UNIVERSITY OF CALIFORNIA, SAN DIEGO

Honest signaling? Testing the toxic pheromone hypothesis in the highly social bees, *Lestrimelitta niitkib and Apis mellifera*

A Thesis submitted in partial satisfaction of the requirements
For the degree Master of Science

in

Biology

by

Chase Chandler James

Committee in charge:

Professor James Nieh, Chair
Professor David Holway
Professor Joshua Kohn

2016

The Thesis of Chase Chandler James is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

Chair

University of California, San Diego

2016

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

Many thanks to my advisor Dr. James Nieh, Dr. Daniel Sanchez and the rest of my collaborators in Tapachula, Mexico, as well as volunteers in the Nieh Lab at UCSD (Karen Santos, Danielle Nghiem, Alex Neskovic, Anna Dipaola, Thomas Leung, and many more). My work in Tapachula was made possible with funding through UC MEXUS.

ABSTRACT OF THE THESIS

Honest signaling? Testing the toxic pheromone hypothesis in the highly social bees, *Lestrimelitta niitkib* and *Apis mellifera*

by

Chase Chandler James

Master of Science in Biology

University of California, San Diego, 2016

Professor James Nieh, Chair

Species in the stingless bee genus, *Lestrimelitta*, like *L. niitkib*, are all obligate cleptoparasites. Rather than foraging for resources, these bees rob the colonies of other social bees, native and introduced. Multiple hypotheses have been proposed as to why *L. niitkib* and other *Lestrimelitta* species are so successful at robbing. In all hypotheses, the copious release of mandibular gland pheromone (MGP) during robbing plays a key role. We propose a new hypothesis, that MGP is a toxin that also honestly signals the greater physical fighting ability of *L. niitkib*. To test this hypothesis we examined the fighting ability and mandible morphology of *L. niitkib*. We also injected natural and synthetic MGP into victims. In both fighting and injection trials, victims increased their rate of falling, abnormal movement, and time spent

motionless. Given that *Lestrimelitta* can repeatedly raid the same colonies, victims should be able to learn to associate MGP with attacks. We therefore propose that multiple MGP hypotheses can be considered under the umbrella of honest signaling, in which the honestly superior attack ability of *L. niitkib* is associated with MGP. In addition, we also have conducted preliminary experiments examining whether honey bee mandibular gland secretions (MGS) are an alarm pheromone. We hypothesize that marking predatory targets at the entrance of the hive with MGS should elicit more attention if MGS is indeed an alarm pheromone. We also tested the toxicity of MGS and found weak support for the hypothesis that it is toxic.

CHAPTER 1: HOW DOES *LESTRIMELITTA NIITKIB* WIN? TESTING THE EFFECTS OF COMBAT AND MANDIBULAR GLAND PHEROMONE.

INTRODUCTION

Insect chemical warfare is pervasive. In social insects such as termites, bees, and ants, chemicals are used in both aggression and defense (Sobotnik et al. 2010; LeBrun et al. 2014). Chemical warfare can often create an evolutionary arms race in which species must constantly evolve to develop better methods of both attacking and defending (LeBrun et al. 2014). Insect chemical warfare in particular provides a unique glimpse into how species may adapt in order to overcome their predators, prey, and competitors.

Lestrimelitta, a genus of neotropical eusocial bees found throughout both Central and South America (Michener 2000), provides an intriguing potential example of chemical warfare. The literature focuses upon two widespread species. *L. limao* and *L. niitkib*, which exhibit similar behaviors and emit large amounts of similar citral-based alarm pheromones during recruitment and robbing (Sakagami et al. 1993; Quezada et al 1999). These obligate robber bees do not forage for nectar or pollen and have lost the corbicular hairs that allow bees to transport pollen (Michener 2000). Instead, *Lestrimelitta* forages by ingesting and stealing brood resources from other bee colonies by carrying these resources in their mouths or crop (Sakagami 1963). Their ability to successfully raid other bee colonies is therefore essential to their fitness, but this phenomenon also represents a longstanding mystery because we do not have a clear understanding of how and why they usually are successful in robbing.

Victim species can consist of physically larger bees, species with colonies with a defense force that outnumbers *L. limao* raiding parties, or both, but *L. limao* usually wins interspecific confrontations (Sakagami et al 1993). Several hypotheses have been proposed for the interference ability of *L. limao*: the size of raids (swarm size), individual fighting strength, and alarm pheromone release (Sakagami et al 1993, Johnson 1987, Wittman et al. 1990). In the swarm size hypothesis, *Lestrimelitta* attacks, such as those launched by *L. limao,* include a large number of raiders (up to 600 bees) that overrun host colonies (Sakagami et al 1993). However even in large raids, *L. limao* can be outnumbered by the host colony (Sakagami et al 1993). Therefore, superior numbers alone may not always explain the success of *L. limao*. More recently, researchers demonstrated that *L. niitkib* may be able to evade detection, at least during the scouting portion of a raid, by mimicking cuticular hydrocarbons produced by some victim species (Quezada-Eúan et al. 2013).

Lestrimelitta limao foragers are excellent fighters (Grüter et al. 2012) and their physical ability to dominate may arise from multiple traits such strength, endurance, or increased robustness of their mandibles, the main physical weapon of stingless bees (Roubik 1992). In ants, changes in jaw morphology and robustness, dramatically illustrated by soldier castes, facilitate cutting or crushing of enemies (Hölldobler and Wilson 2009, Hölldobler and Wilson 1990, Helanterä and Ratnieks 2008). Biting also provides a route for stingless bees, like *Oxytrigona* to spread their mandibular gland pheromone (MGP) onto the bodies of victims and into the bite wound (Blum 1981, Roubik et al. 1987). In *L. limao*, biting plays a key role in their ability to raid (Nogueira-Neto 1970), particularly when attacking *Scaptotrigona postica* (Sakagami et al. 1993).

MGP plays a key role in these raids. *Lestrimelitta limao* and *L. niitkib* are known for the strong and distinctive odor of their alarm pheromones, which are released in large amounts during a raid (Sakagami et al 1993). This raiding/alarm pheromone is produced in the mandibular glands, and consists of two citral isomers: geranial and neral (Blum et al. 1970). There are four traditional hypotheses reguarding this pheromone. (1) The “skunking hypothesis” states that the pheromone overloads the olfaction of victim species, disorienting them (Blum et al. 1970; Sakagami et al. 1993). (2) Similarly, the “masking or supersedure hypothesis” holds that this raiding pheromone reduces the ability of victim species to perceive their own pheromones and thus compromises their defense (Kerr 1951; Moure et al. 1958; Johnson 1987). (3) The “tranquilizer” hypothesis is that the raiding pheromone induces submission in victim species (Sakagami et al. 1993). In *Scaptotrigona pectoralis*, the initial release of MGP elicited frantic activity among the victims for about 10 min, followed by torpor (Sakagami et al. 1993). Finally (4) the citral in MGP may function as a venom (Sakagami et al. 1993). Sakagami et al. (p273, 1993) write, “biting is frequently (sic) only when robbers are first arriving or after they begin to depart, and it at such times that odor concentrations appear highest”. For MGP, we therefore focus on the toxicity hypothesis because it provides a mechanism for the torpor effect described by Sakagami et al. (1993) and can be simply and directly tested. Throughout this process, the strong citrus-like odor of *L. limao* alarm pheromone pervades the nest.

We also propose a different perspective on this the venom hypothesis: MGP is an inherently honest signal of the fighting ability of *L. niitkib*. MGP may be associated, innately or learned, with a genuinely superior fighting ability, chemical toxicity, or both. Stingless bee colonies do not often relocate, and *L. niitkib* usually does not kill

the victim colony (Sakagami et al.1993). A colony of *L. niitkib* is therefore likely to exploit the same local victim colonies repeatedly, giving their victims the opportunity to associate the alarm pheromone of *L. niitkib* with a successful raid (Sakagami et al.1993). *Lestrimelitta limao* is known to regularly attack the same colonies in its territory (Sakagami et al. 1963). Such interspecific honest signaling is widespread and can be evolutionarily robust, particularly when the costs of ignoring honest information are high. For example, aposematic visual signaling warns that a signaler is toxic and occurs in a wide variety of taxa: insects, mammals, fishes, amphibians, reptiles, and molluscs (Blount 2008). Although toxic defensive or offensive compounds are not classically considered to be examples of honest communication, these olfactory signals should be learned by survivors and associated with peril, a process that could also evolve into innate recognition and avoidance of such odors.

Consider the fascinating behavior of raided bee colonies. Sakagami (1993) describes a mass raiding phenomenon in which a swarm of hundreds of *L. limao* foragers descend upon the raided colony. This raid can last for hours or days (Sakagami & Laroca 1963). Raided colonies initially resist and fight the raiders to varying degrees, depending upon the victim species. As the raid progresses, workers evacuate their nest and others hide in far reaches of the nest and avoid contact with the pillaging raiders. Once the brood food has been removed, the raiders depart and victims usually return (Sakagami 1993, Nogueria-Neto 1970). In some cases, colonies do not survive being raided, but in many cases, they live to be raided again (Sakagami et al. 1993; Nogueria-Neto 1970).

Why *Lestrimelitta* generally wins, and the role of MGP in these raids are therefore fascinating questions. We studied *L.* niitkib, which ranges from the Yucatan Peninsula through the state of Chiapas, Mexico (Quezada et al 1999). Little is known

about the raiding behavior of this species, because large raids do not occur frequently and their documentation has typically relied upon chance observations (Quezada et al 1999). Sakagami et al. (1993) conducted the most extensive study on mass raiding by *Lestrimelitta,* focusing on *L. limao*. Although mass raids may be the most spectacular aspect of *L. limao* foraging, in *L. niitkib*, we observed foragers returning to their nests throughout the day to unload food to nestmates on and inside the nest entrance. We used time-lapse photography of the nest entrances of two colonies to measure finer scale fluctuations in this foraging activity.

To test the effects of fighting, we began with paired battles between *L. niitkib* and *Scaptotrigona mexicana* individuals. Both species are sympatric, and L. *niitkib* commonly attacks *S. mexicana* (Quezada et al. 2002). We first measured the behavioral effects of fighting. Because *L. niitkib* usually won these bouts after biting the victim, we used light microscopy and scanning electron microscopy to measure the morphology and thickness of attacker and victim mandibles. During these fights, we detected the characteristic citrus-like odor of *L. niitkib* mandibular alarm pheromone (Blum 1970) on victim bodies. We used gas chromatography-mass spectrometry (GC-MS) to chemically analyze this pheromone and then measured the effects of natural pheromone, synthetic pheromone, and individual synthetic pheromone components applied to victim wounds.

