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Exploration of Argentine ant (*Linepithema humile*) Biology for Pest Management

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ABSTRACT OF THE DISSERTATION

Exploration of Argentine ant (*Linepithema humile*) Biology for Pest Management

by

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Dr. Dong-Hwan Choe, Chairperson

The invasive Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), has successfully established itself in many urban, agriculture and natural habitats worldwide, causing economic damage and disruption of ecosystem processes. Current management strategies for the Argentine ant often rely on insecticidal sprays that contribute to environmental contamination. The overall goal of this dissertation was to develop novel pest control techniques that utilize the Argentine ants' chemistry and unique genetic makeup to help eliminate or reduce pesticide applications for their control, and to explore their chemical defense system as a possible cause of their ecological dominance over native ants worldwide.

A novel baiting technique, "pheromone-assisted baiting technique", was developed and tested against Argentine ant populations in the laboratory and residential sites. The technique relies on commercially available bait that has been treated with the Argentine ants' trail pheromone, (*Z*)-9-hexadecenal. Laboratory results indicate an increase in foraging activity and final morality of Argentine ants with the incorporation of (*Z*)-9-hexadecenal into a commercially available bait. Field results demonstrate that

the pheromone-baiting technique achieves a 74% reduction in Argentine ant activity by the end of 4 weeks. The pheromone-assisted baiting technique demonstrates effective ant control while reducing the amount of insecticides applied in the environment.

RNA interference (RNAi) has the potential to become an effective alternative to conventional pest control baiting techniques. RNAi is a post-transcriptional gene-silencing technique which is triggered by the presence of double-stranded RNA (dsRNA). Laboratory experiments indicate 52% mortality after 7 days when the RNAi bait was consumed by Argentine ants. The study provides proof-of-concept of an RNAi baiting technique for Argentine ant pest management.

Argentine ants utilize pygidial gland secretions for inter- and intra-specific communication during aggressive interactions with a heterospecific competitor, the California harvester ant (*Pogonomyrmex californicus*). Chemical analyses indicated that Argentine ants deploy two iridoids, dolichodial and iridomyrmecin, during aggressive interactions. The compounds cause high levels of irritation to the harvester ants and elicit alarm and attraction of Argentine ant nestmates. The results demonstrate semiochemical parsimony in Argentine ants for various colony-level tasks and help us to understand the mechanisms underlying their successful establishment among various competitors outside their native ranges.

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Chapter 1. Introduction

Exploring the behavior and biology of the Argentine ant may suggest novel, effective pest control strategies that can reduce the negative environmental effects of using insecticides for ant control. Argentine ants are global invaders, specifically known as a "tramp ant" species, that exhibit the following characteristics: their polygynous, unicolonial colonies reproduce by budding, they have been widely dispersed throughout the world by human commerce, and they frequently live in close association with humans (Hölldobler and Wilson 1990). These characteristics have permitted the Argentine ant to become a worldwide pest resulting in numerous detrimental effects (Holway et al. 2002). Once established, Argentine ants negatively affect the native fauna, causing reductions in arthropod diversity, native ant populations, and vertebrate populations (Cole et al. 1992, Human and Gordon 1999, Bolger et al. 2000, Suarez et al. 2000). Their propensity for sugary liquids has established them as "shepherds" of phloem feeding hemipterans, allowing these plant pests to proliferate by protecting them from natural enemies (Lach 2005, Tena et al. 2013). Floral visitation by Argentine ants reduces pollinator visitation, thus negatively affecting vital pollination ecosystem services (Lach 2008, Levan et al. 2014, Hanna et al. 2015). The Argentine ant is recognized as one of the major nuisance pests in the United States; for example it makes up 85% of urban pest control service accounts in California (Knight and Rust 1990, Field et al. 2007).

To combat Argentine ants, pest management professionals (PMPs) primarily use residual insecticide sprays (Rust et al. 2003). Residual insecticide sprays are simple to apply and effective, making them a popular choice among PMPs to control Argentine ant

infestations. However, heavy use of these residual insecticide sprays has resulted in frequent detection of the active ingredients and their toxic degradation products in urban waterways and aquatic sediments. Furthermore, government regulations are reducing or prohibiting current residual insecticide sprays, potential limiting the current pool of available technologies to combat Argentine ants (Greenberg et al. 2017). As the negative environmental effects associated with pesticide sprays come to light and new regulations are implemented in response, new or alternative control methods need to be developed so that control would not rely on conventional pesticides alone.

Baiting is an effective alternative to residual insecticide sprays for managing Argentine ants. When properly designed for a target pest ant, toxicant baits can improve target specificity while reducing non-target exposure and off-site movement of active ingredients into the environment (Greenberg et al. 2006). Nevertheless, baits must adhere to several requirements in order for them to be effective tools for practical applications in pest management. Toxicants that are incorporated into ant baits must maintain prolonged delayed toxicity over a wide dosage range and remain palatable (Rust et al. 2004). Furthermore, ant workers must survive long enough as to maximize feeding, trail following and mass recruitment to toxic baits. Developing improved baiting strategies that provide effective control while minimizing environmental contamination will be necessary for future Argentine ant pest management strategies.

Understanding the mechanism that allows Argentine ants to become successful global invaders may help to improve IPM strategies. Argentine ants' success is attributed to their highly aggressive nature, resulting in the displacement of native ant populations

(Human and Gordon 1999). Their strong interspecific competition relies on two key competitive mechanism: exploitation and interference (Human and Gordon 1996). Studies have shown that Argentine ants are consistently better than native ants at exploiting food sources by locating and recruiting to bait more consistently and with higher numbers (Human and Gordon 1996, Holway 1999). Furthermore, they are able to forage for longer periods of time (Human and Gordon 1996). When Argentine ants and native ants encounter each other at a food source, the native ants were displaced the majority of the time. These competitive mechanism help explain their invasion successes worldwide and subsequent establishment, even though the details of their behaviors and chemical defenses have not been fully elucidated.

My research focuses on three areas: a novel chemical baiting technique, an RNA interference (RNAi) baiting technique, and the chemistry of Argentine ant aggression and its influence on Argentine ant / opponent behaviors. The results from these studies can be either incorporated into conventional control methods or used to develop novel control technologies, eventually allowing more effective Argentine ant management.

In my first research chapter, I focus on improving current Argentine ant baiting using (*Z*)-9-hexadecenal, a pheromone that elicits a trail response. My results indicate that addition of the pheromone improved the efficacy of a commercial bait for Argentine ant control. This is the first empirical study that shows existing insecticidal bait can be successfully enhanced by the addition of (*Z*)-9-hexadecenal.

In the second chapter, I focus on a genetic technique, RNAi, to explore its potential for Argentine ant management. This technique interferes with protein

translation in Argentine ants, using engineered double-stranded RNA (dsRNA) and the species' own genetic mechanisms. Even with current limitations in the RNAi technology, this technique has potential to become an alternative approach for pest management with relatively high specificity. The RNAi technique consists of a sugary liquid bait combined with a specific dsRNA unique to the Argentine ant. Laboratory results indicate that 52% mortality is achieved when the RNAi bait is consumed for 7 days.

In the last chapter, I focus on the aggression of the Argentine ant, specifically against a heterospecific competitor. The high competitiveness of Argentine ants in exploitation and interference competition is due, at least in part, to their ability to communicate effectively via semiochemicals. Here, we report that Argentine ants utilize pygidial secretions for inter- and intraspecific communication during aggressive interactions with a heterospecific competitor, California harvester ant (*Pogonomyrmex californicus*). The headspace / cuticular analyses indicated that Argentine ants deploy pygidial gland secretions containing two volatile iridoids, dolichodial and iridomyrmecin, on the competitor's cuticular surface via the gaster-bending response. Bioassays with live harvester ants and the crude pygidial gland secretion indicated that the secretions applied on the cuticular surface function as a defensive allomone, causing high levels of irritation in heterospecific competitors. Furthermore, the same secretions elicited alarm and attraction of conspecific nestmates, potentially enabling more rapid / coordinated defense by the Argentine ants. Two major volatile constituents of the pygidial gland secretion, dolichodial and iridomyrmecin, were sufficient to elicit these responses in conspecifics (as a mixture or individual compounds). The current study suggests that invasive

Argentine ants' success in exploitative and interference competition may rely on the species' effective semiochemical parsimony. Understanding how Argentine ants utilize the limited chemistry of their pygidial gland secretions for various colony-level tasks will help us to understand the mechanisms underlying their successful establishment among various competitors outside their native ranges.

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Chapter 2

Development of a pheromone-assisted baiting technique for Argentine ants (Hymenoptera: Formicidae)

Abstract

Current control measures for Argentine Ants, *Linepithema humile* (Mayr), in urban settings typically include perimeter applications of insecticides around structures, resulting in potential problems with insecticide runoff and environmental contamination. Insecticidal baits can be an effective alternative to perimeter spray applications and are largely considered target-specific with minimal non-target impact and environmental contamination. We report a "pheromone-assisted baiting technique" as an economically viable approach to maximize the efficacy of conventional baits targeting Argentine ants. Laboratory experiments with a commercially available gel bait indicated that foraging activity and final mortality of Argentine ants were significantly improved by incorporating (Z)-9-hexadecenal in the bait. The field study demonstrated that the pheromone-treated gel bait achieved a 74% reduction in Argentine ant activity by the end of 4 weeks when it was compared with its own pre-treatment value. This was a significant improvement over the untreated gel bait which provided a 42% reduction over the same period of time. The pheromone-assisted baiting technique has the potential in providing effective ant control with reduced amount of insecticides applied in the environment.

Introduction

The invasive Argentine ant, *Linepithema humile* (Mayr), has become one of the major nuisance pests in many urban areas in the United States over the past several decades (Silverman and Brightwell 2008, Gilboa et al. 2012). It is the dominant urban ant pest in California and Georgia and a significant localized pest in the other southeastern states such as South Carolina, Alabama, Mississippi, Louisiana, Florida, Tennessee, and North Carolina (Bambara et al. 2006). For instance, one survey indicated that 85% of urban pest control services are allocated solely to the control of Argentine ants in California (Field et al. 2007). According to another survey conducted in North Carolina in 2000, Argentine ants were present in over 20% of the samples collected by the local pest management professionals (PMPs) from urban areas (Bambara et al. 2006).

Residual insecticide sprays are widely used to control the Argentine ant in urban settings. Their simple application and effectiveness in providing rapid control make them a popular choice among PMPs (Rust et al. 2003, Klotz et al. 2008a). Pyrethroids (e.g., bifenthrin, cyfluthrin, cypermethrin, and permethrin), and phenylpyrazole (e.g., fipronil) are among the most common active ingredients that are used in these residual insecticide sprays (Rust 2001, Rust et al. 2003, Silverman and Brightwell 2008). Based on 2012 data, 25,445 kg of bifenthrin (active ingredient) and 24,166 kg of fipronil (active ingredient) were formulated into various spray products used by licensed PMPs for urban pest management in California (CDPR 2012). Consequently, some of these active ingredients, and their toxic degradation products, are frequently detected in urban

waterways and aquatic sediments (Weston et al. 2009, Lao et al. 2010, Delgado-Moreno et al. 2011).

Frequent detection of these active ingredients in urban water ways is concerning because of the potential effects on non-target species (Bonmatin et al. 2015). For example, bifenthrin was identified as the primary causative agent of toxicity to an amphipod indicator species, *Hyalella azteca*, with additional toxicity being attributed to the presence of cyfluthrin and cypermethrin (Weston et al. 2005). *Hyallela azteca* is an aquatic grazer that is often used to determine the non-target effects of pesticide contaminants in aquatic systems (Weston et al. 2005). Additionally, fipronil was detected in urban waterways in amounts which exceeded the LC50 values for many indicator arthropod species such as mysid shrimp and grass shrimp (Gan et al. 2012). Developing new ant control strategies which are not solely dependent upon residual sprays will help mitigate the negative impact on water quality.

Baiting is an effective alternative to insecticide sprays for managing pest ants (Klotz et al. 2003). When properly designed and used, insecticidal baits can provide several potential advantages over the insecticide sprays. For example, baits can be designed to achieve target specificity and minimal off-site movement of insecticidal active ingredients (Klotz et al. 2010). However, for a bait to be acceptable as an effective tool for a practical ant pest management program, the baits must meet several requirements. For example, the toxic baits should maintain palatability, nonrepellency, delayed toxicity, and transferability over the baiting period (Silverman and Roulston 2001). Maintaining these bait characteristics depends on not only the chemical

constituents (i.e., active and inactive ingredients) of the baits, but also their concentrations. Additionally, baits should restrict access of non-target organisms while eliminating or reducing the target ant populations (Silverman and Brightwell 2008).

One of the potential ways to improve current baiting technologies is to use insect pheromones in conjunction with existing bait products (Vander Meer 1996). By incorporating species-specific insect pheromones to the bait matrix, the bait can be designed to be quickly discovered and consumed by the desired pest species before any detrimental changes of the bait matrix or active ingredient(s) occurs. In particular, fermentation and increased viscosity can quickly occur for the sugar-containing liquid or gel baits placed under warm and dry conditions, negatively impacting the palatability of the baits for liquid-feeding ants (Cooper et al. 2008 and Choe et al. 2010). Several studies have explored the possibility of using a synthetic trail-following pheromone to develop practical management strategies for the Argentine ant. Choe et al. (2014) reported that the addition of (Z)-9-hexadecenal, a putative trail pheromone component of Argentine ants, into insecticide sprays improved the sprays efficacy because (Z)-9-hexadecenal attracts worker ants from nearby locations to the insecticide spray deposits. Greenberg and Klotz (2000) showed that (Z)-9-hexadecenal, when mixed with a sugar solution, increases Argentine ant consumption. However, this study did not determine if the efficacy of insecticidal bait can be improved by incorporating the pheromone into the bait.

In this study, we explored if the incorporation of Argentine ant pheromone, (Z)-9-hexadecenal, could improve the efficacy of existing gel baits. We used a gel bait product that is broadly marketed throughout California (CDPR 2012) and other parts of the US

for residential ant control. Laboratory studies were conducted to determine if the addition of (Z)-9-hexadecenal into a gel bait could increase the Argentine ants' foraging activity on that gel bait and thus, increase the resulting mortality. A field study was also conducted to determine if the addition of (Z)-9-hexadecenal into that same gel bait could increase the bait consumption and consequently, provide improved Argentine ant control in the urban residential settings.

Materials and Methods

Ants. Argentine ants were collected from the biological control grove on the University of California, Riverside campus. Ant nests were excavated from the ground and transported to the laboratory, where the ants were extracted from the soil (Hooper-Bui and Rust 2000). Laboratory stock colonies were maintained in plastic boxes (26.5 by 30 by 10 cm); the inner sides were coated with Teflon (Fluoropolymer resin, type 30, DuPont Polymers, Wilmington, DE) to prevent the ants from escaping. Each colony was provided with two or three artificial nests constructed from plaster-filled petri dishes (9 cm diam by 1.5 cm depth) with a smaller cylindrical area (5 cm diam by 1 cm depth) in the center of the dish, to serve as a nesting space. The colonies had free access to fresh water and 25% (wt:vol) sucrose solution. Freshly killed American cockroaches, *Periplaneta americana* (L.), were provided three times a week as a protein source.

Insecticide. Optigard© ant gel bait (Syngenta Crop Protection, Inc. Greensboro, NC) containing 0.01% (wt/wt) thiamethoxam as the active ingredient was used in the study. The gel bait was chosen based on its effectiveness in preliminary laboratory trials (unpublished data) and its common use by PMPs in California. Thiamethoxam requires

consumption by the ants to become most effective. Additionally, the thiamethoxam ingested by one individual could be transferred to other individuals within the population via oral food exchange (i.e., stomodeal trophallaxis) (Rust et al. 2004).

Laboratory study. Laboratory studies were conducted to determine if the addition of (Z)-9-hexadecenal (Bedoukian Research, Inc., Danbury, CT) into a gel bait could increase the Argentine ants' short-term feeding activity on that gel bait and thus, increase the resulting mortality. A group of Argentine ant workers (0.5 g), reared from laboratory stock colonies (see above), was aspirated, anesthetized with CO_2, and placed in an empty plastic box arena (20 by 35 cm). The inner sides of the arena box were coated with Teflon (Fluoropolymer resin, type 30, DuPont Polymers, Wilmington, DE) to prevent the ants from escaping. This method of preparation resulted in relatively similar-sized experimental colonies [745 ± 28 ants (mean ± SEM), n = 36, range 714-901]. One reproductive (queen) was added to each of the experimental colonies. Each experimental colony was provided with one nest tube, constructed from a 50-ml Falcon plastic tube (Fisher Scientific, Waltham, MA) which was filled with 15 ml water and stopped with a cotton ball; and one sugar water dish containing 25% sucrose solution. The nest tube was placed in one end of the box and the sugar water dish was placed next to the nest.

We compared the following gel bait treatments: gel bait with pheromone (treatment gel bait), and gel bait only (standard gel bait). In addition to these two gel bait treatments, we also included a control group which only received 25% sucrose solution without any toxicant (untreated control), to estimate the natural mortality of laboratory colonies of Argentine ants. The gel bait was provided in a small plastic dish (cap of 1.5-

ml microcentrifuge tube). For the treatment gel bait, 10µl of (Z)-9-hexadecenal preparation, dissolved in acetone (10 µg/ml), was applied in the center of the dish. The solvent was allowed to evaporate for 1 min before placing 1 g of gel bait in the dish. Then, the gel bait was gently stirred with a stick, mixing the gel bait with the pheromone residue. The approximate final concentration of the pheromone in the gel bait was 0.1 µg/g. For the standard gel bait, 10 µl of clean acetone was applied to the dish before placing 1 g of gel bait. The standard gel bait was also stirred in a manner similar to the preparation of treatment gel bait. The bait dish containing either the standard or treatment gel bait was then placed in the plastic box arena at the opposite end from the nest tube, approximately 20 cm away from the nest entrance. Each experimental colony was randomly assigned to treatment gel bait, standard gel bait, or untreated control. Two gel bait treatments and the untreated control were replicated 12 times. The experimental colonies were provided with 25% (wt:vol) sucrose solution throughout the entire experimental period.

