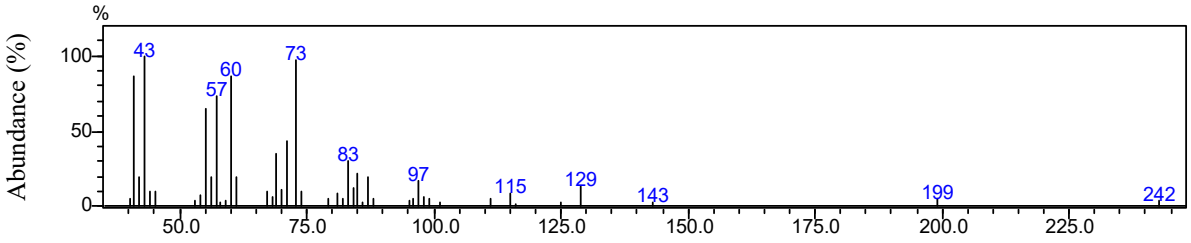


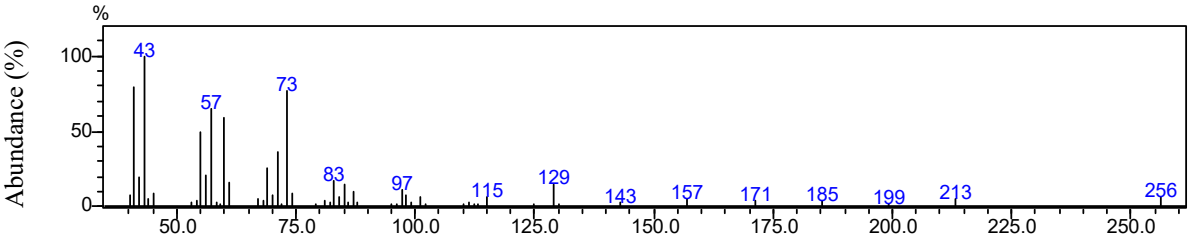
Figure 1. Electron impact mass spectra of compound I (A), compound II (B) & compound III (C) found in female mantid SPME sample. X-axis shows mass charge ratio (m/z).



(A)



(B)



(C)

Figure 2. Electron impact mass spectra of authentic standards tetradecanoic acid (A), pentadecanoic acid (B), hexadecanoic acid (C). X-axis shows mass charge ratio (m/z).