METHODS

Colonies and study site

We conducted our study on the campus of El Colegio de la Frontera Sur (ECOSUR) in Tapachula, Chiapas, Mexico. Although *L. niitkib* is widespread, it is relatively rare, as expected of a cleptoparasite that usually contains several thousand

bees per colony (Roubik 1992). We obtained two natural *L. niitkib* colonies from the surrounding area. For each colony, we cut down the tree to obtain a trunk section that housed the colony. Colonies were placed on opposite sides of the ECOSUR campus. We never observed the *L. niitkib* colonies raiding each other. Victims consisted of *S. mexicana* colonies obtained from trees around Tapachula and housed in wood hives at the ECOSUR campus.

Hive entrance activity

Our earlier attempts to transplant *L. niitkib* colonies into observation boxes were not successful, resulting in their rapid death. This is a common problem with attempts to re-house many species of stingless bees (Roubik 1992). Although the *L. niitkib* colonies could not be moved to observation nests, we were able to visualize activity inside the nest entrance of each colony by inserting a clear polycarbonate tube (35 mm diameter x 200 mm length x 25 mm inner diameter) supported on a tripod between the bee-built nest entrance and the nest base (Fig. 1). By taking photos of nest entrance activity before and while the tube was in place, we observed that the tube had no effect upon activity after an initial adjustment period of 1 hr.

For each colony, we placed a GoPro Hero 3 camera to take time-lapse photos of the colony entrance each minute over a 24 h period. Multiple time series were captured throughout the field season, with a total of 40 days captured throughout a 6-month period. Photos were taken at two angles: a side profile view and a frontal view (Fig. 1). The number of bees at the entrance was counted for the frontal view and the number of bees in a 10 cm section was counted for the profile view. We measured light intensity, humidity, and temperature each minute with HOBO sensors (UA-002-

064 and U12-012, Onset, Bourne, Massachusetts 02532) that were synchronized with the cameras.

Mandible Comparisons

To compare mandible morphology, mandibles from *L. niitkib* and *S. mexicana* and *Tetragonisca angustula* were detached from the head capsule at their joints. Some mandibles were cut open with a razor to measure the thickness of the exoskeleton using scanning electron microscopy (SEM). Using calibrated photos taken with a dissecting microscope, we made four measurements: proximal width (near head), medial width, distal width (near the biting edge), and thickness of the exoskeleton at the distal width measurement (Fig. 2.1).

Fight trials

To determine the outcomes of individual fights, we paired one *L. niitkib* with one *S. mexicana* worker. We captured guards of both species by approaching their respective nest entrances with a clean glass vial and capturing bees that flew at the vials. The vials were then capped with cotton and immediately brought back into the lab for testing. In the lab (about 1 min after capture), we removed the cotton, checked for the odor of *L. niitkib* MGP (bees that premature released MGP were not used), brought both open vial ends together, and briefly and lightly agitated the vials to bring the bees together. If no attack occurred after 3 min, the trial ended, bees were chilled to reduce their motion, painted with permanent acrylic paint on their thoraces to insure that they would not be reused, and then released. Once an attack occurred, defined as the bees grappling or biting each other, we allowed the attack to continue for 5 s before gently and carefully separating the bees with tweezers and placing

them in small petri dishes 35 mm x 10 mm. The bees were then observed for 20 min. We recorded the following: presence of absence of *L. niitkib* alarm pheromone release (easily detected because of its strong, characteristic odor), if a bee was bitten, if any limbs or wings were lost, the rate of major falls per time spent moving, whether the bee exhibited abnormal movement (spinning in circles, not using all of its legs to walk), and total time spent motionless. Based upon our preliminary trials, we defined a major fall as a bee flipping over and remaining on its thorax for ≥3 s.

At the end of each trial, we chilled, painted and released the bees. In our 31 fight trials, *S. mexicana* died during or after 32% of trials. In contrast, *L. niitkib* only died during or after 6% of trials. However, the timing of death was inconsistent. Therefore we chose to not analyze bee deaths in our results. In control trials, individuals were identically captured, but were briefly agitated, as in the fight trials, and then placed in identical separate containers and observed for 20 minutes. No bees died in any of these control trials.

Chemical analysis of MGP

Synthesis of chemical standards

Neral and geranial were synthetized in good yields (95% and 94%, respectively) from their respective alcohols (nerol and geraniol) by a Corey's oxidation/SiO_2 (Fernandes & Kumar, 2003; Luzzio, Fitch, Moore, & Mudd, 1999). For this procedure, pyridinium chlorochromate was freshly prepared. Pure aldehydes were obtained after purification of crude extracts with a flash column chromatography using hexane-acetone (95:5) as eluent. All chemicals were purchased from Sigma Aldrich.

Chemical analyses

Lestrimelitta niitkib foragers were collected from nest entrances as described above. The mandibular glands (two glands per bee) of six foragers from colony one and four foragers from colony two were carefully dissected out under a stereoscopic microscope and then macerated with 1 mL of hexane for 5 min. Each sample consisted of material obtained from a single bee. The extracts were concentrated using a gentle stream of dry N_2 to a volume of 400 µL per sample and were stored in a -20°C freezer until analysis.

Extracts were analysed on a CG-MS Varian Star model 3400 CX GC (Palo Alto, CA, USA). A DB-5 column (30 m x 0.25 mm ID) was temperature programmed from 50°C (held for 2 min) to 280°C at 15°C min^{-1} and held at 280°C for 10 min. The temperature of the injector was held at 250°C. The GC was coupled to a Varian Saturn 4D mass spectrometer and integrated data system. Ionization was carried out by electron impact at 70 eV, 250°C. Compounds were verified and quantified with pure, previously synthesized neral and geranial standards. Pheromone components were quantified by measuring the area under each peak in comparison with external standard curves. To prepare the calibration curves, neral was diluted to 3 ng/□L, 9 ng/□L, 14 ng/□L, 29 ng/□L, 57 ng/□L, 287 ng/□L, and 574 ng/□L. Geranial was diluted to 6 ng/□L, 17 ng/□L, 28 ng/□L, 56 ng/□L, 112 ng/□L, 557 ng/□L, and 1114 ng/□L.

Testing the toxicity of MGP

During fights, we often observed *L. niitkib* using its mandibles to puncture *S mexicana,* as described by Sakagami et al. (1993) for *L. limao* attacking *S. postica*. These small puncture wounds smelled strongly of *L. niitkib* alarm pheromone. We

therefore conducted injection trials to explore the toxicity of *L. niitkib* MGP on *S. mexicana* and test the hypothesis of MGP toxicity (Sakagami et al. 1993).

We injected individuals of *S. mexicana* with five types of treatments in a total volume of 1 µl of insect ringer's solution (Yamasaki and Narahashi 1959): control (ringers only), 1 bee-equivalent (1 BE) of natural MGP extract, 1 BE of synthetic mixture of the main components (geranial and neral in a 5.25:1 natural ratio), and different levels of pure synthetic neral (1 BE) and geranial (0.1, 0.5, or 1 BE). Natural MGP extracts were collected from dissected mandibular glands. We did not test lower levels of pure neral because 1 BE of neral had no effect (see Results). However, we tested the effects of lower geranial levels because 1 BE of geranial significantly impaired *S. mexicana* guard bees.

For these trials, guard bees were captured in glass vials as described previously. For the injections, they were transferred into a holding tube in the lab. The tube (30 mm diameter x 80 mm long) had a mesh cover on one side and an opening on the other. Bees were placed into the opening and a soft foam plunger was then inserted into the tube until the bee gently rested against the mesh. This technique allowed bees to be injected with solutions without being chilled or injured. Bees were injected in the abdomen with a very fine Hamilton syringe (#701) and then placed in a small petri dish 35 mm diameter x 10 mm high and observed for 20 min. As in the fight trials, we recorded the rate of major falls per time spent moving, whether the bee exhibited abnormal movement (spinning in circles, not using all of its legs to walk), and total time spent motionless.

Statistical analyses

For our analysis of nest entrance activity, we ran Pearson correlations comparing entrance abundance time series versus our light, temperature, and humidity variables. We also correlated entrance activity between the two colonies. These correlations were run with a time lag of 0 using R v 3.2.3

To test for differences between mandible morphology, we ran a repeated measures analysis of variance (ANOVA) for each measurement, species and measurement as fixed effects, and the interaction species*measurement.

For fight and injection trials, we used an ANOVA to compare continuous variables with treatment as a fixed effect and colony as a random effect. For nominal variables, we used Nominal Logistic regression (Pearson's) in the analysis of fight data and injection data. We used Tukey's Honest Significant Difference (HSD) tests to make multiple pairwise comparisons. We applied the Sequential Bonferroni correction to our analyses of the number of major falls and the rate of major falls (k=2) and denote significant P-values as ^{SB}. These analyses were run with JMP Pro v11.

RESULTS

Hive Entrance Activity

We observed returning foragers exchanging food with nestmates inside the clear entrance tube, suggesting that they had just returned from a raid (Sakagami 1993). The level of activity was episodic throughout the day (Fig. 1). Colony one and colony two time series were highly correlated with one another (r = 0.645, P < 0.0001), suggesting that raiding activity was synchronized with external factors. Activity in both colonies was positively correlated with temperature (r > 0.492, P <

0.0001) and light ($r > 0.232$, $P < 0.0005$) and negatively correlated with humidity ($r < -0.493$, $P < 0.0001$). However, it is important to note that these three variables were intercorrelated ($|r| > 0.678$, $P < 0.0001$).

Mandible Comparisons

In total we analyzed 17 *L. niitkib* mandibles, 20 *S. mexicana* mandibles, and 12 *T. angustula* mandibles. *Lestrimelitta niitkib* mandibles lack the slender medial width of mandibles of the two victim species, *T. angustula* and *S. mexicana* (Fig. 2). Moreover, all measurements are significantly different for each species (Proximal Width $F_{2,46}= 302.56$, $P < 0.0001$; Medial Width $F_{2,46}= 332.05$, $P < 0.0001$; Distal Width $F_{2,46}= 100.99$, $P < 0.0001$). Preliminary data on the thickness of the mandible exoskeleton is also suggestive: *L. niitkib* (24.85 μm) appears to have thicker mandibles than *T. angustula* (15.61 μm) and *S. mexicana* (10.69 μm), but further replicates are planned because of the small sample sizes (two bees per thickness measurement).