For the gel bait treatments, starting immediately after the introduction of the gel bait dish, each experimental colony was photographed every 3 min in order to accurately record the number of ants feeding on the gel bait over time. Photographs were taken for a total period of 30 min, resulting in 10 observations per trial. After 30 min, the bait dish was removed from the plastic box arena. The timed removal of the bait from the experimental colony was necessary because the continued presence of the gel bait in the colony box would rapidly satiate the experimental colonies contained in a small space (i.e., 700 cm^2); limiting the potential differentiation between treatment and standard gel

baits (unpublished data). All experimental colonies were maintained at 23-25°C and 34-45% relative humidity for 7 d. Dead ants were removed daily from each box and total cumulative number of the dead ants was counted on day 7.

Field study. Field experiments were conducted to determine if the addition of (Z)-9-hexadecenal into the gel bait could increase the gel bait consumption over a long term (i.e., 4 wk). Control efficacies were also compared between treatment gel bait (gel bait with pheromone) and standard gel bait (gel bait only). The study was conducted in Riverside, CA, using residential houses that local homeowners voluntarily provided as research sites. Because the main focus of current study was examining the efficacy difference between treatment gel bait (with pheromone) and standard gel bait (without pheromone), we did not include untreated control houses. Additionally, it was impossible to find homeowners that were willing to cooperate in a study like this without receiving some kind of ant control measures.

Prior to the start of the experiment, houses were monitored for overall ant activity levels based on number of ant visits at monitoring tubes. The 15-ml Falcon plastic tubes (Fisher Scientific) containing 12 ml of 25% sucrose solution were used as monitoring tubes. For each house, 10 monitoring tubes were placed along the perimeter, and the other 10 tubes were placed about 3 m away from the house perimeter. After 24 h, the tubes were capped to prevent liquid loss during transportation, returned to the laboratory and weighed. The weight data was corrected for evaporative loss of the liquid during the 24 h monitoring period (Klotz et al. 2008b). By calculating the difference between the initial weight of the sucrose solution placed into the monitoring tube and the adjusted

weight of the remaining sucrose solution, we obtained the amount of sucrose solution consumed by the foraging ants over the 24-h monitoring period. A single Argentine ant typically consumes 0.0003 g of sucrose solution per visit; therefore, we assumed a direct relationship between amount of sucrose solution consumed and number of ant visits at the monitoring tube (Reierson et al. 1998). Based on this assumption, the number of ant visits per tube was estimated by dividing the consumption (g) by 0.0003 (g per visit). One advantage of this type of monitoring is it reflects an overall foraging activity of the ants over a relatively long period of time (i.e., 24 h) rather than estimating the ant activity based on a snapshot observation (e.g., counting ant numbers on a trail for a fixed amount time), in which the estimations could vary greatly depending upon the times of the day or ambient weather conditions. Based on preliminary monitoring, ten houses with significant levels of Argentine ant activity (i.e., a minimum of total 84 ml loss from the 240 ml originally distributed in 20 monitoring tubes) were selected for this study.

Five of ten houses were treated with treatment gel bait and the other five houses were treated with standard gel bait. Bait stations were constructed from graduated 15-ml Falcon plastic tubes (Fisher Scientific) with a small hole (3 mm diam) drilled in the cap. The small entry hole in the cap allowed the foraging ants to access the gel bait inside of the bait station, while preventing non-target organisms and water (from irrigation and natural precipitation) from entering into the bait station. Each bait station was loaded with 2 ml of thiamethoxam gel bait. For the treatment gel bait, 0.2 µg of (Z)-9-hexadecenal dissolved in 20 µl of acetone was applied on the top of the gel bait inside the tube and mixed by gently agitating the bait station. The standard gel bait consisted of

thiamethoxam gel bait dispensed in the tube without any modification. Application of acetone on the bait did not alter normal foraging behavior of ants and overall mortality rates, compared with the standard gel bait (unpublished data).

Twenty bait stations were evenly distributed around each house, 10 along the perimeter of the house and 10 in the yard. We also referred to product label instructions for the placement of the gel bait. Each bait station was labeled and buried with only the entry hole exposed to soil surface. For the locations where bait stations could not be buried (e.g., hardscapes like the concrete patio), the tubes were laid on the ground in the notch of a small Lincoln Log™ (K'NEX Industries Inc., Hatfield, PA) and covered with plastic flower pots (15.5 cm diam by 11.5 cm ht) to protect it from sprinkler irrigation, pets, and direct sunlight. The bait stations were checked once a week for 4 wk. The amount of bait reduction per bait station was estimated by reading the 0.1-ml measured increments on the side of the bait stations. Stations that showed a substantial amount of reduction (i.e., >50% in vol) were replaced with new bait stations.

To determine the control efficacy, the activity level of Argentine ants was estimated using the monitoring tubes at weeks 1, 2, and 4 post-treatment. The bait stations were removed prior to placing monitoring tubes. Twenty monitoring tubes, 10 near the house and 10 away from the house, were placed in similar locations to where the bait stations had previously been located. After 24 h, the monitoring tubes were sealed and returned to the laboratory for weight measurement to estimate the number of ant visits (see above).

Statistical analyses. All statistical analyses were performed in R version 3.0.3 (R Development Core Team 2008). For the laboratory foraging study, the count data from 10 consecutive observations were averaged for each trial, and the average values were compared between treatment gel bait and standard gel bait only with a Welch Two-Sample T Test (R Development Core Team 2008). For the laboratory mortality data, an ANOVA followed by a Tukey honest significant test (HSD) test (R Development Core Team 2008) was used to compare the total cumulative numbers of dead ants at day 7 for both treatments and untreated control.

For the field study, amounts of gel bait consumed (ml) were compared between treatment and standard gel baits for each week of the experiment using a repeated measures ANOVA, with the fixed effect of treatment (treatment vs. standard gel baits) and a repeated effect of week. Average values from the 20 bait stations were used as data. A generalized mixed model (GLMM) was used to compare ant activity levels. The model contained fixed effects for week (pre-treatment and weeks 1, 2, and 4 post-treatment) and treatment (treatment gel bait and standard gel bait). The interaction of week and treatment was also included as was a random effect term for "house". GLMM was performed using the "lmer" function from the lme4 package (Bates et al. 2014) and the "Anova" function of the package "car" was used to generate type II tests (Fox and Weisberg 2011). The levels of ant activity from the number of ant visits were compared between the treatment and standard gel bait houses for each week using a Wilcoxon rank sum test.

Results

Laboratory study. Foraging activity of the Argentine ant was significantly greater in the treatment gel bait when compared with standard gel bait (Fig.2.1A). On average, the treatment gel bait had twice as many foragers compared with the standard gel bait [142.3 ± 24.4 and 55.5 ± 8.5 (mean ± SEM) ants for the treatment and standard gel baits, respectively] over 30-min observation period ($T = 3.8$; df = 22; $P < 0.001$). The ANOVA with day 7 total cumulative mortality indicated significant variation among the 3 treatments ($F = 42.3$; df = 2,4; $P < 0.001$ (Fig. 2.1B). Day 7 mortality was significantly greater in the treatment gel bait (453.4 ± 65.8 ants) compared with standard gel bait (273.3 ± 44.9 ants) (Tukey HSD: $P < 0.001$). Day 7 total cumulative mortality from the untreated control (45.6 ± 8.6) was low, significantly different from both gel bait treatments (Tukey HSD: $P < 0.001$).

Field study. The average amount of gel bait consumption was significantly higher for treatment gel bait than for standard gel bait in post-treatment monitoring trials ($F = 98.9$; df = 1,35; $P < 0.001$) (Fig. 2.2). The number of ant visits at monitoring tubes varied significantly among weeks (GLMM: $X^2 = 74.5$; df = 3,7; $P < 0.001$), but the main effect of treatments (treatment gel bait vs. standard gel bait) on averaged ant visits was not significant, indicating treatment alone did not influence overall ant visits ($X^2 = 0.82$; df = 1,7; $P = 0.37$). However, when the interaction of the fixed effect of week and treatments was considered, there was a significant effect ($X^2 = 20.2$; df = 3,7; $P < 0.001$) (Fig. 2.3).

The levels of ant activity estimated from the number of ant visits were compared between the treatment and standard gel bait houses for each week (Fig. 2.3). The

Wilcoxon rank sum test indicated that the houses assigned for treatment and standard gel bait applications were similar in Argentine ant activity for the pre-treatment week [19,945 ± 1,757 vs. 21,939 ± 1,848 (mean ± SEM) ant visits for the treatment and standard gel bait houses, respectively; $Z = 0.4$, $P > 0.5$]. In week 1, ant activity level of the treatment gel bait houses increased to 21,823 ± 1,498 ant visits while that of the standard gel bait houses decreased to 16,091 ± 1,456 ant visits. Consequently, the houses treated with treatment gel bait had significantly higher ant activity compared with the houses treated with standard gel bait by the end of week 1 ($Z = -2.8$, $P < 0.01$). In week 2, the ant activity level at the treatment gel bait houses decreased to 16,918 ± 1,590 ant visits while the standard gel bait house had a slight increase to 16,727 ± 1,644 ant visits, leaving the treatment and standard houses similar in their ant activity levels ($Z = -0.8$, $P = 0.4$). By week 4, the ant activity level of the treatment gel bait houses further decreased to 5,108 ± 753 ant visits, being significantly different from that of the standard gel bait houses (12,739 ± 1,253 ant visits) ($Z = 4.7$, $P < 0.01$). When compared with their own pre-treatment values, the treatment and standard gel baits achieved 74 and 42 % overall reductions in the Argentine ant activity level, respectively.

Discussion

Data from the laboratory study clearly indicates that the addition of (Z)-9-hexadecenal into the gel bait increases Argentine ant foraging activity on the gel bait. This result is corroborated by the findings of Greenberg and Klotz (2000), in which consumption rate of Argentine ants was 33% higher when the sucrose solution was dispensed from vials which were treated with (Z)-9-hexadecenal. Furthermore, higher

foraging activity for the pheromone-treated gel bait translates to higher final mortality. Because (Z)-9-hexadecenal is not insecticidal (Choe et al. 2014), the increase in the final mortality found in the pheromone-treated gel bait is attributed to the increasing number of Argentine ant foragers that are discovering and consuming the gel bait within the fixed amount of time. The consumption data from the field study also indicates that Argentine ants consistently consume more gel bait when it is treated with (Z)-9-hexadecenal (Fig. 2.2); the total amounts of gel bait consumed were 238 g and 95 g for the treatment and standard gel baits, respectively.

The field study suggests ant activity levels from the treatment and standard gel bait houses behave differently over time. Prior to the gel bait application, the houses assigned to either treatment or standard gel baits had similar levels of ant activity (Fig. 2.3). By the end of week 1 post-treatment, the houses with the treatment gel bait had higher ant activity compared with the houses with standard gel bait. This phenomenon was primarily caused by the fact that ant activity level at the houses with the treatment gel bait increased between the pre-treatment and week 1 post-treatment monitoring trials, while the houses with the standard gel bait showed a reduction in ant activity level during this period. It is possible that (Z)-9-hexadecenal originating from the treatment gel bait stations might have initially increased the number of foraging ants around the house including areas where the monitoring tubes were placed. Argentine ants are known to be attracted towards the source of (Z)-9-hexadecenal via odor-mediated anemotaxis (Van Vorhis Key and Baker 1982a,b; Choe et al. 2012; Choe et al. 2014). Suckling et al.

(2008) reported that the number of Argentine ants significantly increased when (Z)-9-hexadecenal was released from a point source within the field site.

The ant activity level in the treatment gel bait houses dropped substantially during the second week of baiting, resulting in similar levels of ant activity between the treatment and standard gel bait houses (Fig. 2.3). By the end of week 4, the treatment and standard gel baits achieved 74 and 42 % reductions in the ant activity level, relative to their pre-treatment values. Based on weekly monitoring of an untreated field site, ant activity levels within Riverside area did not experience any natural decline throughout the entire field study period (unpublished data). Additionally, the average temperature range throughout the study was 21-29.5 °C, which is well within the temperature range which would allow normal foraging activity of Argentine ants (Abril et al. 2010). Throughout the 4-wk baiting period, the amount of gel bait consumption was consistently higher for the treatment gel bait compared with the standard gel bait (Fig. 2.2). Based on this information and the data from the laboratory study, the higher control efficacy achieved by the pheromone-treated gel bait by the end of week 4 could be attributed to the higher amount of thiamethoxam delivered to the Argentine ant populations compared with the standard gel bait. However, it is also possible that (Z)-9-hexadecenal incorporated into the gel bait might have influenced the spread of thiamethoxam throughout the population of Argentine ants. For example, the presence of (Z)-9-hexadecenal in the ingested gel bait might influence the frequency of the trophallaxis between the original forager and other recipient ants. This aspect warrants further study.

The 74% reduction achieved in the current field study using the "pheromone-assisted baiting technique" in only 4 wk is particularly remarkable when considering the efficacy levels from other previous baiting trials with commercial products targeting urban ant populations. For instance, one field study indicated that the same gel bait product used in the current study (0.01% thiamethoxam) provided an average of 46% reduction in ant activity around the houses after 30 d (APVMA 2007). This level of control efficacy is similar with what the current study has observed for the standard gel bait by the end of week 4 (42%). Klotz et al. (2007) reported that houses treated with liquid borate bait only attained a 44% reduction in ant activity around the house after 4 wk.

For practical applications in pest management, achieving an initial attraction of Argentine ants to low concentrations of (Z)-9-hexadecenal incorporated into the gel bait is paramount. Even though Argentine ants are generally attracted to a source of (Z)-9-hexadecenal from a distance, it has been also shown that high concentrations of (Z)-9-hexadecenal applied in point sources are repellant or disruptive to the foraging ants (Greenberg and Klotz 2000, Suckling et al. 2010). Maintaining an optimal concentration range of (Z)-9-hexadecenal being released from the gel bait (or the gel bait station) is necessary to maximize the attraction and subsequent bait consumption by the foraging Argentine ants. Once the bait is discovered and consumed by a group of foragers, it is likely that the initial foragers will recruit more ants using their own communication system (Flanagan et al. 2013). The current study demonstrates that 0.1µg of (Z)-9-hexadecenal per 1 g of gel bait is effective in maintaining attraction within the small

laboratory colonies for 30 min without being repellent. The same rate of (Z)-9-hexadecenal was effective in attracting the foragers into the bait stations in the field settings. That is, we often observed that foraging Argentine ants readily discovered the pheromone-treated gel bait inside of the bait stations within 5-10 min after installing the bait stations.

The results of this study demonstrate the proof-of-concept for the pheromone-assisted baiting technique, suggesting its potential as an effective management tool for Argentine ants in urban settings. This pheromone-assisted baiting technique builds upon existing Argentine ant baiting technology by maintaining or improving important bait attributes such as delayed toxicity, transferability, and nonrepellency through the addition of (Z)-9-hexadecenal. Considering the current price of synthetic (Z)-9-hexadecenal (US $36.75 for 1 g, Bedoukian Research, Inc.), the pheromone-assisted baiting technique could be an economically viable modification for existing bait products. Other bait products containing different active ingredients (with different modes of action or transfer) need to be examined to determine their potential for the pheromone-assisted baiting approach. Also, an attribute of the bait station to consider in the current study is that the insecticidal gel bait is contained in a 15-ml plastic tube, thus eliminating the potential of insecticide run off into urban watersheds. The bait tube cap had a small circular hole (3 mm diam) drilled in the middle of the cap to limit the entry of nontarget organisms and water while permitting Argentine ant access. Underground bait stations were protected from homeowners, children and pets, and 100% of the bait stations were retrieved at the end of study. As federal and state laws become more stringent on residual

insecticide spray use, the pheromone-assisted baiting technique may provide an effective improvement to current control strategies.

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Figures

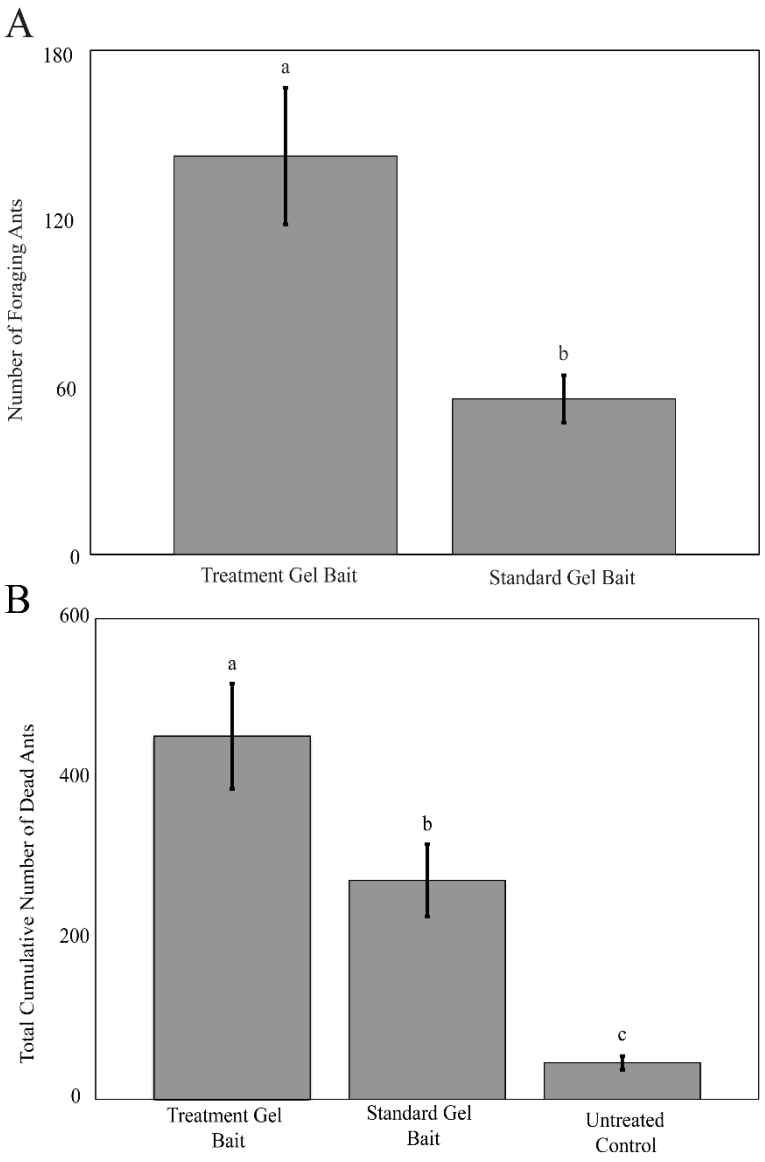

Fig. 2.1. Laboratory data results. (A) Number of foraging ants for treatment and standard gel baits. The height of each bar represents the number of foraging ants (mean ± SEM). (B) The total cumulative number of dead ants for treatment gel bait, standard gel bait, and untreated control after day 7. The height of each bar represents the total cumulative number of dead ants after day 7 (mean ± SEM). For each treatment, means with different letters are significantly different. See text for details of statistical analyses.

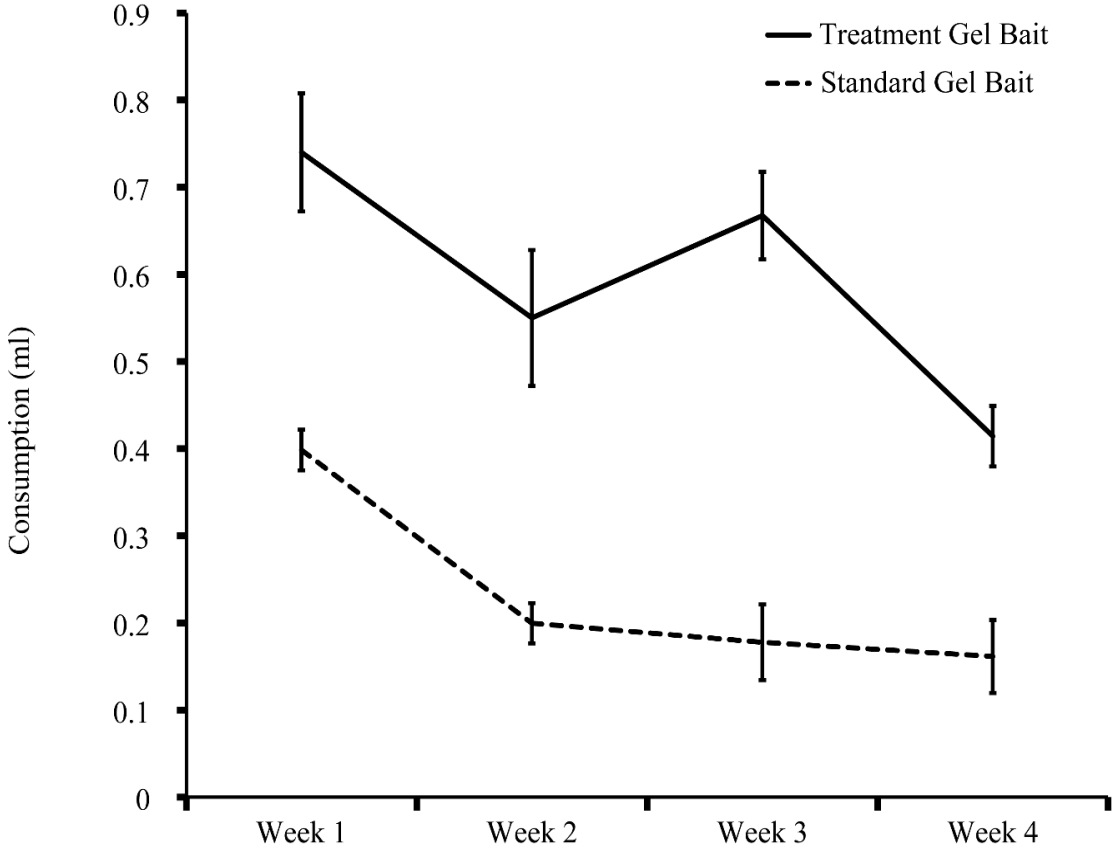

Fig. 2.2. Field study results showing amount of consumption (ml) of treatment and standard gel baits. Each point on the line graph represents the amount of consumption (mean ± SEM) for each week. See text for the details of statistical analyses.

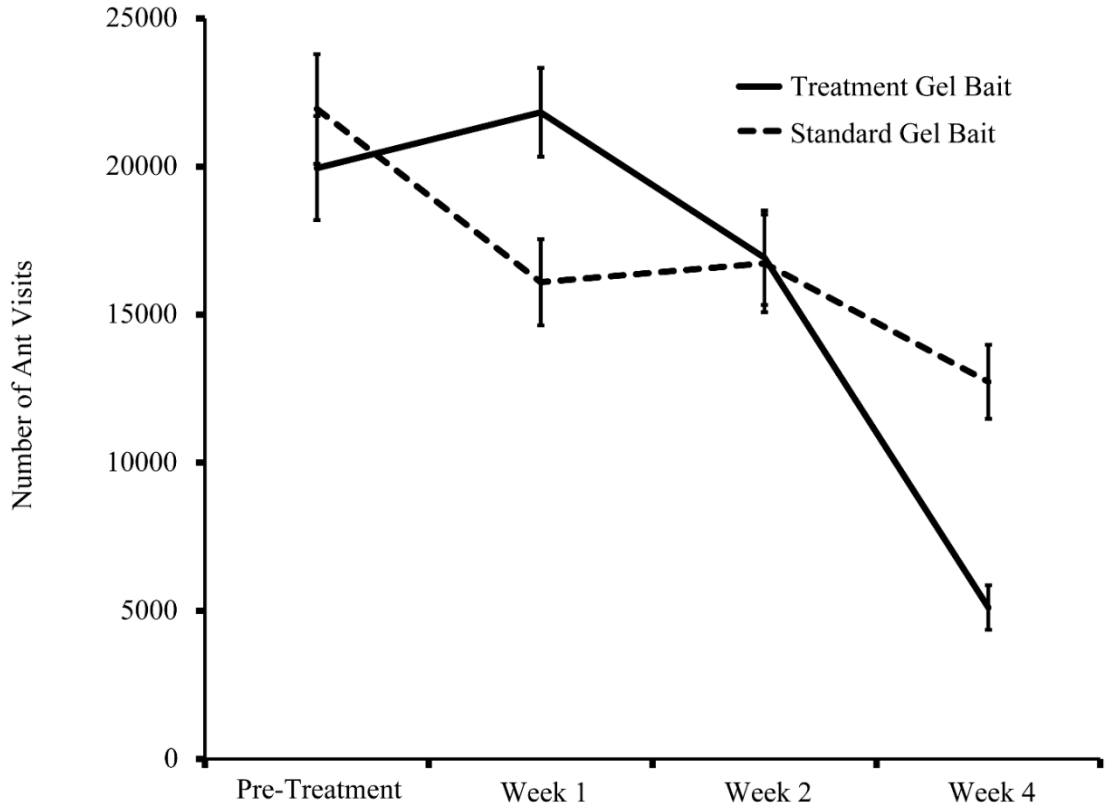

Fig. 2.3. Field study results showing the comparison between treatment and standard gel baits on the ant activity level. Each point on the line graph represents the number of ant visits (mean ± SEM) for each of the 24-h monitoring periods (i.e., pre-treatment and week 1, 2, and 4 post-treatment). See text for the details of statistical analyses.

Chapter 3.

RNAi for Argentine ant management: insecticidal bait with novel modes of action

Abstract

The Argentine ant *Linepithema humile* (Mayr), has been spread by human commerce to Mediterranean and subtropical regions throughout the world. Current control methods for the Argentine ants, in urban settings typically rely on perimeter applications of insecticides around infested structures. Insecticide applications result in potential problems with groundwater contamination and non-target effects. Baiting is often considered an effective alternative to insecticide sprays for managing Argentine ants since baits are largely target-specific with minimal environmental contamination. However, most baiting techniques use toxic chemicals as their active ingredients. RNA interference (RNAi) has the potential to become an effective alternative to conventional pest control baiting techniques. RNAi is a post-transcriptional gene-silencing technique which acts by double-stranded RNA (dsRNA). In this study, we developed a RNAi baiting technique that uses Argentine ant-specific dsRNA in a liquid sugary bait. Laboratory experiments indicated a 52% increase in mortality after 7 days when the RNAi bait was consumed by Argentine ants. The qPCR results confirm knockdown of target gene. Our study provides proof-of-concept of an RNAi baiting technique for Argentine ant management.

Introduction

The invasive Argentine ant is a notorious urban and agricultural pest that has become established worldwide (Suarez et al. 2001, Roura-Pascual et al. 2011). Argentine ant colonies are tremendously successful due to polygyny (the presence of numerous queens) and their ability to undergo fission or budding that eventually creates a network of interrelated nests that form a super colony (Aron 2001, Suarez et al. 2008, van Wilgenburg et al. 2010). Once established in new habitats, Argentine ants contribute to reduced reduce arthropod diversity, enhance phloem-feeding plant pests, compete with pollinators and invade buildings and homes (Knight and Rust 1990, Suarez et al. 2000, Lach 2005) In California, surveys indicate that Argentine ants are the most common urban pest encountered by pest management professionals (PMPs) (Knight and Rust 1990), and 85% of urban pest control services are allocated to the control of Argentine ants in California (Field et al. 2007).

Control of Argentine ant infestations primarily relies on residual insecticide sprays that are simple to apply and provide rapid control, making them a popular choice among PMPs (Rust et al. 2003, Silverman and Brightwell 2008). However, the active ingredients in commonly used residual insecticide sprays occur in urban waterways at levels that could be toxic to aquatic invertebrates (Jorgenson et al. 2012, Ensminger et al. 2013, Weston et al. 2013, Weston and Lydy 2014, Greenberg et al. 2017). Baiting is an effective alternative to insecticide sprays for managing pest ants and provides numerous advantages, such as better target specificity and minimal off-site movement of insecticidal active ingredients (Klotz et al. 2003, Klotz et al. 2010). Insecticidal baits

have the potential to provide adequate Argentine ant control when properly designed and used. For example, the “pheromone-assisted baiting technique” for Argentine ants is designed to achieve higher specificity and efficacy while minimizing the amount of insecticide applied (Welzel and Choe 2016). However, baiting for Argentine ants still relies on conventional chemical insecticides to provide control.

RNA interference (RNAi) has the potential to provide a new class of insecticide that does not rely on conventional chemicals as the toxicant. RNAi is a technique used to switch off gene function that is triggered by the presence of double stranded RNA (dsRNA). The dsRNA is then processed into small interfering RNA’s (siRNA) By an RNase III enzyme called Dicer. These siRNAs become incorporated into a protein complex known as the RNA induced silencing complex (RISC). The RISC is then guided to a specific mRNA that is complementary to the siRNA causing mRNA degradation (Dykxhoorn et al. 2003, Meister and Tuschi 2004). RNAi is being developed as a powerful weapon in the fight against agricultural insect pests, such as plant-mediated RNAi that is used to produce and provide dsRNA to target pest. (Zha et al. 2011). The target gene must be associated with vital biological processes and provide a strong RNAi response once ingested. A drawback of plant mediated RNAi is that there is no guarantee of sufficient uptake of dsRNA or siRNA by insects due to the variation in dsRNA synthesis and concentration in the transgenic plant (Mao and Zeng 2014). Incorporating dsRNA into artificial diets can potentially provide stability and higher concentrations of dsRNA for insect uptake.(Zhang et al. 2013).

RNAi has the potential to become an effective alternative to conventional pest control techniques. Many studies emphasize that dsRNA-mediated gene knockdown may be useful for developing high efficiency and low toxicity pesticides (Zhang et al. 2013). In pest control, RNAi shows great potential because of the numerous advantages including high target specificity (Price and Gatehouse 2008), lack of environmental contamination (Zhang et al. 2013), less potential for insects to develop resistance (Price and Gatehouse 2008) and it can be engineered into a target pest's food source for delivery (Katoch et al. 2013, Zhang et al. 2013).

The current study was designed to verify whether the RNAi pathway is functional in the Argentine ant and whether the ingested dsRNA could induce the level of mortality necessary for pest management. A candidate gene was selected for its potential to cause mortality and confirm that the RNAi machinery was functional. The results of this study provide proof-of-concept for future development of RNAi for the control of the Argentine ant.

Materials and Methods

Gene Selection. The candidate gene was selected based on its potential to exhibit insecticidal like properties. (Zhang et al. 2013). Criteria included feeding application method to adult insects and high potential for toxicity. Genes that require an application method such as injection, or feeding of nymphal or larval stages were omitted. Because the target sequences were not available in the database for *L. humile*, they were downloaded from the *Drosophila* database of the National Center of Biotechnology

Information (NCBI). BLAST (Basic Local Alignment Search Tool) searches of target gene sequences against the *L. humile* genome were conducted using the Hymenoptera Genome Portal (Elsik et al. 2016) for preliminary identification/annotation. Gene sequences having high similarity, including low E-values and matching global homology, were selected. These were BLAST against the database of other insects for confirmation. Conserved regions were not omitted in the designing of primers. Primers were designed using Primer3Plus (Untergasser et al. 2007). Bases on this criteria, . ADP, ATP carrier protein, accession number XP_012227985, was chosen. RNAi knockdown of ADP, ATP carrier protein with similar homology in the whitefly (*Bemisia tabaci*) resulted in 32.5% mortality after day 6 compared to only 13.3% in controls (Upadhyay et al. 2013).

Synthesis of double stranded RNA. A MEGAscript®RNAi Kit (Life Technologies, CA) was used to synthesize dsRNA according to the manufacturer's instructions. Template DNA for dsRNA production was generated by PCR amplification using gene-specific primers containing T7 promoter sequences tailed at the 5' end of each primer (Table 3.1). After synthesis, dsRNA concentrations were measured using a NanoDrop™ (ND1000 spectrophotomter (Thermo-Fisher Scientific, USA).

Feeding Bioassay. A laboratory study was conducted to determine if a sugary bait with dsRNA the addition of dsRNA into a sugary bait could be consumed by the Argentine ant and induce the RNAi pathway, resulting in mortality. A group of 10 Argentine ant workers, reared from laboratory stock colonies, was aspirated, anesthetized with $C0_2$, and placed in an empty soufflé cup (167 ml vol). The inner sides of the cup were coated with Teflon (Fluoropolymer resin, type 30, Dupont Polymers) to prevent escape. Each

experimental colony was provided with a harborage tube, constructed from a 1.5 ml microcentrifuge tube filled with 0.5 ml of water and stoppered with a cotton ball. The harborage was glued down in the center of the soufflé cup with Elmer's glue (Elmer's Products Inc., USA) to prevent movement and allowed to sit for 24 h prior to Argentine ant introduction.

We compared the following bait solutions: A dsRNA sugary bait solution containing 500 ng dsRNA in 1 ul of 25% sucrose solution (dsRNA treatment) and 25% sucrose only bait (Control). For each of the baiting solutions, 10 experimental colonies were used (described above). Experimental colonies were given the bait solutions once every day for 6 d. Dead ants were removed once a day and overall mortality scored at the end of the 7 dday trial period.

RNA extraction and cDNA synthesis

RT-PCR, RNA extraction followed by cDNA synthesis was performed to prepare samples for quantitative RT-PCR (qRT-PCR). A single Argentine ant was collected from the following time points: 4 hr, 8 hr, 24 hr and 3 d after beginning bait solution feeding (dsRNA treatment and control). A total of 3 replicates were collected for each time point. Red food coloring (McCormick & CO., INC., USA) was added to bait solution (10% by volume) confirm uptake of baiting solution during collection. Collected ants were immediately placed on dry ice and total RNA was extracted from a single Argentine ant using the RNeasy™ Plant Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. Quality of RNA was determined by 1.2% agarose gel electrophoresis, and concentration was determined by using NanoDrop™ (Thermo Scientific, USA). cDNA

synthesis was performed using the SuperScript™ III First-Strand Synthesis System (ThermoFisher Scientific, USA) following the manufacture's protocol. After synthesis, cDNA was diluted 10 fold with molecular grade water.

Quantitative RT-PCR

The extent of target gene silencing was assessed by employing quantitative RT-PCR. The housekeeping gene *ef1-alpha* rRNA was used as a reference (Table 3.1), as in previous work (Cheng et al. 2013). qRT-PCR reactions were carried out in biological and technical triplicate replicates for each of the following time points: 4 hr, 8 hr, 24 hr and 3 day after beginning dsRNA bait feeding. For each sample, the total reaction volume was 10 µl, containing 5 µl SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD, USA), 0.3 µl each of forward and reverse primers (10 µM each, Table 3.1), and 2 µl diluted cDNA template mixed with 2.4 µl nuclease free water. Cycling was performed using the following parameters: 95° C for 3 min, followed by 40 cycles of 95° C for 30 s, 56° C for 30 s and 72° C for 1 min. Melt curve analysis of amplicons was performed by continuous capturing of fluorescence while temperature increases from 65 C 95° C with ramp rate of 0.5° C/s. qRT-PCR analysis was performed on a CFX96™ Real-Time System (BIO-RAD, USA).

Data analysis

The relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All qRT-PCR assays were designed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). The effect of silencing the target gene expression was

analyzed statistically by two-sample *t* test at $P < 0.05$ with C_q values of three replicates for each time period. Data for total mortality was analyzed with a Wilcoxon Rank Sum Test. All statistical tests were performed in R (R Development Core Team 2016).