Fight trials

In total, we analyzed 21 control trials and 31 fight trials with *S. mexicana* from four different colonies and *L. niitkib* from two different colonies (Fig. 3). We detected *L. niitkib* alarm pheromone on the bodies of *S. mexicana* victims in 100% of fight trials and none in any of the control trials. In the fight trials, *S. mexicana* was bitten in 100% of trials and *L. niitkib* was bitten in 90.3% of trials, significantly less ($\chi^2_1 = 4.311$, $P = 0.0379$).

In fight trials, *L niitkib* was more active and tried to fly significantly more often than did *S. mexicana* (4.5-times more, $\chi^2_1 = 25.833$, $P < 0.0001$). For trials in which no

fights occurred there was no significant difference between *L. niitkib* and *S. mexicana* trying to fly (χ^2_1= 0, *P* = 1.000). *Scaptotrigona mexicana* had nearly 12-times more body parts cut off than *L.* niitkib (χ^2_1= 13.461, *P* = 0.0002), and *S. mexicana* exhibited 2.6-times more uncoordinated movements compared to *L. niitkib* in fight trials (χ^2_1= 17.953, *P* < 0.0001). Neither species showed uncoordinated behavior in the no-fight trials. *Scaptotrigona mexicana* also was more likely to have paralyzed body parts (legs or wings) (χ^2_1= 9.04, *P*= 0.0026).

There was a significant effect of treatment on the time spent motionless (seconds) (*P* < 0.0001, $F_{1,101}$= 83.397), a significant effect of species ($F_{1,100}$= 127.726, *P* < 0.0001), and a significant interaction of treatment*species ($F_{1,100}$= 83.397, *P* < 0.0001, 3% colony effect) because *S. mexicana* spent 24-fold more time motionless than *L. niitkib* in fight trials.

There was a significant effect of treatment on the number of major falls per time spent in motion ($F_{1,101}$= 11.581, *P* = 0.0014 ^{SB}), a significant effect of species ($F_{1,100}$= 10.809, *P* = 0.0014 ^{SB}), and a significant interaction of treatment*species($F_{1,100}$= 36.0305, *P* < 0.0001 ^{SB}, 0% colony effect) because *S. mexicana* fell 4.7-fold more per time spent in motion than *L. niitkib* in fight trials.

L. niitkib MGP Analysis

Using gas chromatography-mass spectrometry, we analyzed extracts of *L. niitkib* mandibular glands to assess the chemical composition of MGP (Fig. 4). All values represent the sum of both glands per bee, yielding 1 bee equivalent. Both colonies yielded workers with similar amounts and ratios of geranial and neral: colony one (0.282±0.054 µl geranial, 0.056±0.003 µl neral, geranial/neral ratio=5.06) and colony two (0.282±0.032 µl geranial, 0.055±0.003 µl neral, gernial/neral ratio=5.18)

Overall, each bee had an average of 0.283 μL (253 µg) of geranial and 0.054 μL (48 µg) of neral (g/n ratio of 5.25 ± 0.56:1). These volumes would be our standard for 1 bee equivalent (BE) in the injection trials.

Injection Trials

In total we injected 159 bees with varying concentrations. For each injection type, we used 20 bees (except for natural MGP extracts from colony two, which used 19 bees). MGP treatment significantly increased abnormal movement (χ^2_6= 89.497, P < 0.0001). Each of the following increased: number of major falls ($F_{6,152}$= 35.156, P > 0.0001^{SB}, colony accounted for 2% of model effect), the rate of major falls per time spent moving ($F_{6,152}$= 19.779, P > 0.0001^{SB}, 0% colony effect), time spent motionless ($F_{6,152}$= 3.637, P = 0.0021, 4% colony effect) with increasing MGP dose (Fig. 5). In our trials, 1 BE of MGP, 1 BE of synthetic MGP, 1 BE of geranial, and 0.5 BE of geranial caused the most detrimental effects.

DISCUSSION

Lestrimelitta niitkib evidently uses multiple strategies to successfully raid nests and appears to do so, at least at low level, on a daily basis. Pulses in foraging may vary daily depending on weather conditions and resource availability, as it does for many bee species (Vicens and Bosch 2000). Due to the high correlation we found between colonies, external and internal drivers may similarly affect colony raiding perhaps because *Lestrimelitta* foraging, like that of non-cleptoparasitic bees, is strongly shaped by environmental variables like light, temperature, and humidity (Vicens and Bosch 2000).

Our fight trials demonstrated the combat efficacy of *L. niitkib*. Following fights, *S. mexicana* exhibited more crippled behavior than did *L. niitkib*, perhaps reflecting greater injuries. For example, 39% of all *S. mexicana* lost limbs in fights, and approximately 32% had paralyzed body parts. *Scaptotrigona mexicana* also had 4.7-fold more major falls per time spent moving than *L. niitkib* after fights (Fig. 3). *Scaptotrigona mexicana* exhibited 2.6-fold more abnormal, uncoordinated movement than *L. niitkib* in fight trials. Finally, in fight trials *S. mexicana* tried to fly 5-fold less than did *L. niitkib*.

Lestrimelitta niitkib mandibles may be better suited to combat whereas its victims, similar to honey bees (*Apis mellifera*), may have “spoon-shaped” mandibles that are capable of multiple tasks, yet may be disadvantageous when in combat (Winston 1991). This difference may especially be important when having to deal with a more robust fighter such as *L. niitkib*, which has significantly thicker mandibles proximally and medially (Fig. 2). We are currently collecting more data on mandible exoskeleton thickness and mandible size relative to body size. However, our results show that *L. niitkib* clearly has bigger mandibles at the proximal and medial points than does either victim species.

Our data support the toxic pheromone hypothesis because similar behavioral changes were elicited by injecting relevant doses of MGP into *S. mexicana*. One bee equivalent of natural MGP, Synthetic MGP, and geranial significantly impaired *S. mexicana,* increasing the number of major falls, the rate of major falls, and the amount of time spent motionless. Although 1 BE is a relatively large amount, we observed significant impairment in number of falls following exposure to a lower dose of 0.1 BE of geranial.

Although 100% of *S. mexicana* victims in all fight trials were bitten and all bore the strong, characteristic odor of *L. niitkib* MGP on their body, it would be desirable to determine the amount of MGP that typically penetrates into a victim. We are currently conducting an experiment to measure this amount. However, a large amount of MGP was not necessary. In fact, 0.1 bee equivalent of MGP was sufficient to significantly impair a victim (Fig. 5).

Unlike most venom compounds, both geranial and neral are structurally simple compounds (Casewell et al. 2013). In fact, citral and its isomers, geranial and neral, are widespread in the mandibular gland pheromones of multiple stingless bee species, where they are also used in alarm communication (Blum 1970). However toxicity also depends on the dose. Even compounds with low inherent toxicity, can be poisonous if present in high enough concentrations. For example, the pygidial glands of some ants produce benzaldehyde, which may act as an alarm pheromone. Furthermore, in these ant species, this gland is larger and produces larger volumes of this pheromone that can potentially act as a toxin (Hölldobler et al. 2013, Hölldobler and Engel 1978). In honey bees (*Apis mellifera*), the mandibular gland pheromone, whose key active component is 2-heptanone, is injected into victims like nest parasites and is also toxic, leading to paralysis, similar to our observation of increasing time spent immobile (Shearer and Boch 1965; Papachristoforou et al. 2012).

While we believe that honest signaling plays an important role in the success of raiding by *L. niitkib* and *L. limao,* we acknowledge that the previous hypotheses of skunking, masking, and tranquilizing are still credible. Rather than discard these previous hypotheses, we believe that an honest signal interpretation provides a useful

context to consider multiple MGP hypotheses because it clarifies why victims may choose to hide or retreat upon detecting *Lestrimelitta* MGP.

Cleptoparasitism is a derived trait in stingless bees (Michener 2000), and thus the ancestor to *Lestrimelitta* was a floral foraging species. However, *Lestrimelitta* appears to use its alarm pheromone to help activate and direct its raids, something that, we speculate, could have favored an increase in pheromone volume per bee. This increase in alarm pheromone produced per bee, in conjunction with its use of mandibles as weapons, could have further favored an increase in alarm pheromone, with the additional benefit of increased toxicity via dosage.

In general, this process may have occurred repeatedly and suggests that similar studies would be useful for other aggressive social insects. If attackers have an alarm pheromone, which is selected to increase in amount per individual as part of its signaling efficacy, then the pheromone may achieve a toxic level for victims. At a certain point, a semiochemical therefore evolves the additional and complementary function of a poison. This processes is particularly true if the alarm pheromone is closely associated with a physical weapon that pierces the victim's exoskeleton, such as mandibles or a sting. In stingless bees, mandibles are the main weapon and nearly all studied species appear to use their mandibular gland pheromone as an alarm pheromone (Roubik 1992). Thus, toxic alarm pheromone could have arisen in multiple stingless bee species.

For instance, stingless bees in the genus *Oxytrigona* (fire bees), secrete compounds like formic acid (Roubik 1987) in their mandibular glands (Kerr and Costa Cruz, 1961) that cause skin lesions in their victims. These compounds are typically viewed as defensive compounds because *Oxytrigona tataira* and *Oxytrigona*

mellicolor are not obligate cleptoparasites. They have corbicular hairs and forage for floral nectar and pollen (Schwartz 1948)

However, in line with our hypothesis about the linked evolution of toxic pheromones and cleptoparasitism, these *Oxytrigona* species steal honey from honey bee nests. Rinderer et al. (p496, 1988) wrote, “During nest plundering, the fire bee produces a cephalic secretion which has a strong but, to humans, pleasant floral odor…honeybees do not defend their nest but remain motionless on the comb, hang in a cluster of bees outside the entrance of the colony, or appear to “wander” in a seemingly disoriented manner over the surface of the comb.” This discription is almost exactly the behavior of victims of *Lestrimelitta* attacks (Sakagami et al. 1993).

Beyond *Oxytrigona*, there are other genera in which to test our hypothesis. The African genus *Cleptotrigona* forages in nest of *Hypotrigona* and does not visit flowers (Michener 2000). It attacks in a very similar way to *Lestrimelitta* (de Portugal-Araújo 1958).

We have shown that *L. niitkib* may in fact be producing an honest signal with its mandibular gland pheromone, which warns victims such as *S. mexicana* of its fighting strength and potential toxicity. This may be beneficial for both the host colonies and robbers and mortality rates decrease with less conflict. Finally, this research is an important first step towards better understanding interspecific honest signaling within the context of insect pheromones.