Results

Feeding Bioassay

Both bait solutions were provided every day for six days (see methods). The overall Argentine ant mortality was significantly higher in the dsRNA treatment compared to the control at the end of the seventh day (z=3.8 , df=18, P< 0.001). Argentine ant mortality was 52% (±6.9, SEM) in dsRNA treated sucrose water trials and 10% (±5.2, SEM) in sucrose solution only (Fig. 3.1).

Quantitative RT-PCR

In qRT-PCR, the melt curve analysis of target gene, ADP, ATP carrier protein and reference gene, *ef1-alpha* exhibited a single melt peak, indicating specific amplification of the respective genes. Further, resolving of qRT-PCR products on agarose gel reconfirmed the presence of a single band. The present study showed that a sucrose bait containing dsRNA triggered RNAi in the Argentine ant. We observed a significant reduction in the mRNA levels of the target gene using our specific dsRNA concentration after a single feeding. Argentine ants collected at 4 h, 8 h, 24 h, and 3 d post feeding showed a 81%, 83%, 79% and 84% reduction in ADP, ATP carrier protein 2 mRNA levels, respectively, (Fig. 3.2). Relative mRNA levels were significantly lower in 8 hr, 24 hr and 3 d dsRNA treatments when compared to controls [(T=-2.84, df= 4, P= 0.02), (T=-3.44, df= 4, P= 0.01), (T=-2.75, df= 4, P= 0.03), respectively.]

Discussion

The study provides evidence of a functional RNAi pathway in the invasive Argentine ant. Argentine ants that were fed RNAi bait resulted in target gene suppression and death. We demonstrated a 52% reduction in Argentine ant numbers in 7 d using dsRNA in a sugary liquid bait. Furthermore, mRNA levels were significantly reduced after 8 h, 24 h, and 3 d after beginning RNAi feeding. RNAi is likely to be a valuable tool for pest management of the invasive Argentine ant.

Double-stranded RNA mediated knockdown of specific genes by the RNAi process has been reported in a plethora of insect species including Coleoptera, Lepidoptera, Diptera, Himiptera, Hymenoptera, Isoptera, and Orthoptera (Huvenne and Smagghe 2010, Asokan et al. 2013). The core RNAi machinery found in these insect orders consists of Argonaute endonucleases, Dicer enzymes and dsRNA binding protein that make up three different RNAi pathways based on the types of Dicers, Argonauts and small RNAs involved (Carthew and Sontheimer 2009, Jinek and Doudna 2009, Moazed 2009, Siomi and Siomi 2009). Uptake of dsRNA into cells and systemic spread are suspected to be the most limiting factors of RNAi. Developing a successful RNAi based approach for pest management requires identification of suitable target gene(s) and methods of delivery that that will maintain specific concentrations and the integrity of dsRNA in the baiting medium.

Oral delivery of dsRNA, while a popular mechanism for practical pest management strategies, still has many drawbacks to overcome. For example, continuous feeding over several days is a major requirement to induce significant mortality. Previous

studies were unsuccessful when dsRNA was administered through diet, even with continuous feedings, e.g. 1 µg day^{-1} in *Schistocerca gregaria* and 6 µg day^{-1} in *Locusta migratoria* for 8 d (Luo et al. 2013, Wynant et al. 2014). Another study achieved mortality and target gene suppression after continuous feeding of 0.5 µg day^{-1} for 16 d in *B. germanica* (Lin et al. 2017). The presence of dsRNA degrading enzymes in the midgut may lead to less effective gene silencing in some insects (Christiaens et al. 2014, Wynant et al. 2014). However, our study has demonstrated that continuous feeding of a relatively low amount of dsRNA (0.5 µg day $^{-1}$) for 7 d depletes target gene expression within 8 h and causes 52% mortality in the Argentine ant, indicating a possible lack of dsRNA degrading activity in the midgut. Further investigation into the dsRNA degrading activity in the Argentine ant will be necessary in developing a novel baiting technique utilizing RNAi.

To translate RNAi to field applicability in the pest control industry, RNAi baiting techniques will need to combine biotic or abiotic systems that mediate both protection and uptake of essential RNAi components. The success of RNAi technology is largely dependent on the stability of dsRNA or siRNA during and/or after oral delivery that may be remedied using nanoparticles. dsRNA-nanoparticles are being developed to control *Anopheles Gambiae* and show great potential for development into novel strategies for pest management (Zhang et al. 2010, Zhang et al. 2015). Furthermore, genetically modified yeast was designed to produce and deliver dsRNA to *Drosophila*, resulting in decreased fitness in the *Drosophila suzukii* (Murphy et al. 2016). Transgenic yeast may provide a possible delivery solution for developing RNAi bating for ant management.

Advances in technology that aim to improve the efficacy of RNAi baiting will help cement this technology as a potential alternative to current control methods in the pest industry.

RNAi by oral delivery of dsRNA in insects has great potential as a tool for integrated pest management (IPM), especially with respect to addressing the need to reduce off-target effects and slow down resistance development to chemical insecticides. Our results indicate 52% in Argentine ants after 7 d and drastic target gene suppression. In order this technique to become an effective tool We provide proof-of-concept for the development of a potential RNAi baiting technique for Argentine ant management.

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Figures

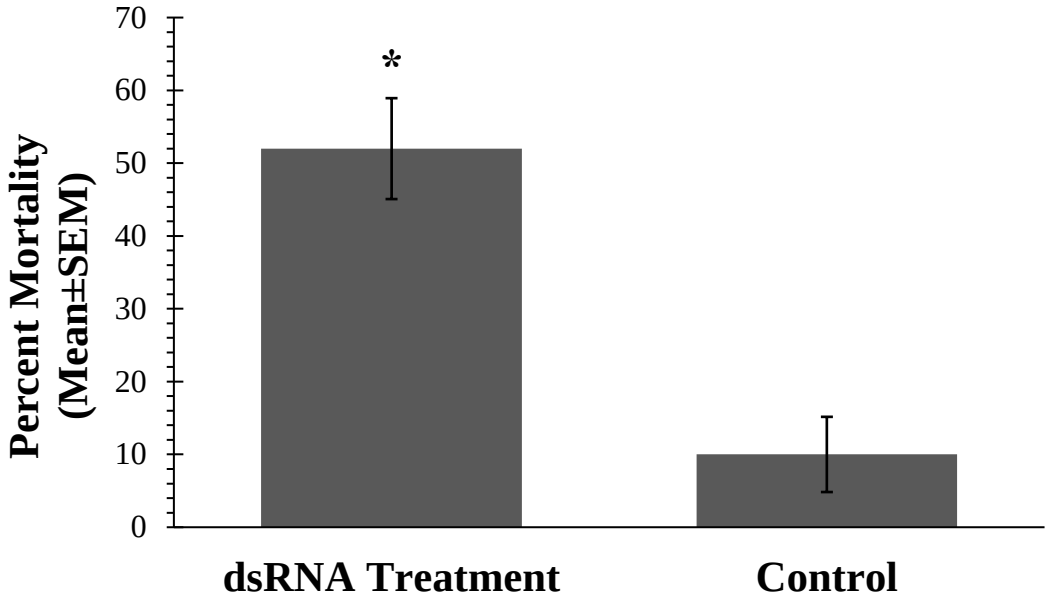

Fig. 3.1. Effect of ADP, ATP carrier protein dsRNA sucrose bait on Argentine ant mortality after 7 days of continual feeding. dsRNA treatment contained 500ng ADP, ATP carrier protein in 1 µl of sucrose water and control consisted sucrose water only. Error bars indicated standard error of the mean. Asterisks indicates dsRNA treatment is significantly different from control, $P< 0.001$.

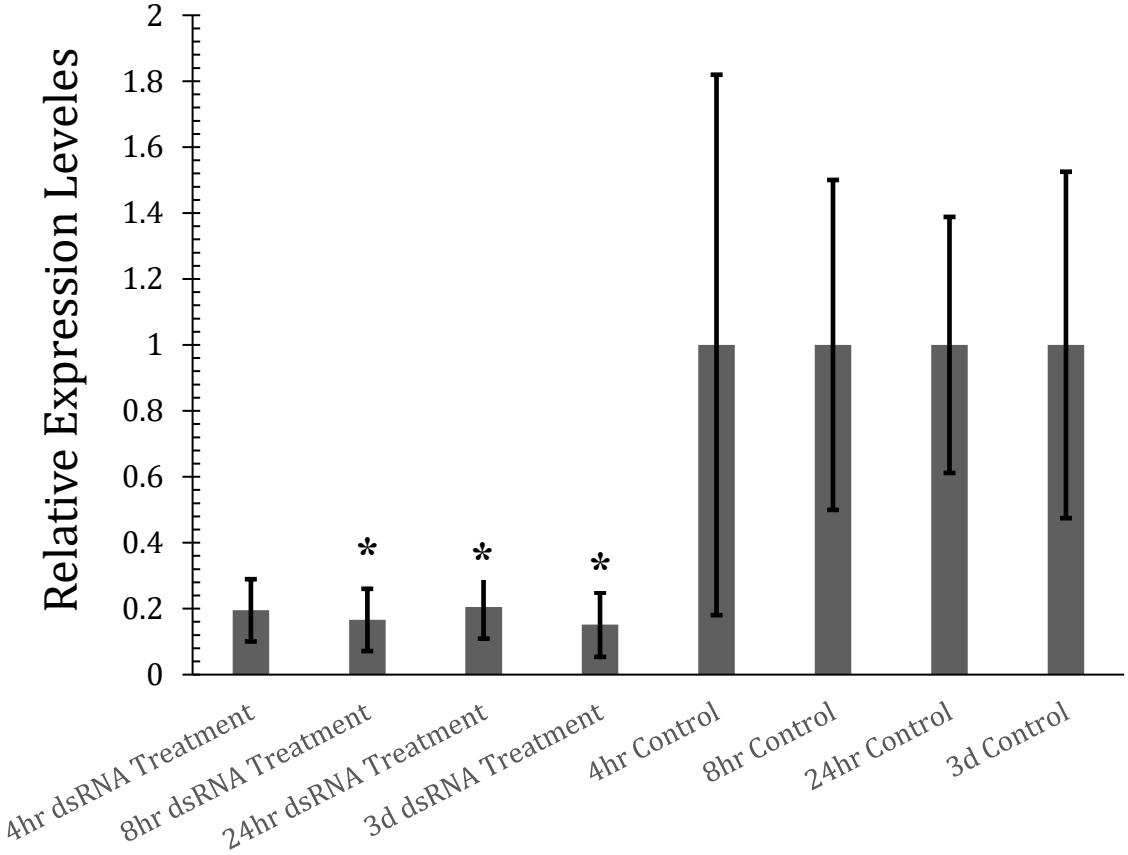

Fig. 3.2. Relative quantification of target gene expression by employing qRT-PCR. The effect a single application of 500ng ADP, ATP carrier protein in 1 µl of sucrose water (dsRNA Treatment) and sucrose water only (Control) on expression levels. Error bars indicate standard deviation of the triplicate samples. Asterisk indicates statistically significant differences ($P < 0.05$).

Table 3.1. Description of primers used in this study. The T7 promoter sequence is Italicized.

Gene	Primer sequence (5' -> 3')	Amplicon Length
ds- ADP, ATP carrier protein	For: *TAATACGACTCACTATAGGG*AGATAGGCAAAGCTGGTGGTGAG Rev: *TAATACGACTCACTATAGGG*AGAACCACGGAGGACGTTAGAGA	406
qRT- ADP, ATP carrier protein	For: TAGCGATTCCCAAAAGCCGA Rev: AGCTGCTACGTCCAACAACAT	94
qRT- ef1-alpha	For: AAGGGCTCGTTCAAGTACGC Rev: CGTCTCGAACTTCCACAGGG	96

Chapter 4.

The identification of Argentine ant defensive compounds and their behavioral effects on heterospecific competitors and conspecific nestmates

Abstract

The invasive Argentine ant (*Linepithema humile*) has become established worldwide in regions with Mediterranean or subtropical climates. The species typically disrupts the balance of natural ecosystems by competitively displacing some native ant species via strong exploitation and interference competition. Here we report that Argentine ants utilize glandular secretions for inter and intra-specific communications during aggressive interactions with a heterospecific competitor, California harvester ant (*Pogonomyrmex californicus*). Chemical analyses indicated that Argentine ants deploy glandular secretions containing two major volatile iridoids, dolichodial and iridomyrmecin, on the competitor's cuticular surface during aggressive interactions. Bioassays indicated that the glandular secretions function as a defensive allomone, causing high levels of irritation in the heterospecific. Furthermore, the same glandular secretions elicited alarm and attraction of conspecific nestmates, potentially enabling more rapid / coordinated defense by the Argentine ants. Two major volatile constituents of the glandular secretion, dolichodial and iridomyrmecin, were sufficient to elicit these responses in conspecifics (as a mixture or individual compounds). The current study suggests that invasive Argentine ants' superior exploitative and interference competition may rely on the species' effective semiochemical parsimony.

Introduction

From its native range in South America, the Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) has become a global invader, particularly in areas with Mediterranean climates (Vega and Rust 2001). Argentine ant colonies interact and freely mix over long distances, form super colonies, and maintain a high rate of reproduction via multiple queens (Holway 1999). These traits of the Argentine ant, unicoloniality and polygyny, have likely contributed to their successful establishment in locations around the globe (Hölldobler and Wilson 1990, Helantera et al. 2009). The successful establishment of Argentine ant populations in their non-native ranges typically results in numerous detrimental effects, such as reduction of arthropod diversity (Bolger et al. 2000), proliferation of phloem-feeding plant pests (Lach 2005, Tena et al. 2013), competition with pollinators (Buys 1987, Levan et al. 2014), and invasion of residential structures (Knight and Rust 1990).

Once introduced into new habitats, Argentine ants must compete with existing native species to successfully establish (Suarez et al. 1998). Exploitation competition (i.e., depleting resources that would otherwise be used by other species) and interference competition (i.e., prohibiting access to resources by other species via direct interactions) have been studied to understand their roles in the Argentine ant's successful establishment in non-native habitats, especially against other native ant species (Holway 1999, Thomas and Holway 2005, Walters and Mackay 2005). Some of these empirical studies have indicated that invasive populations of Argentine ants have a higher competitive ability than most native ant species in exploitation and interference

competition, not only discovering and dominating resources more quickly than the native ants, but also outcompeting native ants via highly aggressive interactions at the resources (Holway 1999, Buczkowski and Bennett 2008).

In many social insects, semiochemicals play an integral role in organizing colony-level activities such as foraging and defense (Hölldobler and Wilson 1990, Vander Meer 1998). Argentine ants' high competitiveness in exploitation and interference competition might be, at least in part, due to their efficiency in communicating via semiochemicals (Wilson 1971). For example, the rapid mass-recruitment of nestmates to the new resource via the use of trail pheromone may have contributed to their success in exploitation competition by allowing them to numerically dominate the resource more quickly than competitors (Human and Gordon 1996, Holway 1999). Dolichodial, iridomyrmecin, and (Z)-9-hexadecenal have been studied as components of the trail pheromone of Argentine ant (Cavill et al. 1980, Choe et al. 2012). Argentine ants also defend the resource from other competitors by directly preventing the competitors' physical establishment at the resource (Holway 1999). For example, aggressive interactions between Argentine ants and other native ant species have been described in several studies (Ward 1987, Holway 1999, Human and Gordon 1999, Sorrells et al. 2011), suggesting that Argentine ants' high competitiveness in aggressive interactions might play a role in their ability to outcompete and displace native ant species.

Previous studies have speculated that Argentine ants use "defensive compounds" during aggressive interactions with other species of ants (Brown 1973, Human and Gordon 1999, Buczkowski and Bennett 2008). In particular, Argentine ants have been

observed to display a behavior known as “gaster bending” or “gaster flexing” during aggressive interactions (Liang et al. 2001, Dejean et al. 2010). In this behavior, a worker Argentine ant typically bends its gaster ventrally to place the tip of the gaster onto the opponent (Fig. 4.1). It was also speculated that irritant chemicals may be “sprayed” on the opponent via similar behaviors (Lieberburg et al. 1975). Several studies have suggested that Argentine ants might use pygidial (anal) gland compounds for defensive functions, like other dolichoderine ants (Pavan 1951, Cavill and Cark 1971, Hölldobler and Wilson 1990). Historically, the pygidial glands of dolichoderine ants have been believed to produce defensive secretions that provoke the alarm response, or chemicals with antibiotic/insecticidal effects (Wilson and Pavan 1959, Cavill et al. 1976, Blum and Hermann 1978, Kugler 1979a). Two iridoids, dolichodial and iridomyrmecin, have been described in the pygidial gland secretions of Argentine ant (Pavan 1951, Cavill et al. 1976). However, detailed behavioral and chemical investigations have not been conducted to elucidate the chemical ecology of Argentine ant aggression and the possible defensive functions of the pygidial gland chemicals.

Competition between Argentine ants and harvester ants (*Pogonomyrmex* spp.) has been documented in field sites in California, and typically resulted in the displacement or decline of harvester ant populations in areas with both species (Erickson 1971, Suarez et al. 2000). The competitive relationship between these two species is unique because they naturally occupy different ecological niches. Argentine ants prefer relatively high moisture content in the soil for nesting, and they are omnivorous, collecting insect prey and tending phloem-feeding insects for their sugary secretions (Erickson 1971). In

contrast, harvester ants tolerate higher temperatures and drier conditions in semi-desert habitats, primarily relying on diets consisting of gathered seeds (Erickson 1971, Tschinkel and Kwapich 2016). However, Argentine ant populations often penetrate drier semi-desert areas via surrounding urban edges where they become abundant due to irrigation from the developed areas (Suarez et al. 1998, Menke and Holway 2006). In addition, coastal environments with fresh water from natural springs or ephemeral waterways may provide suitable environmental conditions for Argentine ant establishment (Randall et al.). This habitat overlap between harvester ants and the Argentine ants results in competitive interactions between the two species (Mackay 1995, Menke and Holway 2006). Nevertheless, due to differences in their preferred diets and nesting conditions, competition for identical food resources and nest sites do not appear to be among the main reasons for the decline of harvester ant populations (Erickson 1971, Zee and Holway 2006). Instead, competitive interactions between these species often take the form of nest raiding by Argentine ants, which also has been observed between Argentine ants and other native ants (Rowles and O'Dowd 2007, Buczkowski and Bennett 2008). In California, invasive Argentine ant populations have been observed to exploit the brood of *Pogonomyrmex subnitidus* (Emery) colonies, resulting in the eventual decline of harvester ant populations(Zee and Holway 2006).