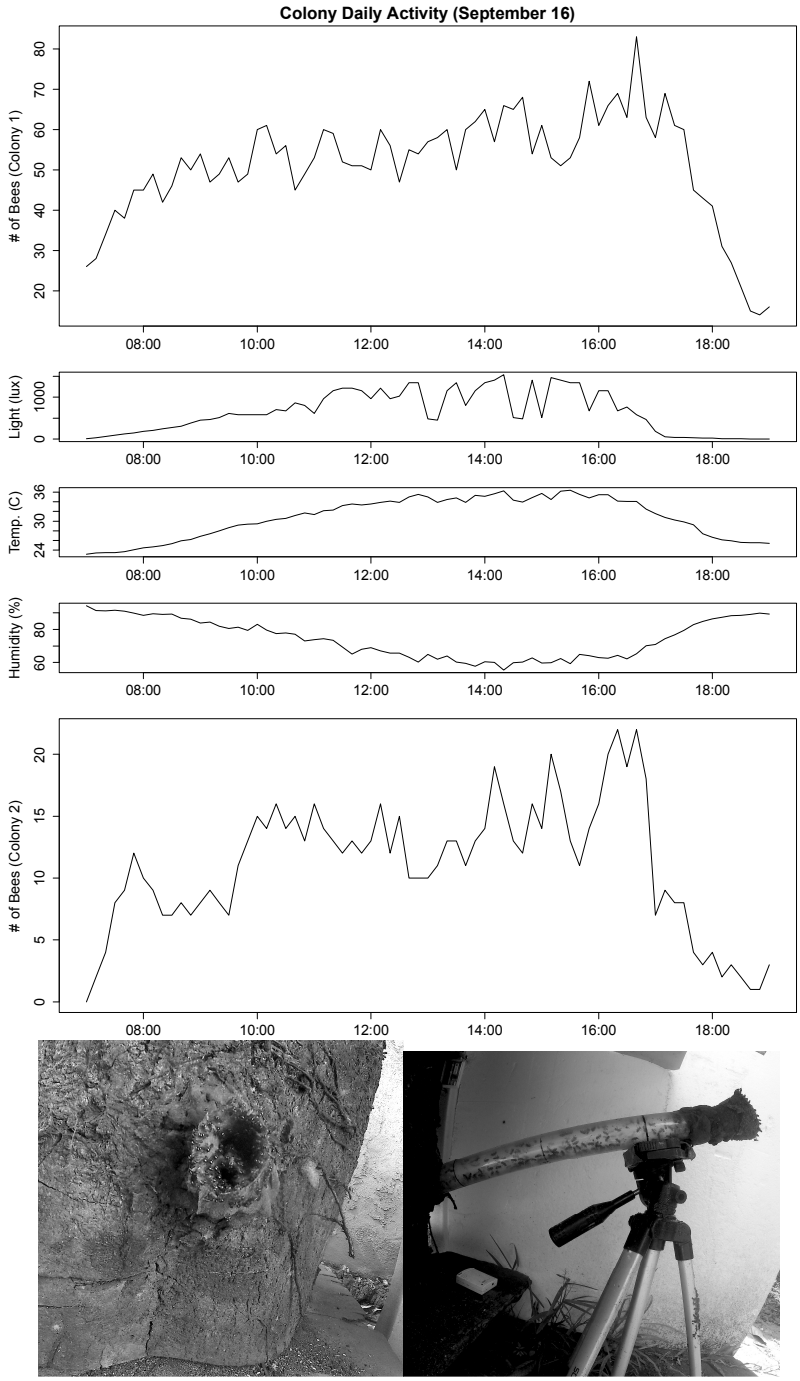

Figure 1. Foraging and entrance activity of *L. niitkib*. Typical foraging activity and correspond abiotic data for both colonies of *L. niitkib*. The number of bees for two colonies (upper and lower graphs) are compared to temperature, light intensity, and relative humidity. Photos show both the frontal and profile views of a colony with and without the observation tube attached.

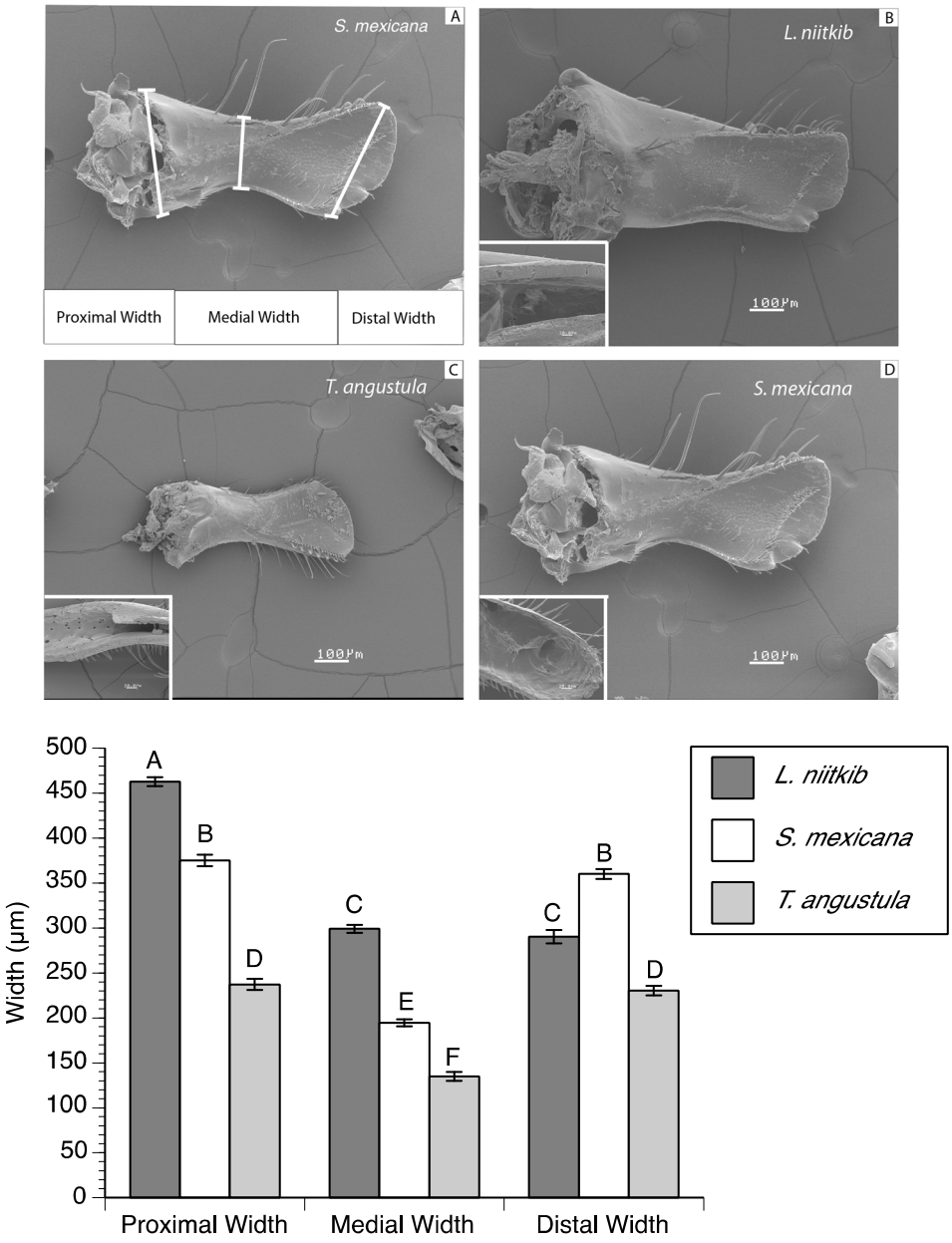

Figure 2. The morphology of stingless bee mandibles. (A) scanning electron micrographs of mandibles of *S. mexicana*, *L. niitkib*, and *T. angustula* (scale bars shown). Inset photos show typical mandible cross-sections of mandibles, revealing exoskeleton thickness. (B) Mean mandible measurements ± 1 standard error. Significant differences shown with different letters (Tukey's HSD test, $P<0.05$).

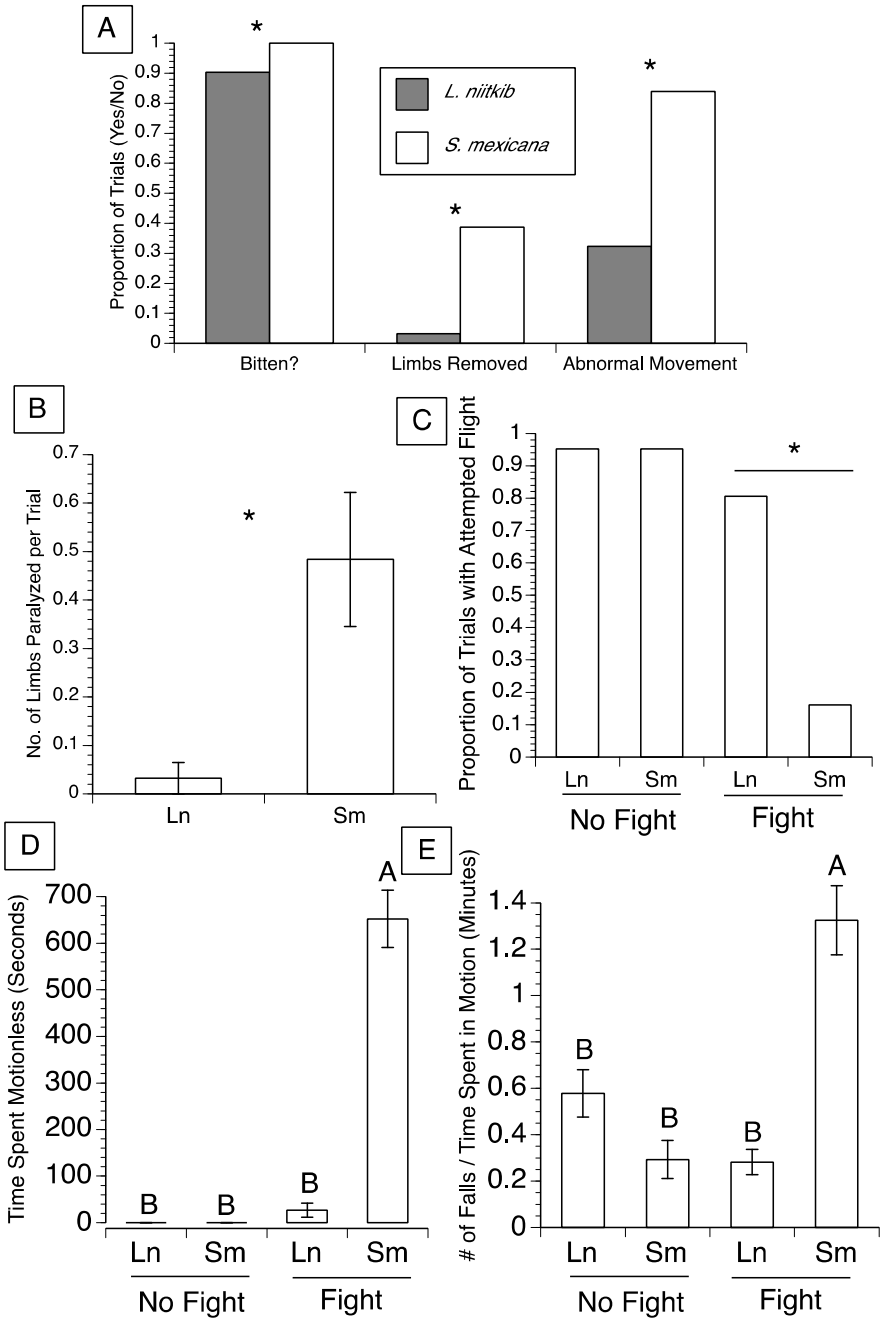

Figure 3. Results of fight trials. (A) Proportion of trials in which participants were bitten, had limbs cut off, and had abnormal movements and (B) the average number of limbs paralyzed per trial. Significant differences indicated with asterisks. (C) Proportion of trials with attempted flight, (D) time spent motionless, and (E) rate of major falls per time spent in motion. Different letters indicate significant differences (Tukey's HSD test, $P<0.05$).

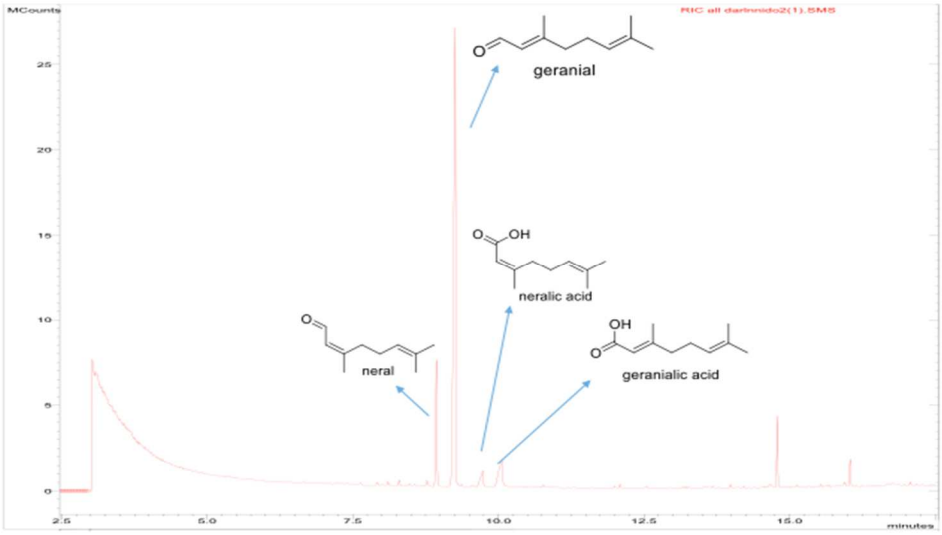

Figure 4. Chromatogram of *L. niitkib* MGP. The two largest peaks correspond to geranial and neral. Neralic acid and geranalic acid appear in only trace amounts.

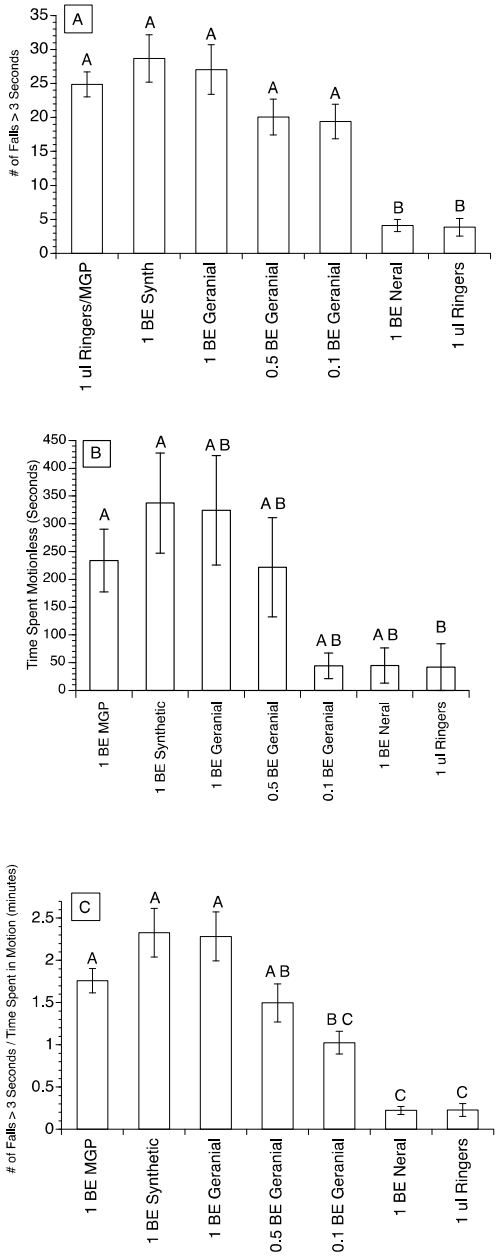

Figure 5. Results of the injection trials. (A) The number of major falls > 3 seconds (B) Amount of time spent motionless (seconds) (C) The number of major falls > 3 seconds per time spent in motion (minutes). Different letters indicate significant differences (Tukey's HSD test, $P<0.05$).

CHAPTER 2: IS HONEY BEE MANDIBULAR GLAND SECRETION AN ALARM PHEROMONE? PRELIMINARY RESULTS

INTRODUCTION

Pheromones play an important role in regulating and defending colony life (Pankiw et al. 1998; Pankiw et al. 1998). Alarm pheromones in the context of social insects can be defined as pheromones that cause mobilization throughout the colony within the context of defense. In honey bees, (*Apis mellifera*) sting venom includes compounds that elicit pain, inflammation, tissue necrosis, and alarm behavior. Prior research has suggested that honey bees may also have a second alarm pheromone, mandibular gland secretions (MGS). While less effective at eliciting classic alarm responses than sting alarm pheromone, MGS is potentially useful for colony defense because it attracts nestmates to a foreign body at the colony nest entrance (Maschwitz 1964; Shearer and Boch 1965). Some studies refer to the secretions of the honey bee mandibular gland as a pheromone (Maschwitz 1964; Shearer and Boch 1965). However, because it remains unclear if MGS is a pheromone, we will use the term MGS.

The simplest way for alarm pheromones to evolve is from defensive compounds. For example, Formicine ants and at least one species in the stingless bee genus *Oxytrigona* spray formic acid against invaders (Hölldobler & Wilson 1990; Michener 2000). Such defensive compounds can evolve to become alarm pheromones because they (1) are consistently produced in contexts that require colony mobilization, and (2) have a genuinely damaging effect against opponents. Although honey bee sting venom is clearly both a venom and alarm pheromone

(Breed et al. 2004), relatively little research has been conducted upon honey bee mandibular gland secretions (Shearer and Boch 1965).

Stingless bees are closely related to honey bees (Ramírez et al. 2010) and stingless bee mandibular gland secretions are, in all known species, alarm pheromones (Roubik 1992). This observation may have led to the hypothesis that honey bee mandibular gland pheromone is also an alarm pheromone (Blum 1972). In fact, honey bees and multiple species of stingless bees share a common, abundant component in their mandibular glands, 2-heptanone (Schorkopf 2009). In honey bees, Shearer and Boch (1965) identified 2-heptanone as the main compound in MGS. Butler (1966) proposed that this compound may be used to deter food store robbers, which is a common problem facing social insect colonies like honey bees that store large amounts of resources.

Boch and Shearer (1970) conducted a behavioral assay in which they applied hexane extracts of heads, which may contain more than just the mandibular gland contents, or 2-heptanone in paraffin oil, (0.07 µl - 0.875 µl) ranging from 2-23 bee equivalents (based upon Papachristoforou et al. 2012), to small corks placed at the hive entrance. Guard bees reportedly became alerted and agitated at throughout this range of concentrations. Control corks with paraffin oil alone did not elicit such reactions (Boch and Shearer 1970).

Collins and Kubasek (1982) tested a 1/10 dilution of 2-heptanone in paraffin oil and presented 30 µl of this mixture under a cage with bees. They then showed that 2-heptanone would elicit consistent and significantly intense alarm responses, although the amount presented (3 µl) corresponds to 77 bee equivalents and thus the high concentration alone may have contributed to this response. Simpson (1966) tested attraction of bees to crushed heads of bees presented at a feeder and found a

repellant effect, although this could be due to the hemolymph included in the crushed heads (Goodale and Nieh 2012) and compounds from other cephalic glands.

Recently, researchers demonstrated that honey bees may inject and apply their mandibular gland pheromone (2-heptanone) to paralyze intruders into the nest, primarily parasites (Papachristoforou et al. 2012). GC/MS analysis showed that the average honey bee forager has 0.0386 µL (1 bee equivalent) of 2-heptanone in its two mandibular glands (Papachristoforou et al. 2012). In wax moth larvae, an average of 0.65 nL of 2-heptanone was found in wax moth larvae that were bitten. These larvae became paralyzed for several minutes after a bite. In *Varroa* mites, 0.025 μL. of 2-heptanone was topically applied and altered the rhythmic expansion of the thorax and abdomen.

Discovery of these effects suggests that honey bee MGS is a kind of toxin, but also suggests an explanation for the results of previous studies. MGS has been shown to paralyze intruders, but it has not been shown to elicit removal behavior, though the early studies on bees removing MGS-marked papers and corks are suggestive. However these prior studies used 2-heptanone at levels up to 77 times higher than the amount now known to found in a single bee (Papachristoforou et al. 2012). We therefore decided to investigate the effects of realistic quantities of MGS upon nest and individual behavior.

We first tested the hypothesis that MGS marks hive invaders or parasites for removal. To do so, we used natural levels of MGS to mark wasp predators (*Vespula pensylvanica*) at the nest entrance to the hive. *Vespula pensylvanica* occurs naturally west of the Rocky Mountains from southern California to southwestern Canada (Visscher & Vetter, 2003) and can attack and capture live honey bees (Wilson & Holway 2010; Jack-McCollough and Nieh 2015). Because of this predatory behavior,

wasps sometimes visit the hive entrance of bee colonies (Tan et al. 2007), particularly in the fall (observed at our apiary). Therefore, wasps appear to be a good candidate to use as targets for removal at the entrance of colonies.