The present study explored the chemical ecology of aggressive interactions between Argentine ant and the California harvester ant, *Pogonomyrmex californicus* (Buckley). In particular, we hypothesized that Argentine ants release and apply pygidial gland secretions through the gaster-bending response during aggressive interactions with other

species. By exploring the functions of the pygidial gland secretions (and chemicals) in Argentine ants' competitive interactions with heterospecific competitors, we attempted to elucidate some of the key mechanisms behind their remarkable competitive ability. The objectives of this study were: (1) to determine if the pygidial gland secretion is produced by Argentine ants during aggressive interactions with harvester ants, (2) to determine if the pygidial gland secretion is applied on the harvester ants' cuticle during aggressive interactions, (3) to understand the behavioral effects of the crude pygidial secretion on the harvester ant when it is topically applied to the cuticle, and (4) to understand the behavioral effects of the crude pygidial secretion and its volatile constituents on Argentine ants when the chemicals are presented from a nearby point source.

Materials and Methods

Insects. Argentine ants were collected from the biological control citrus grove on the University of California, Riverside campus. Ant nests were excavated and transported to the laboratory, where the ants were extracted from the soil (Hooper-Bui and Rust 2000). As the soil dried, workers moved the entire colony into moistened disks made of plaster of paris where they were carefully shaken off into plastic boxes (26.5 by 30 by 10 cm). Laboratory colonies were maintained in plastic boxes (26.5 by 30 by 10 cm); the inner sides were coated with Teflon (Fluoropolymer emulsion, type 30, DuPont Polymers, Wilmington, DE) to prevent ants from escaping. Each colony was provided with two or three artificial nests constructed from plaster-filled petri dishes (9 cm in diameter by 1.5 cm in depth) with a smaller cylindrical area (5 cm in diameter by 1 cm depth) in the center of the dish, to serve as a nesting space. The colonies had free access to water and

25% (wt:vol) sucrose-water solution. Freshly killed American cockroaches (*Periplaneta americana*) were provided to the colonies three times a week as a protein source.

A nest of *P. californicus* was located on the University of California, Riverside campus. Foragers were collected from the soil surface around their trails or nest entrances. The collected harvester ants were temporarily kept in plastic boxes (26.5 by 30 by 10 cm) with the inner sides coated with Teflon, before being used for the bioassays on the same day.

Chemicals. The authentic standard of *trans, trans*-dolichodial (>99% pure) collected from *Anisomorpha buprestoides* stick insect defensive secretion (Dossey et al. 2008) a was provided by A.T. Dossey (Gainsville, Florida). Synthetic *cis,trans*-iridomyrmecin (94% pure) was provided by K.R. Chauhan (USDA-ARS, Beltsville, Maryland). Authentic standards of dolichodial, iridomyrmecin and *n*-dodecane (Sigma-Aldrich) were used for identification of the volatile compounds and subsequent behavioral assays. The authentic standards of dolichodial (0.325 µg/ml) and iridomyrmecin (1mg/ml) were originally obtained in methylene chloride and acetone, respectively. Thus, the same solvent was used to prepare the diluted samples for each of the standards to avoid any uncontrolled change or loss of the standard compounds during the evaporation of the original solvent and reconstitution in a new solvent. Also, a mixture of methylene chloride and acetone was used as the solvent control in some of the behavioral assays.

Headspace volatile analyses. Headspace volatile analyses were conducted to determine if the pygidial gland chemicals are emitted by gaster-bending Argentine ants during aggressive interactions with a harvester ant. Samples (i.e., presence of aggressive

interactions) were prepared and analyzed in the following manner. Five Argentine ants were briefly anesthetized with carbon dioxide and placed in a 2-ml glass vial (Agilent Technologies) coated with Teflon. Once the ants recovered from the anesthesia (after 10 min), one live harvester ant was placed in the vial. The behavioral interactions between the two species were observed. Once any Argentine ant performed the gaster bending behavior on the harvester ant, a solid phase microextraction (SPME) fiber [Supelco Inc., 100-mm polydimethylsiloxane (PDMS)] was exposed in the headspace of the glass vial through the septum vial cap. Once gaster bending ceased (up to 2 min) the SPME fiber was removed from the vial and immediately injected into a coupled gas chromatograph-mass spectrometer (GC-MS) for analysis. For GC-MS, electron impact mass spectra (70 eV) were taken with an Agilent 5975C mass selective detector interfaced to an Agilent 7890A gas chromatograph fitted with a DB-5 column (30 m x 0.25 mm inner diameter, Agilent Technologies). Samples were injected in splitless mode, with a temperature program of 50˚C for 1 min, then 10˚C min^{-1} to 280˚C with a 5-min hold. Helium was used as the carrier gas. The compounds were identified based on comparisons (mass spectra and retention times) with standards of natural (dolichodial) or synthetic (iridomyrmecin) origin (Choe et al. 2012). The analysis was replicated 10 times with randomly selected Argentine and harvester ants. To confirm if detectable amounts of pygidial gland volatiles are produced only during aggressive interactions between the two species, the headspace volatiles of control samples (i.e., absence of aggressive interactions) were also analyzed and compared with the treatment including: 1) five Argentine ants only, 2) one harvester ant only, and 3) empty glass vial with no ants. Five

replications were made for each of these controls. All ants were used only once for volatile collections.

Cuticular extract analyses. Cuticular extracts of the harvester ants were analyzed to determine if the pygidial gland chemicals were applied to them by the gaster-bending Argentine ants during aggressive interactions. A group of 100 Argentine ants was briefly anesthetized using carbon dioxide and placed in a glass petri dish (150 mm in diameter by 20 mm in height). A thin layer of Teflon was applied to the inner side of the dish to prevent the ants from escaping. After the Argentine ants recovered from the anesthesia (~10 min), one harvester ant was placed in the dish. From the time point when an Argentine ant showed the first gaster bending towards the harvester ant, the ants were allowed to continue interacting for an additional 2 min. During this period, multiple Argentine ants showed gaster-bending responses. The total number of Argentine ants showing the gaster-bending response was recorded. After the 2-min observation, the harvester ant was removed from the glass petri dish and immediately placed on dry ice. Dry ice killed the harvester ant instantly and kept it at a low temperature (-78.5 °C), potential limiting dissipation of any volatile chemicals from its cuticle.

Thirty harvester ants were individually prepared as described above, and pooled for subsequent solvent extractions. The pooled sample was loaded in a glass Pasteur pipette (7 mm in diameter by 14.6 cm in length, Fisher Scientific) with 150 mg of silica gel bed (60Å pore size, 230-400 mesh, Whatman®) for brief extraction and simultaneous fractionation of the extracted chemicals. Two fractions were obtained from the sample. Fraction 1 (non-polar fraction) was obtained by flushing the ants and the column with 2

ml of hexane; Fraction 2 (polar fraction) was obtained by subsequently flushing the column with 2 ml of methylene chloride. Each extraction / fractionation process was brief, allowing the solvent to contact the sample for about 5-10 seconds. The fractions were collected in separate vials, and concentrated to 0.5 ml under nitrogen. Both fractions were examined with normal phase (silica gel) thin layer chromatography (Flexible plates for TLC AL SIL G/UV 250 µm layer, Whatman®). Both fractions were further analyzed with an Agilent 7890 gas chromatograph equipped with a DB-5 column (30 m x 0.25 mm inner diameter, Agilent Technologies) and a flame ionization detector (GC-FID), using an automatic liquid sampler (ALS) that injected 1 µl of sample. Samples were injected in split mode, with a temperature program of 50˚C for 1 min and then 10˚C min^{-1} to 300˚C with a 10-min hold. Helium was used as the carrier gas. In addition to the GC-FID analysis, the samples were also analyzed with a GC-MS using the methods described in the headspace volatiles analysis section. The compounds were identified based on comparisons (mass spectra and retention times) with the standards (Choe et al. 2012). A control sample was prepared from 30 harvester ants that had not interacted with Argentine ants, but were otherwise handled identically.

To estimate the quantities of the compounds per harvester ant, a series of external standards of known concentrations [0.01-0.32 µg/µl for dolichodial in methylene chloride and 0.01-1.00 µg/µl for iridomyrmecin in acetone] were analyzed with the GC-FID. Calibration curves were established for each compound, and the total quantity of each compound in the cuticular extract was estimated based on the corresponding calibration curve. To obtain the quantities per harvester ant, the values estimated for the entire

sample (30 harvester ants combined) were divided by 30, assuming that each of the harvester ants contributed equally to the total quantities.

Collection and chemical analyses of pygidial gland chemicals. The pygidial gland secretion was collected from live Argentine ants for subsequent behavioral assays. First, a group of Argentine ants were briefly anesthetized (carbon dioxide) and subsequently chilled on an ice pack. The pygidial gland secretion was "milked" out of the gland reservoir of each ant by gently squeezing the dorsal posterior region of the gaster with a pair of fine forceps. The droplet of pygidial gland secretion (referred to as "pygidial gland extract" or "PGE" hereafter) was taken up into a glass micropipette (1 µl in capacity, Drummond Scientific Co.) and weighed. Ten separate collections were made from ten different Argentine ants as replicates. The ten collections of PGE were rinsed out of the capillary with acetone and pooled. The amounts of dolichodial and iridomyrmecin in one ant equivalent of PGE were estimated with a series of external standards of known concentrations. For water detection in the PGE we used paper treated with cobalt chloride (IN20A humidity indicator cards small, Conservation Resources UK Ltd.).

Response of harvester ants when topically treated with pygidial gland chemicals. The behavioral effect of Argentine ant pygidial gland chemicals to harvester ants was examined by topically applying PGE on the cuticular surface of the harvester ants. Using a pipette pump, one ant equivalent of PGE was dispensed from the glass micropipette and applied onto the head of the harvester ant, between the compound eyes. The head was chosen as the location for PGE treatment because Argentine ants typically aim their

gasters towards the opponent's head during gaster bending (Lieberburg et al. 1975). The control consisted of harvester ants that were not treated with PGE, but otherwise handled identically. The treatment or control harvester ant was immediately placed in a single well of a 24-well cell culture plate (Corning Inc.) and the behavioral responses of the harvester ant were recorded for 30 min using a video camera (Canon FS11, Canon Inc.). Mortality was recorded after 24 h. Treatments and controls were replicated 10 times each.

The recorded video footages were used to analyze the behavioral responses of the harvester ants. Several distinctive behavioral categories were defined based on pilot laboratory observations (data not shown). The behavioral categories were: uninhibited mobility (UM), mandible clasping (C), mandible clasping with gaster manipulation (CG), grooming (G), grooming with gaster manipulation (GG), turning upside down (UP, insect rolls on its dorsal side), and knockout (KO, insect ceases movement coupled with leg twitches). The total duration for each behavioral category was recorded. For the data interpretation, we considered the categories C, CG, G, and GG as signs of irritation, and the categories UP and KO as signs of insecticidal activity.

Response of Argentine ants to items treated with pygidial gland chemicals. The effects of pygidial gland chemicals on the behavior of Argentine ants were studied by analyzing their responses towards inanimate objects treated with the chemicals. In the first assay, a dead California harvester ant was used as a test item. The dead harvester ant was fixed in the center of the assay arena by adhesive (Elmer's School Glue ®, Borden, Inc., Columbus Oh), preventing the movement of the test item (and the source of the chemicals) by the focal Argentine ant. With this method, several behavioral parameters

that would be significantly influenced by the locational shift of the test items (e.g., travel distance or velocity in certain zones within the arena) could be collected and analyzed in a standardized manner. In the second assay, a dead California harvester ant was used again, but without being fixed in the center of the arena. By allowing the dead harvester ant to be moved from its original place, some of the consequences of the behavioral modifications of the Argentine ants (as they were seen in the first assay) could be observed and quantified. In the third assay, a clean glass bead fixed in the center of the arena was used as a test item. The use of a chemically inert, clear glass bead (3 mm in diameter) as a test item allowed us to examine the behavioral effects of the pygidial gland chemicals with a minimal influence of other preexisting stimuli such as visual, chemical, and tactile cues. With the glass bead as a test item, authentic standards of two iridoids (as individual compounds or a mixture) were also tested in addition to the crude pygidial gland secretion (PGE) obtained from the Argentine ants.

In all assays, the behavior of a single Argentine ant was observed in a circular arena (11 cm in diameter) with a plastic wall. The inner surface of the wall was coated with Teflon to prevent the ant from escaping. The bottom of the arena was lined with a filter paper disc (15 cm in diameter, GE Healthcare Life Sciences, Whatman^{TM}). The arena bottom included two zones - the center and outer zones. The center zone consisted of a central circular area (3.5 cm in diameter) and the outer zone consisted of the remaining radial area surrounding the center zone. For the assays with dead harvester ants as test items, a single freeze-killed harvester ant was placed in the center of the arena with or without an adhesive. For the assays with glass beads as test items, the beads were

fixed in the center of the arena with the adhesive. Completed arenas were left for 24 h prior to an experiment to ensure the complete hardening of the adhesive.

Immediately before initiating the assays, the chemicals were applied on the test items. For the assays with dead harvester ants as test items, one ant equivalent of crude PGE (total weight ≈17 µg containing ≈ 0.05 dolichodial and ≈ 0.15 µg of iridomyrmecin, considering the amount that is not completely removed from the micropipette; see Results) was dispensed from the glass micropipette and applied onto the head of the harvester ant, between the compound eyes (PGE). Control consisted of dead harvester ants without PGE treatment. For the assays with glass beads as test items, the following treatments were tested: (1) application of one ant equivalent of PGE immediately followed by 1 µl of solvent (methylene chloride and acetone solvent mixture at 1:1, vol:vol) (PGE), (2) application of standard mixture containing 0.08 µg dolichodial and 0.25 µg iridomyrmecin in 1 µl solvent mixture (Mix), (3) application of 0.08 µg dolichodial only in 1 µl methylene chloride (Dol), and (4) application of 0.25 µg iridomyrmecin only in 1 µl acetone (Irid). The quantities of the standard iridoids were based on the quantities estimated for one harvester ant in the cuticular extract analyses, and they were also comparable with the quantities found in one ant equivalent of PGE (see Results). The test items treated with solvent only served as the control.

One Argentine ant was randomly selected among the active foragers in the main laboratory colonies and subsequently confined at the release point within the assay arena (3 cm away from perimeter of the center zone) using a small plastic ring (1 cm in diameter) coated with Teflon. After a 5-min acclimation period, the chemical treatment

was made to the test items, and the Argentine ant was immediately released by lifting the small plastic ring confinement. Behavioral responses were recorded for 10 min per trial using EthoVision XT video tracking system (see Behavioral analyses). Argentine ants were used only once, and treatments and controls were replicated ten times.

Behavioral analyses. Because many different factors can affect the type and intensity of the alarm reaction observed in a biological system, a range of behaviors are generally provided to define an alarm behavior instead of relying on a specific definition (Vander Meer 1998). In addition, behavioral responses can vary depending upon a variety of factors, including concentration of pheromone, compounds within the pheromone, colony size, and the spatial context of communication (Hölldobler and Wilson 1990, Norman et al. 2017). Given the complexity of defining the alarm reactions caused by the alarm pheromone, we used a computerized behavior tracking system in combination with manual observation, enabling quantitative analyses for some of the qualitative parameters.

A computerized behavior tracking system, EthoVision XT version 11.0.928 (Noldus Information Technology), was used to capture video images of each trial and record behavioral parameters of interest. The detection method used for acquisition was dynamic subtraction as described below. The detection thresholds for Argentine ant tracking were set so that all objects that were different from the background image by less than 40 or greater than 600 pixels were ignored and therefore recognized as part of the background. The sample rate (rate at which EthoVision analyzes the images to find the subject) was 25 samples s^{-1}. Only the samples with <1% missed samples and <1%

subject not found (no subject detected by the EthoVision) were selected. Velocity or speed (distance moved per unit time) was calculated as cm s^{-1}.

For all three behavioral assays with Argentine ants, overall speed (total velocity in entire arena, cm s^{-1}) and overall distance (total distance traveled in entire arena, cm) were recorded to determine the overall activity levels of the focal Argentine ants within the experimental arena. For the assays with test items (dead harvester ant or glass bead) fixed in the center of the arena, the following behavioral parameters were recorded: center zone distance (cm), center zone velocity (cm s^{-1}), center zone cumulative duration (total time spent in center zone, s) center zone frequency (total number of visits to center zone), and latency to first (time until the first visit to the center zone, s). All parameters were automatically recorded with EthoVision XT software. For the assays with a dead harvester ant not fixed in the arena center, the following parameters were manually recorded based on the recorded video footage: number of physical interactions between the Argentine ant and the harvester ant (e.g., antennation, mandible clasping, gaster bending, and crawling), interaction duration (total time spent in contact with the harvester ant performing the interactions), and movement of the dead harvester ant (total number of separate movements caused by Argentine ant).

Statistical analyses. To compare relative quantities of dolichodial and iridomyrmecin between different samples in the headspace volatile analyses, the areas of the corresponding peaks in the chromatograms were compared (Romeo 2009). A Wilcoxon rank sum test was used due to small ($n < 10$) and unequal sample sizes (Whitley and Ball 2002).

To analyze the differences in total time spent in the behavioral categories between treatment and control groups of harvester ants (i.e., with or without topical treatment with PGE), a permutational MANOVA (multivariate analysis of variance) was performed with the Adonis function in the R package Vegan (Oksanen 2017). The permutational MANOVA is analogous to traditional MANOVA, but more robust to violations of normality assumptions (McArdle and Anderson 2001). Principal component analysis (PCA) was also performed using the R package FactoMineR (Lê et al. 2008) to identify behavioral categories that are correlated with the treatment group.

For the Argentine ant behavioral assays with dead harvester ants, depending on normality and homogeneity of variance of the data, a two-sided two-sample t-test, two-sided Welch two sample t-test or a two-sided Wilcoxon rank sum test was performed to compare PGE treatments and controls for each of the behavioral parameters. Due to their non-normal distribution, the data from the Argentine ant behavioral assays with glass beads were analyzed using a Kruskal-Wallis test, followed by all-pairwise comparisons of mean ranks at $\alpha = 0.05$ using the "PMCMR" package. All statistical tests were performed on R Statistical software (R Development Core Team 2016).

Results

Headspace volatile analyses. Agonistic responses including Argentine ants' gaster bending were observed in all vials containing both species of ants. The headspace analyses indicated that two iridoids, dolichodial and iridomyrmecin, were consistently present in relatively large quantities in the vials where there were aggressive interactions between Argentine ants and harvester ants (Fig. 4.2, Aggression, 10 of 10). The iridoids

were also detected in the headspaces of the vials containing Argentine ants only (Fig. 4.2, Argentine ant control, 5 of 5), but in much smaller quantities when compared with the former. The comparisons between integration values of the corresponding iridoids indicated that much higher amounts of iridoids (i.e., 193 – 197 times larger integration value) were produced by the Argentine ants during aggression compared to the Argentine ant control (dolichodial: 2.9e+07 ± 1.1e+05 area counts vs. 1.5e+05 ± 8.7e+04; iridomyrmecin: 7.9e+07 ± 5.5e+07 vs. 4.0e+05 ± 1.0e+05 for the aggression and control, respectively) (dolichodial: $z = 2.76$, n = 13, $P = 0.003$; iridomyrmecin: $z = 2.76$, n = 13, $P = 0.006$). The iridoids were not detected in the vials containing harvester ant only (Fig. 4.2, harvester ant control, 5 of 5). All control vials did not contain any detectable peaks (5 of 5). A third peak, identified as *n*-dodecane, was present in relatively large quantities in both aggression and harvester ant control samples, but not in Argentine ant control samples (Fig. 4.2), indicating that it was likely produced by the harvester ants. The overall data indicated that the Argentine ants released relatively large quantities of pygidial gland chemicals when they were actively engaged in aggressive interactions with the harvester ants.

Cuticular extract analyses. The total number of Argentine ants that performed the gaster-bending response on the harvester ant during the 2-min observation period was 16.0 ± 1.1 (mean ± SEM, n = 30). The GC-FID analyses of the cuticular extracts indicated that dolichodial and iridomyrmecin were consistently detected in the polar fraction (methylene chloride fraction) of the harvester ant cuticular extracts after the gaster bending of the Argentine ants (Fig. 4.3, Aggression). The nonpolar fraction

(hexane fraction) mostly consisted of cuticular hydrocarbons, without detectible amounts of the iridoids. The cuticular extracts of the control harvester ants (without Argentine ants' aggression) did not contain dolichodial and iridomyrmecin in either the polar or nonpolar fractions. However, *n*-dodecane was present in the control harvester ants' cuticular extracts in both the polar and nonpolar fractions. (Fig. 4.3, harvester ant control). The estimated amounts of dolichodial and iridomyrmecin on the cuticle of a single harvester ant were 0.08 and 0.28 µg, respectively (ratio 1:3.5).

Collection and chemical analyses of pygidial gland chemicals. One ant equivalent of PGE weighed 17.6 ± 2.0 µg (mean ± SEM, n=10) and contained ≈0.05 µg of dolichodial and ≈0.15 µg of iridomyrmecin (ratio 1:2.8). These values were comparable with the quantities of the iridoids estimated for one harvester ant cuticle, justifying the use of one ant equivalent of Argentine ant PGE in the subsequent bioassays. The two iridoids (≈0.2 µg total) accounted for approximately 1.2% of the total weight of PGE. Water detection with paper treated with cobalt chloride indicated the presence of water in the crude PGE.

Response of harvester ants when topically treated with pygidial gland chemicals. Behavioral responses of harvester ants topically treated with Argentine ant PGE were distinctively different from those of the untreated control group. Overall, the amounts of time spent in different behavioral categories differed significantly between the treatment and control groups (Permutation MANOVA: $F= 49.9$, df= 19, $P < 0.001$). Harvester ant mortality after 24 h was 2 and 1 for treatment and control groups, respectively.

The PCA of individual harvester ant timed behavioral responses showed a clear discrimination between the treatment and control groups (Fig. 4.4). Two principal

components (PC) with eigenvalues greater than one [PC 1 (3.12) and PC 2 (1.48)] accounted for 66% of the total variation. PC 1 indicated that "uninhibited mobility" was negatively correlated with all other timed behavioral responses. PC 2 indicated that behaviors "upside-down" and "knockout" were negatively correlated with "clasping" and "grooming".

The timed behavior response "uninhibited mobility" was the primary factor associated with the control group, while all other behaviors that represented irritation and insecticidal effects were associated with the treatment group that received Argentine ant PGE (Fig. 4.4). To infer correlations, there should be clustering in the factor map (Fig. 4.4) and the squared cosine (Cos2) should be greater than one half (Abdi and Williams 2010). Cos2 values determine the strength of between behavioral variables (timed behavioral responses) and a PC. Higher Cos2 values indicate stronger correlation(Abdi and Williams 2010). For PC1, the timed behavioral responses UM, KO, UP, C, CG and GG are demonstrating stronger correlations in having the Cos2 values 0.98, 0.52, 0.64, 0.52, 0.75 and 0.76, respectively. Similarly, for PC2, the responses C, G, KO and UP are displaying stronger correlations in having the Cos2 values 0.58, 0.59, 0.59, and 0.64, respectively.

Response of Argentine ants to the items treated with pygidial gland chemicals.

The behavioral parameters of the Argentine ant to a dead harvester ant fixed in the arena center are summarized in Fig. 4.5. Argentine ants in the PGE treatment and control were similar in their overall speed [1.65 ± 0.12 and 1.48 ± 0.11 cm s^{-1} (mean ± SEM) for PGE treatment and control, respectively; T = -1.01, df = 18, P = 0.33] (Fig. 4.5A), and overall

travel distance [980.7 ± 71.2 cm and 891.3 ± 66.2 cm for PGE and control, respectively; T = -0.92; df = 18; P = 0.37] (Fig. 4.5B).

However, Argentine ants travelled significantly longer distances in the center zone when the center zone had the PGE-treated harvester ant compared to the untreated control harvester ant (center zone distance: 121.2 ± 11.5 cm and 76.1 ± 14.8 cm for PGE and control, respectively; T = -2.4, df = 18, P = 0.02) (Fig. 4.5C). Center zone velocity of the ants was not significantly different between PGE treatment and control (center zone velocity: 1.38 ± 0.11 and 1.37 ± 0.14 cm s^{-1} for PGE and control, respectively; T = -0.03, df = 18, P = 0.99) (Fig. 4.5D). When the center zone had PGE-treated harvester ants, the Argentine ants spent significantly longer time in the center zone compared to the controls (center zone cumulative duration: 114.5 ± 20.0 s and 58.5 ± 9.3 s for PGE treatment and control, respectively; T = -2.5, df = 18, P = 0.04) (Fig. 4.5E). Center zone frequency also was significantly greater in the assays with PGE-treated harvester ants compared to controls (center zone frequency: 34.2 ± 4.1 and 24.8 ± 2.5 for PGE and control, respectively; T = 2.0, df = 18, P = 0.03] (Fig. 4.5F). Latencies to the first entry into the center zone were not significantly different between PGE treatment and control (latency to first: 0.15 ± 0.05 s and 0.76 ± 0.24 s; z= 0, df = 18, P = 1) (Fig. 4.5G).

The behavioral response parameters of Argentine ants to an unfixed dead harvester ant are summarized in Fig. 4.6. Argentine ants in PGE treatment and control were similar in their overall speed [1.17 ± 0.22 and 1.56 ± 0.15 cm s^{-1} for PGE and control, respectively; T = 1.5, df = 18, P = 0.15] (Fig. 4.6A), and overall travel distance (701.6 ± 129.5 and 937.4 ± 89.2 cm for PGE and control, respectively; T = 1.5, df = 18, P

= 0.15] (Fig. 4.6B). The number of interactions between the Argentine ant and the harvester ant was significantly greater in the PGE treatment compared to the control [13.6 ± 2.8 and 8 ± 1.3 for PGE and control, respectively; T = -1.8, df = 18, P = 0.05] (Fig. 4.6C). The Argentine ants interacted longer with the PGE-treated harvester ants compared to the control harvester ants (172.6 ± 61.2 s and 51.9 ± 26.7 s for PGE and control, respectively; z = -2.1, df = 18, P = 0.04) (Fig. 4.6D). The number of movements of the harvester ant was also significantly greater in the PGE treatment compared to controls (2.5 ± 0.9 and 0.4 ± 0.2 for PGE and control, respectively; z= -2.3, df = 18, P = 0.02) (Fig. 4.6E).

The behavioral response parameters of Argentine ants to a glass bead fixed in the arena center are summarized in Fig. 4.7 and Table 1. Argentine ants in all treatments and controls were similar in their overall speed (H = 7.5, df = 4, P = 0.10) (Fig. 4.7A), and overall travel distance (H = 7.6, df = 4, P = 0.10) (Fig. 4.7B). However, several behavioral parameters were significantly different between the treatments and controls. When glass beads were treated either with PGE or with authentic standards of the iridoids (individually or as a mixture), Argentine ants traveled longer distances in the center zone (H = 24.3, df = 4, P < 0.001) (Fig. 4.7C). However, their center zone velocities were similar across the treatments and controls (H = 3.8, df = 4, P = 0.43) (Fig. 4.7D). Furthermore, Argentine ants in the treatments (PGE or authentic standard of the iridoids) spent more time in the center zone (center zone cumulative duration: H = 16.0, df = 4, P < 0.001) (Fig. 4.7E), and entered the center zone more frequently (center zone frequency: H = 20.8, df = 4, P < 0.001) (Fig. 4.7F). Latency to the first entry into the center zone

was also significantly shorter in the treatments compared to the solvent controls (latency to first: $H = 22.2$, df = 4, $P < 0.001$) (Fig. 4.7G).

Discussion

The successful establishment of Argentine ants in numerous locations worldwide is due, at least in part, to its superior ability to exploit and defend resources (Holway 1999). Their trail pheromones play a critical role in resource exploitation by efficiently leading colony members to the resources. Once they arrive at the resources, they maintain their dominance against competing species through aggressive interactions. The current study shows that Argentine ants utilize their pygidial gland secretions during aggressive interactions with a heterospecific competitor. The pygidial gland secretions cause irritation and disorientation to competitors when topically applied on their cuticles. The pygidial gland secretion or its major volatile constituents, dolichodial and iridomyrmecin, also release alarm and aggregation responses for other Argentine ants nearby, potentially facilitating group defense.

Aggressive interactions between Argentine ants and harvester ants resulted in the release of dolichodial and iridomyrmecin in the headspace. The same iridoids were also detected from the headspace of control vials containing Argentine ants only. However, the higher quantities of dolichodial and iridomyrmecin in the headspace were only associated with aggressive interactions. Our data suggest that Argentine ants release small amounts of dolichodial and iridomyrmecin even in the absence of aggression, but strongly increase their release during aggressive interactions. There are possible other behavioral functions for the iridoids at the low concentrations. For example, the presence

of dolichodial and iridomyrmecin on the cuticle of live Argentine ants inhibits necrophoretic behaviors (Choe et al. 2009). In addition, dolichodial and iridomyrmecin are among the chemical constituents of Argentine ant recruitment trails (Choe et al. 2012).

Previous studies had speculated that Argentine ants might deploy defensive compounds during aggressive interactions (Brown 1973, Holway 1999, Human and Gordon 1999, Buczkowski and Bennett 2008). The current study experimentally confirmed that Argentine ants deploy dolichodial and iridomyrmecin from their pygidial glands and deposit them on the cuticles of opponents during aggression, through the gaster-bending response. During the 2-min observation period, multiple gaster-bending responses (average of 16) by Argentine ants were observed. Multiple applications of the pygidial chemicals might be necessary to overcome larger heterospecific competitors. In the current study, the harvester ant did not appear to be significantly affected by the first gaster-bending response. However, after several additional gaster-bending responses (towards the end of the 2-min observations), some evident behavioral effects (e.g., disorientation, signs of irritation) were observed in the harvester ants. One-on-one interactions between Argentine ants and native species usually result in the death of the Argentine ant (Holway 1999, Rowles and O'Dowd 2007), suggesting the importance of numerical dominance by the Argentine ant during aggressive interactions involving the gaster-bending response.

In addition to dolichodial and iridomyrmecin, *n*-dodecane was detected in some of the samples with the harvester ants. *n*-Dodecane was detected only in headspace volatiles

from harvester ants, with larger quantities generally associated with aggression (Fig. 4.2). *n*-Dodecane was also detected in cuticular extracts of harvester ants (aggression as well as harvester ant controls) (Fig. 4.3). Thus, it likely that *n*-dodecane originated from the harvester ants. *n*-Dodecane is the most abundant volatile component found in the Dufour's gland of *P. rugosus* and *P. barbatus* (Regnier et al. 1973). It has been suggested that a pheromone blend containing *n*-dodecane might be used for orientation and nest location by harvester ants (Hölldobler 1971). The production of *n*-dodecane and its potential functions in interspecific interactions of harvester ants (e.g., defense, alarm, etc.) warrant further investigations.

The topical application study confirmed the allomonic role of the Argentine ant pygidial gland secretions. In particular, the pygidial gland chemicals caused significant levels of irritation and disorientation in harvester ants when topically applied. The treated harvester ants typically showed continuous grooming and mandible clasping responses. In some cases, the treated harvester ant lost its ability to right itself, often resulting in going upside down or being temporarily incapacitated. Even if the current study did not examine the effects of pure iridoids on the harvester ants, it is likely that dolichodial and iridomyrmecin in the pygidial gland secretion are among the major causes for the behavioral responses observed in the treated harvester ants. Previous studies have reported that dolichodial and iridomyrmecin have "insecticidal" properties and may serve as defensive allomones in the Argentine ant and other species of ants (Pavan 1951, Cavill and Cark 1971

, Cavill et al. 1976). The current study clearly shows that dolichodial and iridomyrmecin are the primary volatile chemical constituents of the pygidial gland secretion. However, further chemical investigation would be necessary to determine if there are other non-volatile and/or more polar compounds in the pygidial gland secretion, which also maybe bioactive. Besides the iridoids, which are responsible for ≈1.2% of the total weight of the secretion, the pygidial gland secretion also contained substantial amount of water.

It is also important to note that the "insecticidal" effects of the pygidial gland secretions (and associated iridoids) appear to be only temporary for the harvester ants. For example, eight out of the ten harvester ants recovered from the effects of topical application of the pygidial gland secretion after 10 min and subsequently showed uninhibited mobility. Some previous studies have stated that the toxicity of the iridoids are "not great" (Pavan 1951, Attygalle and Morgan 1984); however, these studies do not provide as to how toxicity was measured. Additional studies conducted with other biological systems utilizing identical or similar iridoid chemistries suggest that the iridoids might function as general "aggression suppressants" for ants in interspecific interactions. For example, a blend of iridomyrmecin and iridomyrmecin-like compounds were found in the mandibular gland secretions of the aphid hyperparasitoid *Alloxysta brevis,* and these secretions discourage aggression by aphid-tending ants (*Lasius* sp.) (Volkl et al. 1994). Iridodials and iridomyrmecin were found in the pygidial gland secretions of *Tapinoma* sp., which suppressed the aggression in heterospecific ants when they were daubed with the secretion (Tomalski et al. 1987). The current study did not

continue to track the competitive interactions between Argentine ants and the harvester ants after the treatment with the pygidial gland chemicals. However, the general aggression-suppressant effects of the pygidial gland chemicals would provide an important advantage for the Argentine ants by temporarily disabling the harvester ants during confrontations near resources, or while nest raiding for brood and other resources.

Upon encountering harvester ants treated with the pygidial gland secretions (glued or unglued in the arena center), Argentine ants showed behaviors that are typically associated with an alarm reaction. These behaviors included attraction to the source of chemicals, increased speed of movement, arrested motion, aggressive posture, and aggression such as mandible clasping and gaster bending (Ayre and Blum 1971, Kugler 1979a, Hölldobler and Wilson 1990, Vander Meer 1998). The subsequent assays with inert glass beads treated with iridoid standards indicated that the volatile iridoids in the pygidial gland secretion are primarily responsible for Argentine ants' alarm responses towards the treated items. Historically, the volatile compounds produced in the pygidial glands of dolichoderine and myrmicine ants have been considered to have alarm or defensive functions (Wilson and Pavan 1959, Kugler 1979b, Hefetz and Lloyd 1983). Our study experimentally confirms that Argentine ants actively utilize dolichodial and iridomyrmecin in the pygidial gland secretion for these functions, potentially facilitating a more rapid and coordinated group defense. Similar to the assays with dead harvester ants, Argentine ants increased their travel distance and time spent in the center zone when the glass bead in the center zone was treated with the iridoid(s). Also, Argentine ants

increased their frequency of visitation to the center zone when the glass bead was treated with the iridoid(s).