Because the previous toxic effect study was conducted with parasites (Papachristoforou et al. 2012), little is known about the potential toxic effects that 2-heptanone has for larger Hymenoptera like wasps and bees. Honey bees are not predators, but are known to rob other bee colonies (Free 1977) and could therefore be natural target for the toxic effects of MGS. We therefore tested the toxic pheromone hypothesis for honey bee MGS with yellow jackets and bees.

METHODS

Removal Trials

We conducted these trials during the summer of 2014. Using aspirators, we captured wasps at the nest entrances of honey bee colonies. We immediately froze the wasps and then presented wasps as targets at the nest entrances of 20 honey bee colonies (10 full frames of bees per colony). Wasps were marked with one bee-equivalent of MGS in 10 µl of hexane. Each colony was tested with the MGS obtained from foragers of that colony. To obtain this MGS, we dissected out both mandibular glands from 10 honey bees into 100 µl of hexane. For each trial, we video-recorded nest entrance activity for 15 min. Treatments were glued with cyanoacrylate adhesive to clear acetate squares (5X5 cm) that were placed on the entrance platform separated by a distance of 10 cm. We watched videos and counted the total number of touches on each target. There was a two-hour gap between trials. We tested the following paired treatments: (1) MGS on wasps versus hexane on wasps, (2) MGS on filter paper versus hexane on filter paper, and (3) MGS on filter paper versus an oval

piece of filter paper that was the same size as wasp (wasp outline). In our MGS wasp versus hexane wasp trials, we expected that a natural predator marked with MGS would elicit more attention from bees than a natural predator not marked with MGS. MGS paper versus hexane paper trials were run to determine how bees responded to MGS alone, with no wasp present. Finally, MGS paper versus wasp outline trials were run to control for attraction to MGS or a very simple visual stimulus, the ellipsoidal shape of a wasp.

Injection Trials

These trials were run throughout the summer and fall of 2014. In all trials, 1 µL of solution was injected into victims using a very fine Hamilton syringe (#701). We tested different doses of 2-heptanone, always mixed with insect ringer's solution to obtain a constant injection volume of 1 µL (Yamasaki and Narahashi 1959). Preliminary trials showed that a 1 µL injection of insect ringers solution did not aversely affect bees or wasps. Controls were pure insect ringer's solution with no (0 µL) of 2-heptanone. Doses of 2-heptanone consisted of 0.0386 µL, 0.1 µL, 0.25 µL, and 0.5 µL corresponding respectively to 1, 2.6, 6.5, and 13 bee equivalents (based upon Papachristoforou et al. 2012). Victims were chilled on average for 313 ± 43 s to allow for ease of injection before being video-recorded. For each victim, we recorded the exact chilling time (s).

We recorded the following behaviors: (1) time (s) spent moving abnormally (bee spun around or walks abnormally because some legs were paralyzed), (2) time (s) spent moving normally (walking or flying without impairment), (3) time (s) spent still (immobile), and (4) number of falls (bee flips over on its thorax). Total time varied as observations were stopped after the victim was confirmed dead or if the victim did

not exhibit abnormal behavior for an extended period of time. On average, victims were observed for 664 ± 156 seconds.

Statistical Analyses

In the removal experiment, we used paired t-tests (two-tailed) to examine the differences between how the same colony behaved in a given trial towards the control and experimental treatments. We then wished to compare the three different treatment types and therefore calculated a single value per trial: the proportion of touches in each experiment that were directed towards the object treated with MGS. We ran an Analysis of Variance (ANOVA, REML algorithm) to determine the effect of treatment. Colony was a random effect.

For injection trials we ran an two-way ANOVA looking at how differing concentrations of 2-heptanone affected observed behavior. Treatment (dose of MGS), victim species (wasp or bee) and the interaction treatment* victim species were fixed model effects. We applied the arcsine square root transformation to all proportions. All data met parametric assumptions after residuals analyses. We used Tukey Honest Significant Difference (HSD) tests to make multiple pairwise comparisons.

RESULTS

Removal Trials

In total, we ran 149 trials for three trial types: MGS paper versus hexane paper (15 replicates) MGS paper versus paper outline of wasp (16 replicates), and MGS wasp versus hexane wasp (118 replicates). We first examined the differences within each treatment type. In treatment one, MGS paper did not receive significantly

more touches than did hexane paper (T = -2.12, df = 14, P = 0.052). In treatment two, MGS paper received significantly more touches (3.9-fold greater) than did hexane paper (T = -2.15, df = 14, P = 0.049). However, for treatment three, MGS wasp versus hexane wasp, there was no significant difference in the amount of touches towards each target (T = 0.21, df = 96, P = 0.83) (Fig. 6A).

When we compared the proportion of MGS touches between the three trials, we found that bees were significantly more attracted to MGS when it was applied to filter paper, but not to a wasp ($F_{2,143.5}$ = 6.888, P = 0.0014, colony accounted for <1% of model variance, Fig. 6B). Thus, MGS did not increase potential removal behavior of a wasp predator.

Injection Trials

In our injection trials we injected 28 bees in our control trials and 56 bees at varied concentrations of 2-heptanone. We also ran nine wasps in our control trials and 40 wasps at varied concentrations of 2-heptanone.

For the number of falls, there was no significant effect of species ($F_{1,123}$ = 1.11, P = 0.293), 2-heptanone concentration ($F_{4,123}$ = 1.28, P = 0.281), or the interaction species*concentration ($F_{4,123}$ = 1.42, P = 0.232)

For proportion of abnormal movement, there was no significant effect of species ($F_{1,123}$ = 1.71, P = 0.193), 2-heptanone concentration ($F_{4,123}$ = 1.77, P = 0.137), or the interaction species*concentration ($F_{4,123}$ = 2.02, P = 0.096).

For proportion of normal movement, there was no significant effect of species ($F_{1,123}$ = 0.69, P = 0.406), or the interaction species*concentration ($F_{4,123}$ = 1.80, P = 0.1319). But there was a significant effect of 2-heptanone concentration ($F_{4,123}$ = 2.76, P = 0.0306, Fig. 7).

For proportion of time spent still, there was no significant effect of species ($F_{1,123}$ = 2.87, P = 0.0923), or the interaction of species*concentration ($F_{4,123}$ = 1.64, P = 0.1678). But there was a significant effect of concentration of 2-heptanone ($F_{4,123}$ = 8.78, P < 0.0001, Fig. 7). Because we found no effect of species, we pooled the results for wasps and bees in all figures. (Fig. 7)

DISCUSSION

The role of MGS as a potential alarm pheromone remains unclear. In our nest entrance trials, MGS was most attractive when presented on filter paper. However, MGS did not increase attraction to dead wasps. Thus, the hypothesis that MGS aids in the removal of potential threats was not supported. In these removal trials, bees were inherently attracted to the wasp and MGS did not increase this attraction.

Our results suggest that MGS may not act as a traditional alarm pheromone for *A. mellifera*, as previously assumed (Maschwitz 1964). Instead, the visual presence of the predator, its inherent odor, or both could be more important for bees at the hive entrance when dealing with threats such as wasps. In the Asian honey bee, *Apis cerana,* visual recognition of the hornet, *Vespa velutina*, plays an important role in hornet detection (Tan et al. 2012). Recently, Tan et al. (accepted) demonstrated that these bees initiated defense (heatballing) against a hornet predator at the nest entrance, primarily in response to the hornet odor and to a far lesser degree to the presence of sting alarm pheromone, a proven alarm pheromone.

In our injection trials, there were no significant differences between control trials and trials where natural levels of 2-heptanone were injected (0.04 µl 2-heptanone, Papachristoforou et al. 2012). However, we noticed effects at a 2.6-fold higher dose. This effect corresponds to a target being bitten by approximately three

bees. While this limits the potential for 2-heptanone as a toxin, multiple workers can attack the same intruder because group defense is common in honey bees (Breed et al. 2004).

Thus, our data do not strongly support MGS as an alarm pheromone. MGS attracted bees, but did not activate colony defense (bees attacking the marked target) and did not increase attraction to a marked predator. This failure to attract more attention may have occurred because the wasp already elicited sufficient attraction on its own. However, the hypothesis that MGS enhances attraction to a predator was not supported.

So what does MGS do? Other studies have shown that foragers may use MGS as a repellant scent-mark on previously visited food sources (Giurfa 1993). However, we now know that honey bees and other bees deposit cuticular hydrocarbon footprints on flowers that they visit. These olfactory cues are sufficient to repel (or attract, depending upon the context and nectar rewards) subsequent visitors (Wilms and Eltz 2008). Moreover, it remains unclear why honey bees need an additional alarm pheromone when they already possess a highly effective sting alarm pheromone that attracts the aggressive attention of objects, predators, and intruders scented with sting alarm pheromone (Collins and Kubasek 1982).

Based upon Papachristoforou et al. (2012), we are left with the possibility that MGS is a relatively mild toxin in honey bees that can temporarily paralyze small nest parasites. However, the evidence for a pheromonal function is weak. In honey bee queens, MGS is a true pheromone that plays a key role in colony organization, suppresses worker ovary development (Strauss et al. 2008) and can influence worker foraging activity (Pankiw et al. 1998). However, Queen MGS is chemically distinct and contains multiple distinct semiochemicals not found in worker MGS. For example,

queen MGS contains (E)-9-keto-2-decenoic acid (9ODA), (R,E)-(-)- and (S,E)-(+)-9-hydroxy-2-decenoic acid (9HDA), methyl p-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA), which are all thought to be important in initiating and maintaining retinue behavior in worker bees (Slessor et al. 1990). Further studies are required to better understand the role MGS plays, if any, within worker honey bee communication.

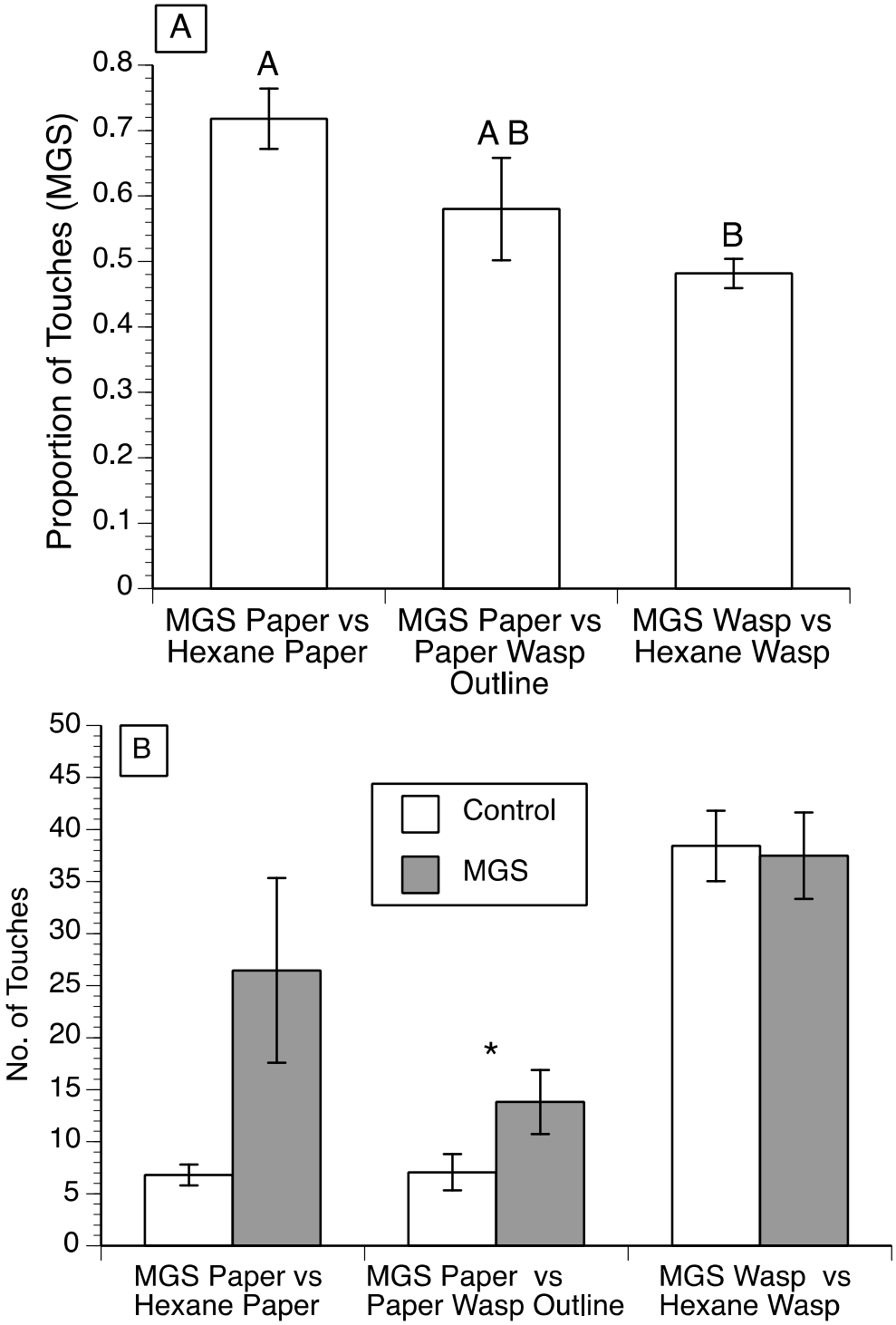

Figure 6. Results of Entrance Removal Trials (A) Proportion of touches towards the MGP object. (B) Differences between control and MGP touches within experiment. Different letters indicate significant differences (Tukey's HSD test, $P<0.05$).

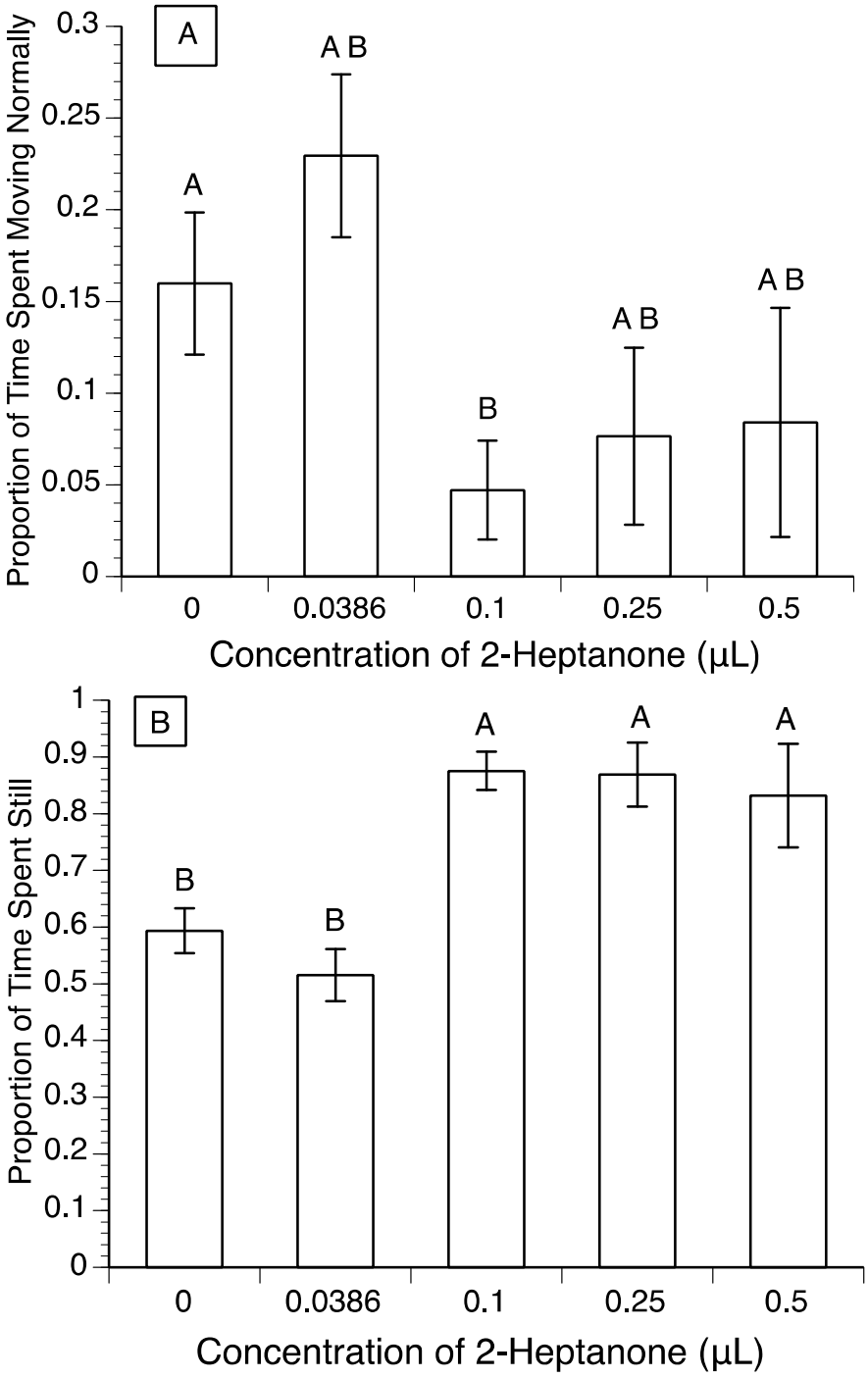

Figure 7. Results of 2-Heptanone Injection Trials. (A) The proportion of time spent moving normally (B) The proportion of time spent still.

REFERENCES FOR CHAPTER 1

Blount, J. D., Speed, M. P., Ruxton, G. D., & Stephens, P. A. (2009). Warning displays may function as honest signals of toxicity. *Proceedings of the Royal Society of London B: Biological Sciences*, *276*(1658), 871-877.

Blum, M. S., Crewe, R. M., Kerr, W. E., Keith, L. H., Garrison, A. W., & Walker, M. M. (1970). Citral in stingless bees: Isolation and functions in trail-laying and robbing. *Journal of Insect Physiology*, *16*(8), 1637-1648.

Casewell, N. R., Wüster, W., Vonk, F. J., Harrison, R. A., & Fry, B. G. (2013). Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecology & Evolution*, *28*(4), 219-229.

de Portugal-Araújo, V. (1958). A contribution to the bionomics of Lestrimelitta cubiceps (Hymenoptera, Apidae). *Journal of the Kansas Entomological Society*, *31*(3), 203-211.

Euán, J. J. G. Q., & Acereto, J. G. (2002). Notes on the nest habits and host range of cleptobiotic Lestrimelitta niitkib (Ayala 1999)(Hymenoptera: Meliponini) from the Yucatan Peninsula, Mexico. *Acta Zoológica Mexicana (nueva serie)*, (86), 245-249.

Fernandes, R. A., & Kumar, P. (2003). PCC-mediated novel oxidation reactions of homobenzylic and homoallylic alcohols. *Tetrahedron Letters*,*44*(6), 1275-1278.

Grüter, C., Menezes, C., Imperatriz-Fonseca, V. L., & Ratnieks, F. L. (2012). A morphologically specialized soldier caste improves colony defense in a neotropical eusocial bee. *Proceedings of the National Academy of Sciences*,*109*(4), 1182-1186.

Hölldobler, B., & Engel, H. (1978). Tergal and sternal glands in ants. *Psyche*,*85*(4), 285-330.

Hölldobler, B., Plowes, N. J., Johnson, R. A., Nishshanka, U., Liu, C., & Attygalle, A. B. (2013). Pygidial gland chemistry and potential alarm-recruitment function in column foraging, but not solitary, Nearctic Messor harvesting ants (Hymenoptera: Formicidae: Myrmicinae). *Journal of Insect Physiology*, *59*(9), 863-869.

Jackson, D. E. (2008). Interspecific Communication: Treehopper Alarms Make Ants Come Running. *Current Biology*, *18*(14), R602-R603.

Johnson, L. K. (1987). The pyrrhic victory of nest-robbing bees: did they use the wrong pheromone?. La victoria pírrica de las abejas ladronas de nidos: utilizan ellas la feromona equivocada?. *Biotropica.*, *19*(2), 188-189.

Kerr, W. E., & Cruz, C. D. C. (1961). Funções diferentes tomadas pela glândula mandibular na evolução das abelhas em geral e em “Trigona (Oxytrigona) tataira” em especial. *Revista Brasileira de Biologia*, *21*(1), 1-16.

Luzzio, F. A., Fitch, R. W., Moore, W. J., & Mudd, K. J. (1999). A Facile Oxidation of Alcohols Using Pyridinium Chlorochromate/Silica Gel. *J. Chem. Ed.*, *76*(7), 974–975.