Overall, the assays with fixed dead harvest ants and fixed glass beads showed similar results in most behavioral parameters (e.g., center zone distance, center zone cumulative duration, and center zone frequency). However, we found a distinct difference between these assays in the outcome with "latency to first" parameter. Specifically, Argentine ants first visited the center zone more readily when the glass bead was treated with the pygidial gland chemicals, compared to the control. In contrast, there was no such effect of the pygidial gland chemicals on Argentine ant behavior when a dead harvester ant was used as the test item. Overall, Argentine ants responded quickly to the dead harvester ant fixed in the center, entering the center zone almost immediately after the assay initiation regardless of the presence of pygidial gland chemicals (e.g., 0.4 and 0.1 s until the first entry for control and PGE, respectively). In contrast, when the clean glass bead was provided in the arena center, Argentine ants took 30.3 s on average to enter the center zone. This latency was reduced to 5.9 s on average by treating the glass bead with the pygidial gland chemical(s). These results suggest that some inherent differences between the test items (i.e., dead harvester ant vs. glass bead) might be responsible for the discrepancy. First, it is possible that some pre-existing chemicals on the harvester ant might have influenced the behavior of Argentine ant, causing them to enter the center zone immediately. For example, Argentine ants might be able to sense *n*-dodecane and other pre-existing cuticular hydrocarbons of the dead harvester ants from a distance (i.e., 4.8-5 cm) and respond to them accordingly. In contrast, the use of inert / clean glass bead

as the test item would have eliminated any possible chemical cues. Secondly, it is possible that Argentine ant might have responded to the visual stimuli provided by the dead harvester ants, promptly entering the center zone. In this case, the lack of color of the glass bead might substantially delay the first entry to the center zone by the Argentine ant. Several studies on orientation behavior of ants, including the Argentine ant, suggest that visual and chemical mechanism interplay in mediating orientation behaviors (Aron et al. 1993, Grüter et al. 2011, Bowens et al. 2013).

The use of the same chemical for two or more functions in different contexts is known in several insect systems, and this phenomenon is referred to as semiochemical parsimony (Blum 1996). In several ant species, the defensive chemicals often have various other functions in other behavioral contexts such as aggregation (Ikan et al. 1970), alarm (Ayre and Blum 1971, Prudic et al. 2008), and recruitment (Gurgel do Vale et al. 2002). The current study reports that Argentine ants utilize their pygidial gland secretions containing dolichodial and iridomyrmecin primarily as defensive allomones against heterospecific competitors, but the same secretions also cause nearby conspecifics to be alerted and quickly aggregate for defense. It is possible that Argentine ants also rely on these semiochemicals during intraspecific aggression (Thomas et al. 2006). Furthermore, dolichodial and iridomyrmecin are known to function in other behavioral contexts of the Argentine ant, such as necrophoresis and recruitment. This functional versatility of dolichodial and iridomyrmecin appears to be related to the quantities produced / released by Argentine ants. For example, only nanogram quantities (per centimeter of trail) of dolichodial and iridomyrmecin were found on the Argentine ant

recruitment trails(Choe et al. 2012) while the quantities of iridoids on the cuticular surface of harvester ants in the current study (after being attacked by several Argentine ants) were in microgram quantities (per harvester ant). Studies suggest that the recruitment trail is continuously and uniformly reinforced over a period of time and the concentration of the trail pheromone may be dependent on the resource quality (Aron et al. 1993, Choe et al. 2012, Latty et al. 2017). Being able to parsimoniously utilize these iridoids in a variety of behavioral / ecological contexts (e.g., colony maintenance, recruitment, defense, and alarm) may contribute to the Argentine ants' ability to outcompete several other ant species (Blum 1996). Recent studies which have examined the chemical ecology of the tawny crazy ant (*Nylanderia fulva*) attributed its ability to displace native ants to the species' superior ability to utilize its limited set of semiochemicals for multiple functions such as defense, detoxification, and recruitment to food resources or sites of conflict (LeBrun et al. 2014, Zhang et al. 2015). This suggests that semiochemical parsimony might play a significant role in the success of several invasive ant species worldwide.

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Figures

Fig. 4.1. An Argentine ant worker performing gaster-bending behavior on the head of harvester ant.

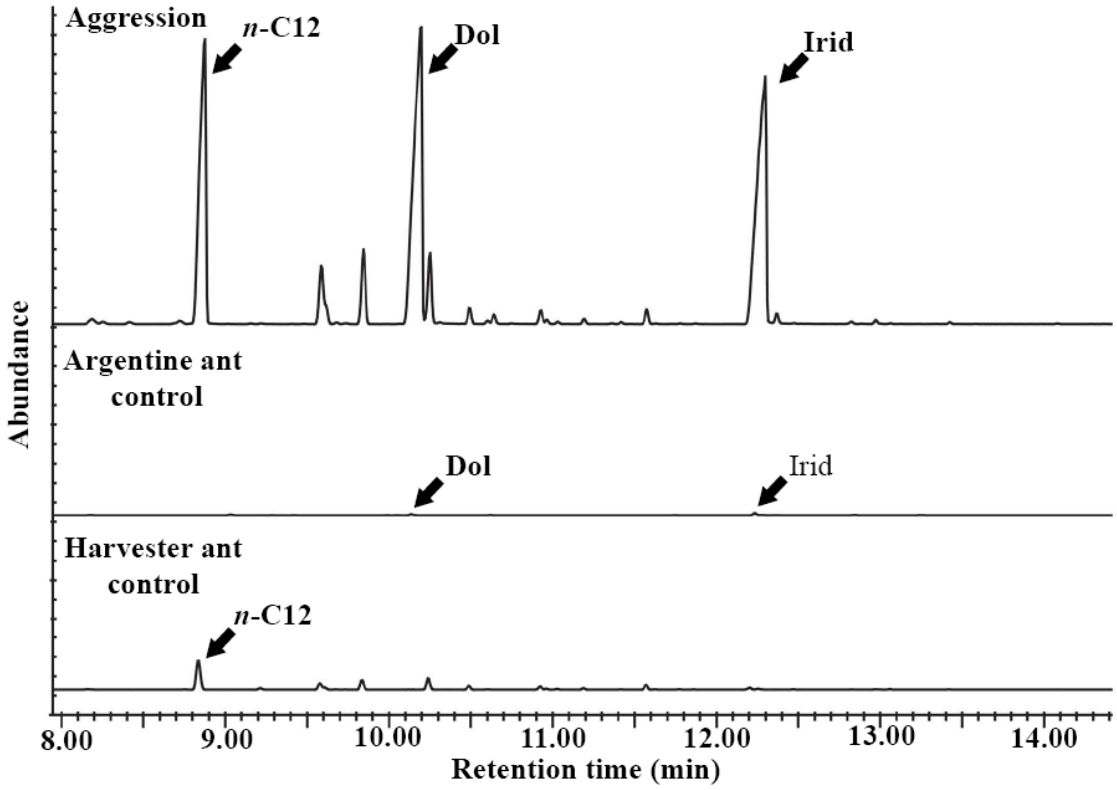

Fig. 4.2. Representative chromatograms (GC-MS) from the headspace volatile analyses. The sample of volatiles were collected from vials containing five Argentine ants and one harvester ant (Aggression), five Argentine ants only (Argentine ant control), or one harvester ant (Harvester ant control). Dolichodial (Dol) and Iridomyrmecin (Irid) were consistently detected in all Aggression samples (n=10) and Argentine ant controls (n=5). Harvester ant controls did not show the presence of dolichodial or iridomyrmecin (n=5). *n*-Dodecane (*n*-C12) was present in Aggression and Harvester ant control samples. *n*-C12 was not present in the Argentine ant control samples.

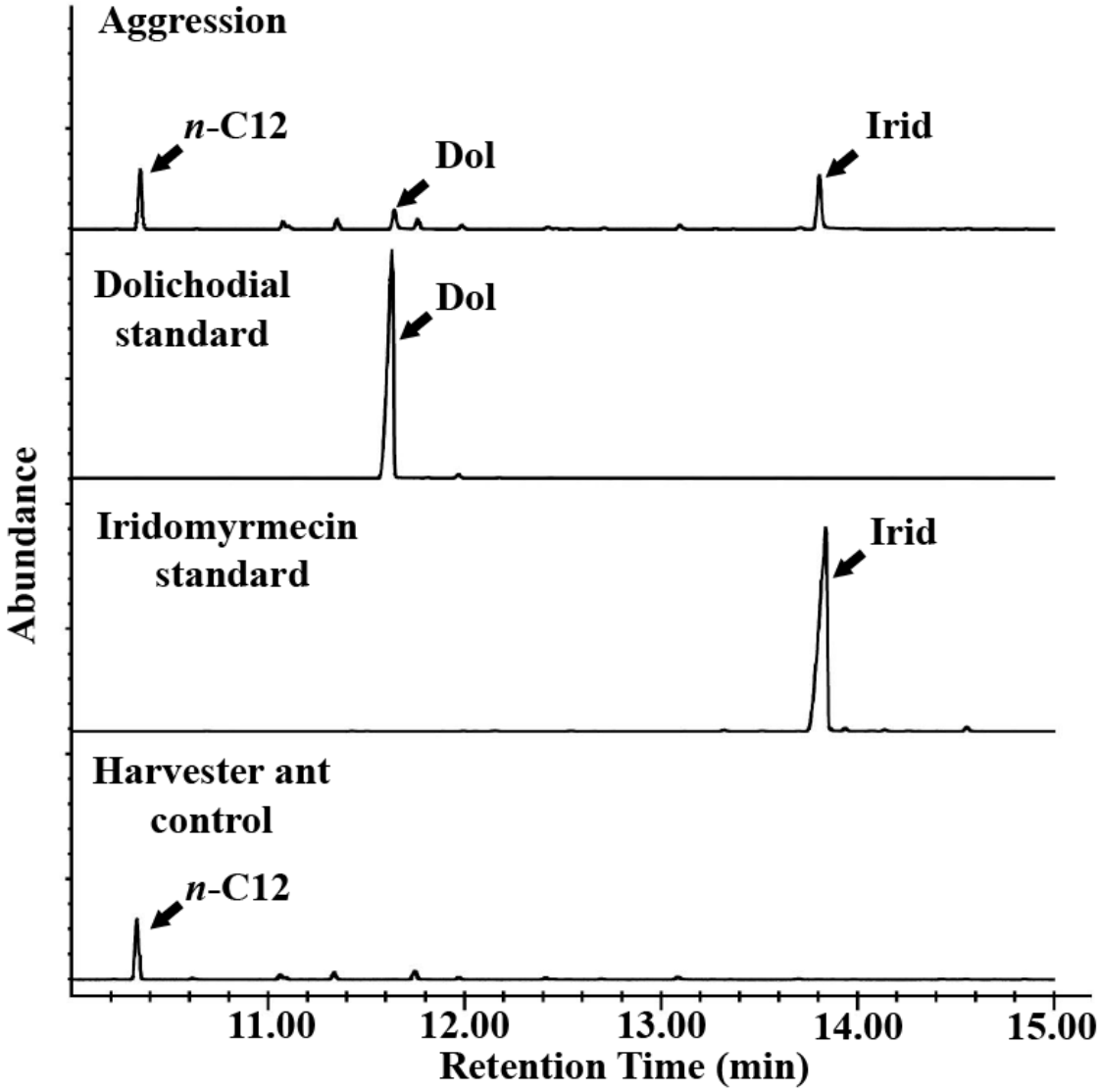

Fig. 4.3. Representative chromatograms (GC-FID) from the cuticular extract of harvester ants. The samples were collected from a pooled sample of 30 harvester ants after Argentine ant aggression (Aggression), two standards for identification (dolichodial standard and iridomyrmecin standard), and the cuticular extract of harvester ants without Argentine ant aggression (Harvester ant control). Peaks for dolichodial (Dol) and iridomyrmecin (Irid) are marked with arrows. *n*-Dodecane (*n*-C12) was only present in Aggression and Harvester ant control samples. Chromatograms represent the polar fraction (methylene chloride).

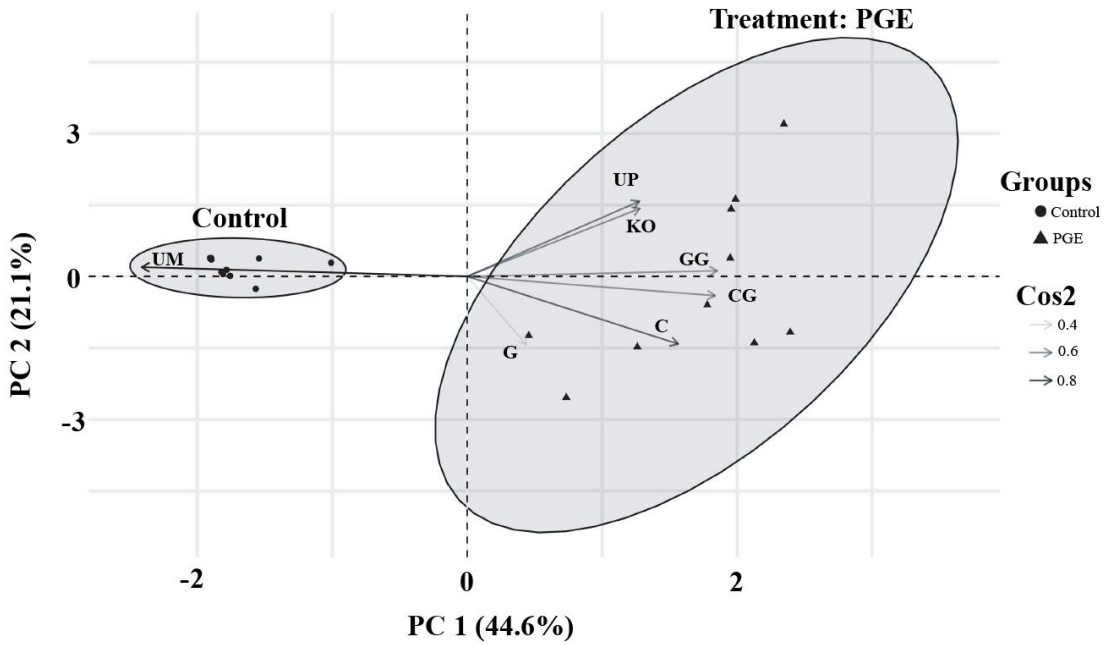

Fig. 4.4. A factor map of the first two principal components PC1 and PC2. Individual harvester ant timed behavioral response categories include: uninhibited mobility (UM), mandible clasping (C), mandible clasping with gaster manipulation (CG), grooming (G), grooming with gaster manipulation (GG), turning upside down, (UP), and knockout (KO). Shaded ellipses illustrate groupings corresponding to PGE (triangles) and control (circles) harvester ants. Arrows represent squared cosine (Cos2) values of behavioral response for a given observation. Behavior responses with cos2 closer to 1 (darker arrows) are considered important for that group.

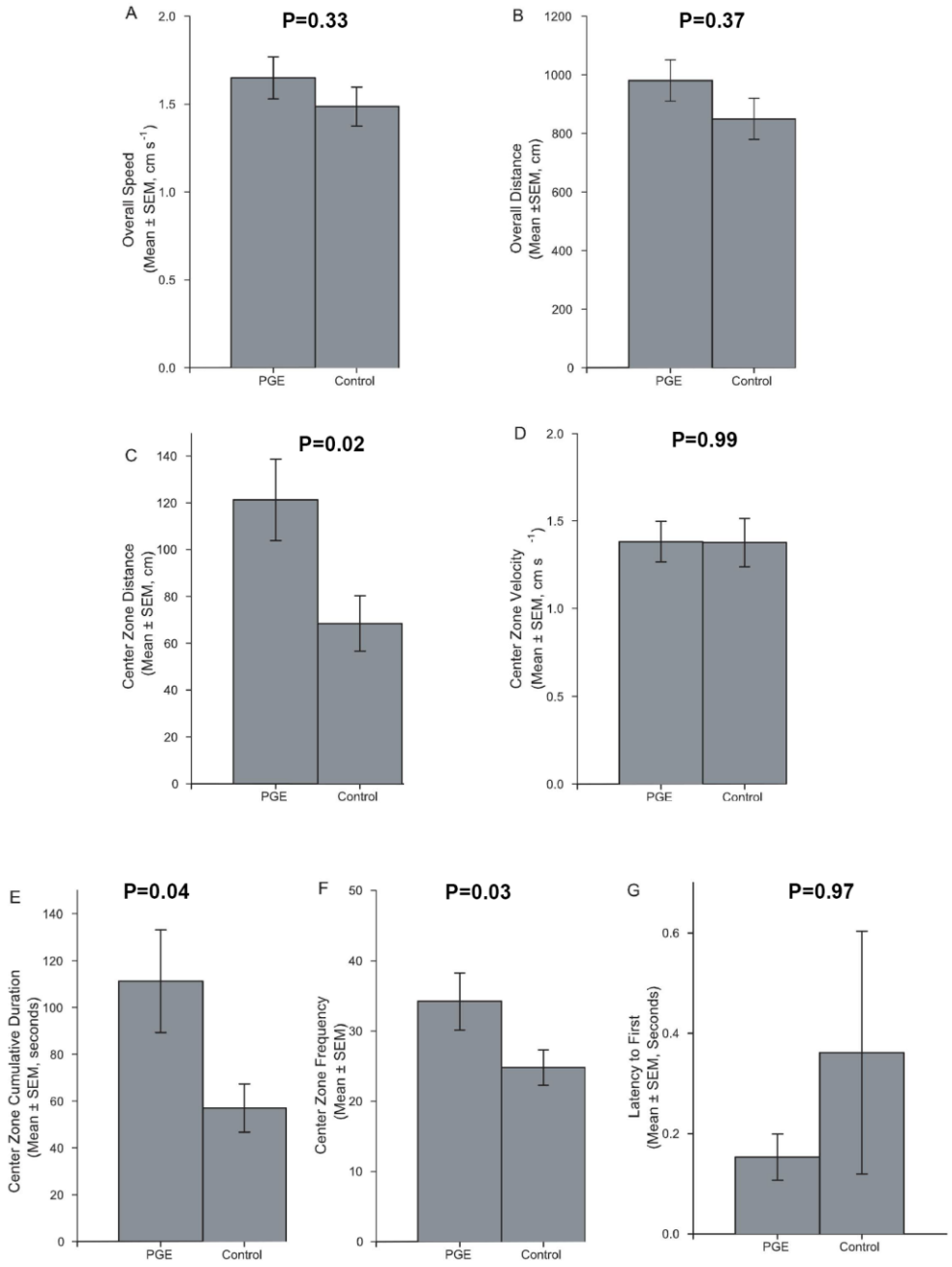

Fig. 4.5. Responses of an Argentine ant to a fixed harvester ant with or without Argentine ant pygidial gland extract (PGE). Ten replications were made for PGE treatment and control. The behavioral parameters analyzed include: overall speed (A), overall distance (B), center zone distance (C), center zone velocity (D), center zone cumulative duration (E), center zone frequency (F), and latency to first (G). P-value is provided for each behavioral parameter. Error bars indicate SEM values.