Merida, J. (2003). Chemical ecology of the robber bee, Lestrimelitta niitkib. Master's thesis, El Colegio de la Frontera Sur.

Michener, C. D. (2000). *The Bees of the World* (Vol. 1). JHU Press.

Nogueira-Neto, P. (1970). Behavior problems related to the pillages made by some parasitic stingless bees (Meliponinae, Apidae). *Development and evolution of behavior: Essays in memory of TC Schneirla.*, 416-434.

Pompeu, M. S., & Silveira, F. A. (2005). Reaction of Melipona rufiventris Lepeletier to citral and against an attack by the cleptobiotic bee Lestrimelitta limao (Smith)(Hymenoptera: Apidae: Meliponina). *Brazilian Journal of Biology*, *65*(1), 189-191.

Quezada-Euán, J. J. G., Ramírez, J., Eltz, T., Pokorny, T., Medina, R., & Monsreal, R. (2013). Does sensory deception matter in eusocial obligate food robber systems? A study of Lestrimelitta and stingless bee hosts. *Animal Behaviour*, *85*(4), 817-823.

Rinderer, T. E., Blum, M. S., Fales, H. M., Bian, Z., Jones, T. H., Buco, S. M., ... & Howard, D. F. (1988). Nest plundering allomones of the fire beeTrigona (Oxytrigona) mellicolor. *Journal of Chemical Ecology*, *14*(2), 495-501.

Roubik, D. W. (1992). *Ecology and Natural History of Tropical Bees*. Cambridge University Press.

Roubik, D. W., Smith, B. H., & Carlson, R. G. (1987). Formic acid in caustic cephalic secretions of stingless bee, Oxytrigona (Hymenoptera: Apidae).*Journal of Chemical Ecology*, *13*(5), 1079-1086.

Sakagami, S. F., & Laroca, S. (1963). Additional Observations on the Habits of the Cleptobiotic Stingless Bees, the Genus Lestrimelitta Friese (Hymenoptera, Apoidea)(With 6 Text-figures). *Journal of the Faculty of Science Hokkaido University Series VI. ZOOLOGY*, *15*(2), 319-339.

Sakagami, S. F., Roubik, D. W., & Zucchi, R. (1993). Ethology of the robber stingless bee, Lestrimelitta limao (Hymenoptera: Apidae). *Sociobiology (USA)*.

Schwarz, H. F. (1948). Stingless Bees (Meliponidae) of the Western Hemisphere. Lestrimelitta and the Following Subgenera of Trigona, Paratrigona, Swarziana, Parapartamona, Cephalotrigona, Oxytrigona, Scaura, and Mourella. Abejas Jicotes (Meliponidae) Del Hemisferio Occidental. Lestrimelitta Y Los Siguientes Subgéneros de Trigona, Paratrigona, Swarziana, Parapartamona, Cephalotrigona, Oxytrigona, Scaura Y Mourella. *Bulletin of the American Museum of Natural History*, *90*, 1-536.

Shearer, D. A., & Boch, R. (1965). 2-Heptanone in the mandibular gland secretion of the honey-bee.

Simpson, J. (1961). The salivary glands of Apis mellifera and their significance in caste determination. In *Symposia Genetica et Biologica Italica* (Vol. 10, pp. 173-188).

Stout, J. C., & Goulson, D. (2001). The use of conspecific and interspecific scent marks by foraging bumblebees and honeybees. *Animal Behaviour*,*62* (1), 183-189.

Vicens, N., & Bosch, J. (2000). Weather-dependent pollinator activity in an apple orchard, with special reference to Osmia cornuta and Apis mellifera (Hymenoptera: Megachilidae and Apidae). *Environmental Entomology*, *29*(3), 413-420.

Winston, M. L. (1991). *The Biology of the Honey Bee*. Harvard University Press.

Wittmann, D. (1985). Aerial defense of the nest by workers of the stingless bee Trigona (Tetragonisca) angustula (Latreille)(Hymenoptera: Apidae).*Behavioral Ecology and Sociobiology*, *16*(2), 111-114.

Yamasaki, T., & Narahashi, T. (1959). The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon. *Journal of Insect Physiology*, *3*(2), 146IN3149-148158.

REFERENCES FOR CHAPTER 2

Blum, M. S. (1969). Alarm pheromones. *Annual Review of Entomology*, *14*(1), 57-80.

Blum, M. S., & Brand, J. M. (1972). Social insect pheromones: their chemistry and function. *American Zoologist*, *12*(3), 553-576.

Boch, R., Shearer, D. A., & Petrasovits, A. (1970). Efficacies of two alarm substances of the honey bee. *Journal of Insect Physiology*, *16*(1), 17-24.

Breed, M. D., Guzmán-Novoa, E., & Hunt, G. J. 3. (2004). Defensive behavior of honey bees: organization, genetics, and comparisons with other bees. *Annual Reviews in Entomology*, *49*(1), 271-298.

Butler, C. G. (1966). Mandibular gland pheromone of worker honey bees. *Nature*, *212*(5061), 530-530.

Collins, A. M., & Kubasek, K. J. (1982). Field test of honey bee (Hymenoptera: Apidae) colony defensive behavior. *Annals of the Entomological Society of America*, *75*(4), 383-387.

Free, J. B. (1977). Social Organization of Honeybees.

Giurfa, M. (1993). The repellent scent-mark of the honeybeeApis mellifera tigustica and its role as communication cue during foraging. *Insectes Sociaux*, *40*(1), 59-67.

Goodale, E., & Nieh, J. C. (2012). Public use of olfactory information associated with predation in two species of social bees. *Animal Behaviour*,*84*(4), 919-924.

Hölldobler, B., & Wilson, E. O. (1990). *The Ants*. Harvard University Press.

Jack-McCollough, R. T., & Nieh, J. C. (2015). Honeybees tune excitatory and inhibitory recruitment signalling to resource value and predation risk. *Animal Behaviour*, *110*, 9-17.

Maschwitz, U. (1964). Gefahrenalarmstoffe und Gefahrenalarmierung bei sozialen Hymenopteren. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *47*(6), 596-655.

Michener, C. D. (2000). *The Bees of the World*. Johns Hopkins University Press. *Baltimore, Maryland, USA*.

Pankiw, T., Page Jr, R. E., & Fondrk, M. K. (1998). Brood pheromone stimulates pollen foraging in honey bees (Apis mellifera). *Behavioral Ecology and Sociobiology*, *44*(3), 193-198.

Pankiw, T., Winston, M. L., & Robinson, G. E. (1998). Queen mandibular gland pheromone influences worker honey bee (Apis mellifera L.) foraging ontogeny and juvenile hormone titers. *Journal of Insect Physiology*, *44*(7), 685-692.

Papachristoforou, Alexandros, Alexia Kagiava, Chrisovalantis Papaefthimiou, Aikaterini Termentzi, Nikolas Fokialakis, Alexios-Leandros Skaltsounis, Max Watkins, Gérard Arnold, and George Theophilidis. (2012). The bite of the honeybee: 2-heptanone secreted from honeybee mandibles during a bite acts as a local anaesthetic in insects and mammals. *PloS One*, *7*(10), e47432.

Ramírez, S. R., Nieh, J. C., Quental, T. B., Roubik, D. W., Imperatriz-Fonseca, V. L., & Pierce, N. E. (2010). A molecular phylogeny of the stingless bee genus Melipona (Hymenoptera: Apidae). *Molecular Phylogenetics and Evolution*, *56*(2), 519-525.

Roubik, D. W. (1992). *Ecology and Natural History of Tropical Bees*. Cambridge University Press.

Schorkopf, D. L. P., Hrncir, M., Mateus, S., Zucchi, R., Schmidt, V. M., & Barth, F. G. (2009). Mandibular gland secretions of meliponine worker bees: further evidence for their role in interspecific and intraspecific defence and aggression and against their role in food source signalling. *Journal of Experimental Biology*, *212*(8), 1153-1162.

Shearer, D. A., & Boch, R. (1965). 2-Heptanone in the mandibular gland secretion of the honey-bee.

Simpson, J. (1966). Repellency of the mandibular gland scent of worker honey bees. *Nature*, *209*, 531-532.

Slessor, K. N., Kaminski, L. A., King, G. G. S., & Winston, M. L. (1990). Semiochemicals of the honeybee queen mandibular glands. *Journal of Chemical Ecology*, *16*(3), 851-860.

Strauss, K., Scharpenberg, H., Crewe, R. M., Glahn, F., Foth, H., & Moritz, R. F. (2008). The role of the queen mandibular gland pheromone in honeybees (Apis mellifera): honest signal or suppressive agent? *Behavioral Ecology and Sociobiology*, *62*(9), 1523-1531.

Tan, K., Radloff, S. E., Li, J. J., Hepburn, H. R., Yang, M. X., Zhang, L. J., & Neumann, P. (2007). Bee-hawking by the wasp, Vespa velutina, on the honeybees Apis cerana and A. mellifera. *Naturwissenschaften*, *94*(6), 469-472.

Tan, K., Wang, Z., Li, H., Yang, S., Hu, Z., Kastberger, G., & Oldroyd, B. P. (2012). An ‘I see you’prey–predator signal between the Asian honeybee, Apis cerana, and the hornet, Vespa velutina. *Animal Behaviour*, *83*(4), 879-882.

Tan, K., Dong, S., Liu, X., Wang, C., Li, J., and Nieh J.C. (accepted) Honey bee inhibitory signaling is tuned to threat severity and can act as a colony alarm signal. PLOS Biology.

Vallet, A., Cassier, P., & Lensky, Y. (1991). Ontogeny of the fine structure of the mandibular glands of the honeybee (Apis mellifera L.) workers and the pheromonal activity of 2-heptanone. *Journal of Insect Physiology*, *37*(11), 789-804.

Visscher, P. K., & Vetter, R. S. (2003). Annual and multi-year nests of the western yellowjacket, Vespula pensylvanica, in California. *Insectes sociaux*,*50*(2), 160-166.

Wilms, J., & Eltz, T. (2008). Foraging scent marks of bumblebees: footprint cues rather than pheromone signals. *Naturwissenschaften*, *95*(2), 149-153.

Wilson, E. E., & Holway, D. A. (2010). Multiple mechanisms underlie displacement of solitary Hawaiian Hymenoptera by an invasive social wasp. *Ecology*, *91*(11), 3294-3302.

Yamasaki, T., & Narahashi, T. (1959). The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon. *Journal of Insect Physiology*, *3*(2), 146IN3149-148158.