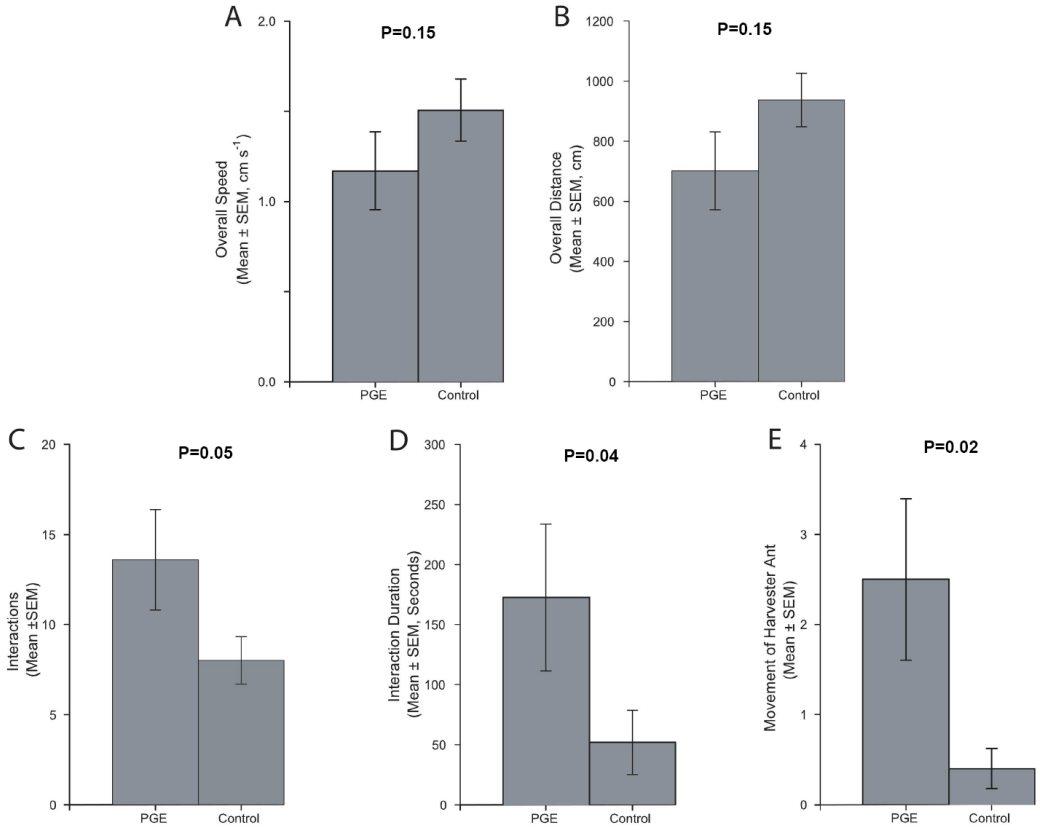

Fig. 4.6. Response of an Argentine ant to a harvester ant with or without Argentine ant pygidial gland extract (PGE). Ten replications were made for PGE treatment and solvent only control. The Behavioral parameters analyzed include: overall speed (A), overall distance (B), interactions (C), interaction duration (D), and movement of harvester ant (E). P-value is provided for each behavioral parameter. Error bars indicate SEM values.

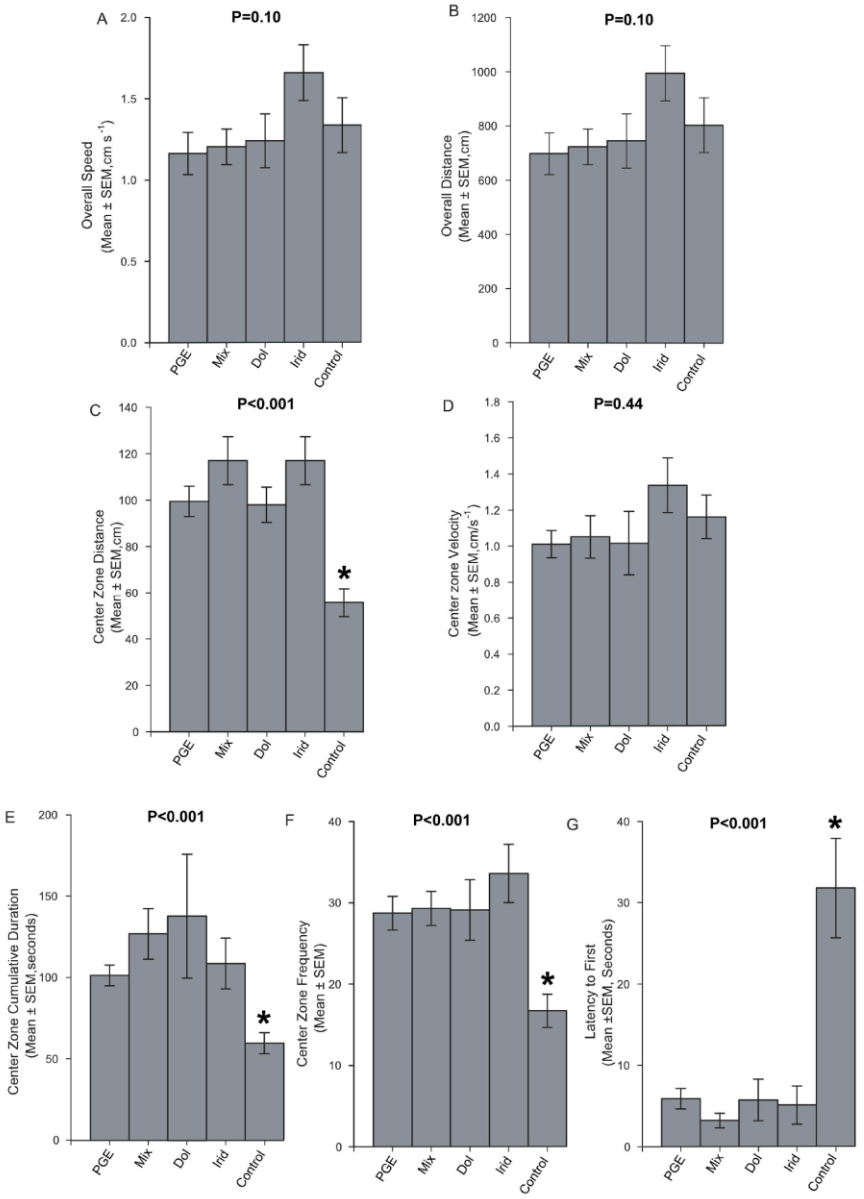

Fig. 4.7. Response of an Argentine ant to a glass bead treated with pygidial gland extract (PGE), a mixture of authentic standards of dolichodial and iridomyrmecin (Mix), dolichodial standard (Dol), iridomyrmecin standard (Irid), and solvent only (Control). Ten replications were made for each of the treatments and control. The behavioral parameters analyzed include: overall speed (A), overall distance (B), center zone distance (C), center zone velocity (D), center zone cumulative duration (E), center zone frequency (F), and latency to first (G). Asterisk (*) indicates control that was significantly different from all of the treatments. P-value is provided for each behavioral parameter. Error bars indicate SEM values.

Table 4.1. Means (± SEM) associated with five treatment groups for the seven Argentine ant's behavioral response parameters in assays with a fixed glass bead. Treatments include pygidial gland extract (PGE), a mixture of authentic standards of dolichodial and iridomyrmecin (Mix), dolichodial standard (Dol), and iridomyrmecin standard (Irid). The control consisted of solvent only (Control). Treatment means followed by the letter "a" are significantly different from "b" by all-pairwise comparisons of mean ranks at $\alpha = 0.05$.

Treatment	Overall Speed (cm s^{-1})	Overall Distance (cm)	Center Zone Distance (cm)	Center Zone velocity (cm s^{-1})	Center Zone Cumulative Duration (s)	Center Zone Frequency	Latency to First (s)
PGE	**1.16 ± 0.17**	**697.6 ± 77.4**	**99.4 ± 6.5 a**	**1.01 ± 0.08**	**101.2 ± 2.1 a**	**28.7 ± 2.1 a**	**5.90 ± 1.25 a**
MIX	**1.20 ± 0.13**	**722.5 ± 65.4**	**117.0 ± 10.3 a**	**1.05 ± 0.12**	**126.7 ± 15.5 a**	**29.3 ± 2.1 a**	**3.22 ± 0.89 a**
Dol	**1.24 ± 0.17**	**744.4 ± 99.5**	**98.0 ± 7.6 a**	**1.02 ± 0.18**	**137.6 ± 38.1 a**	**29.1 ± 3.7 a**	**5.73 ± 2.56 a**
Irid	**1.66 ± 0.17**	**1007.2 ± 101.9**	**134.1 ± 15.3 a**	**1.34 ± 0.15**	**108.5 ± 15.6 a**	**33.6 ± 3.6 a**	**5.12 ± 2.33 a**
Control	**1.38 ± 0.17**	**802.3 ± 101.0**	**55.7 ± 6.1 b**	**1.16 ± 0.12**	**59.5 ± 6.5 b**	**16.2 ± 2.0 b**	**31.8 ± 6.1 b**

Chapter 5

Conclusions

The Argentine ant is one of the most successful invasive species in the world, in practically all temperate climates. Their presence results in loss of biodiversity and degradation of ecosystem services. The economic burden of controlling invasive Argentine ants is further complicated by copious insecticide use, resulting in environmental contamination. For these reasons, developing new Argentine ant control strategies that focus on environmentally responsible and economically viable approaches is necessary. The studies discussed in this dissertation explored the Argentine ants' biology to develop new control strategies and provide insight into their invasive success.

The study in chapter 2 reports a "pheromone-assisted baiting technique" that combines (*Z*)-9-hexadecenal with a commercially available bait. Previous studies confirm that (*Z*)-9-hexadecenal is an Argentine ant trail pheromone component and our laboratory tests confirmed trail-following behavior. The addition of (*Z*)-9-hexadecenal to a bait improves the Argentine ant baiting strategy. Laboratory results demonstrated that the addition of (*Z*)-9-hexadecenal into a commercially available gel bait increases Argentine ant foraging activity. The increase in foraging activity may rely on (*Z*)-9-hexadecenal's ability to decrease the discovery time for individual Argentine ants to locate the bait. By decreasing the discovery time, Argentine ants had more time to recruit to the bait, resulting in higher foraging activity during the 30-min observation period, compared to bait not treated with (*Z*)-9-hexadecenal. More importantly, this significant increase in Argentine ant foraging activity had a positive effect on mortality. Within one week, laboratory colonies fed a single application of (*Z*)-9-hexadecenal- infused gel bait showed significantly higher morality compared to colonies fed control gel bait.

The “pheromone-assisted baiting technique” was tested under field conditions using residential homes with similar ant numbers. The pheromone-assisted baiting technique builds upon existing Argentine ant baiting technology by maintaining or improving important bait attributes such as delayed toxicity, transferability, and nonrepellency. This technique relied on 20 bait stations containing gel bait applied with a specific concentration of (*Z*)-9-hexadecenal. Formulation of the pheromone bait relies on careful calculations of the pheromone and bait ratio, as either too high or too low a concentration of (*Z*)-9-hexadecenal decreased foraging rates. Furthermore, the insecticidal gel bait is contained in a 15-ml plastic tube, thus eliminating the potential of insecticide run-off into the environment while maintaining the pheromone-bait formulation.

Ant activity levels dropped substantially after 4 weeks of using the pheromone-assisted baiting technique when compared to initial ant activity levels. By the end of the 4 week study period, homeowners enjoyed a 74% reduction in ant activity using the pheromone-assisted baiting technique. Throughout the 4 week baiting period, the amount of gel bait consumed was consistently higher with the pheromone-assisted baiting technique compared to the control gel bait stations. The results indicated that (*Z*)-9-hexadecenal was responsible for higher bait consumption and may play a role in the feeding habits of the Argentine ant by decreasing initial discovery time resulting in higher overall bait consumption. It may also influence the frequency of trophallaxis between the initial foragers and subsequent recruited foragers. The results of this study demonstrate the ability of the pheromone-assisted baiting technique to provide adequate

Argentine ant control while minimizing environmental risks associated with residual pesticide sprays. The pheromone-assisted baiting technique has the potential to become an effective and environmentally friendly management tool for Argentine ants.

The pheromone assisted baiting technique provides an improvement in current baiting technology for Argentine ant management. However, this technique still relies on current insecticides that are based on a few modes of action. Chapter 3 demonstrates proof of concept of an altnerative strategy using RNA interference (RNAi) baits that can be developed for Argentine ant pest management. RNAi is a gene silencing mechanism that suppresses specific gene expression levels using a species specific double-stranded RNA (dsRNA). RNAi has great potential to contribute to development of future pest management methods that do not rely on chemical insecticides. Current studies provide evidence that dsRNA can be designed to selectively manage insect pests without affecting non-target insects, even when targeting genes that are highly conserved across a variety of taxa.

However, there are limiting factors for RNAi based approaches of insect pest management. Key factors in the successful use of RNAi as a potential tool for pest management will be the choice of target sequences for the target insect and the mode of delivery. Furthermore, the ability to deliver sufficient amounts of dsRNA is dependent on the deployment method, the stability of dsRNA in the environment and within the insect gut and an economically viable method to synthesize dsRNA on sufficient scale. The current study addresses some of these factors for the successful development of an RNAi baiting technique for the Argentine ant.

A laboratory study measured the mortality of Argentine ants after they ingested sucrose water containing a specific amount of dsRNA that targeted a gene necessary for life. The target gene was chosen based on previous studies that successfully delivered dsRNA to insects by ingestion. The target gene, "ADP, ATP carrier protein", was engineered into dsRNA and formulated into a sucrose solution bait for Argentine ant feeding trails. The dsRNA sucrose bait resulted in 52% mortality in Argentine ants after 7 days of consumption. Furthermore, target gene expression was tested to see if suppression occurred after single feeding events. The results showed that mRNA levels were significantly reduced after 8 hours, 24 hours and 3 days of feeding of dsRNA sucrose bait. The results provide evidence for the existence of the necessary RNAi machinery in the Argentine ant and proof of concept for potential development of this technology for a baiting strategy. While the results are promising for potential application to Argentine ant management, further research and development in the area of dsRNA based insecticides is necessary in order for practical applications in pest management.

An understanding of the behavioral mechanisms responsible for the successful establishment of Argentine ants is key in preventing or predicting future introductions and associated consequences to ecosystems. The success of the Argentine ant has been attributed to its superior competitive ability in resource acquisition and exploitation. Argentine ants rely on various chemical signals for many essential colony tasks, including foraging and defense. Numerous studies have speculated that Argentine ants use a chemical defense system, however, the semiochemicals associated with their defensive behaviors have yet to be elucidated.

In chapter 4, the chemical ecology associated with the invasive Argentine ants' defensive behaviors was elucidated. This study focused on a behavior known as "gaster bending" which is often used during aggressive interactions to help identify potential chemicals used during aggression. During the gaster bending behavior, an Argentine ant will typically bend its gaster ventrally to place the tip of the gaster onto the opponent. During this behavior, relatively large amounts of two iridoid compounds, dolichodial and iridomyrmecin, were present in the headspace and on the cuticular surface of the heterospecific ant. By focusing on a behavior that is only performed during agonistic interactions, we were able to conclude with certainty that the chemicals identified from this behavior have a defensive function.

A framework was established for identifying behaviors associated with defensive compounds by implementing a computerized tracking system. This system, summarized in chapter 4, enabled quantitative analyses of Argentine ant behaviors in response to objects were treated with the chemicals identified from the gaster bending behavior. Previous studies have described behaviors associated with aggression using a grading system or descriptive adjectives, such as "arrestment", "attraction", "faster" or "slower", with minimal quantitative data, consequently making these studies difficult to replicate. This study provides quantitative data for behavioral observations, such as velocity, travel distance, arrestment time, visitation rates and time for initial discovery, that led to the conclusion that dolichodial and iridomyrmecin elicited both alarm and attraction behaviors in Argentine ants.

My studies found that Argentine ants utilize their glandular secretions, containing dolichodial and iridomyrmecin, for multiple functions, providing evidence for semiochemical parsimony, or the use of the same chemical for two or more functions. Argentine ants deploy their glandular secretions during the gaster bending behavior as defensive allomones. Simultaneously, the compounds cause nearby conspecifics to become more alert and quickly aggregate for defense. Furthermore, dolichodial and iridomyrmecin are known to function in necrophoric and recruitment behaviors . The Argentine ants' ability to parsimoniously utilize dolichodial and iridomyrmecin for a variety of behavioral functions has likely contributed to their ability to react quickly to threats while outcompeting native species for resources.

In summary, the studies described in this dissertation provide new knowledge that can be exploited for development of new Argentine ant management strategies. These studies also provide insights into the underlying causes of their worldwide success as invasive species. Two novel baiting strategies have been developed to combat the Argentine ant that have the potential to reduce insecticidal runoff, increase target specificity and provide an acceptable amount of control. The pheromone-assisted baiting technique is not limited to any one baiting medium and can be implemented into future baits such as liquid, gel and hydrogel. Combining the pheromone with RNAi bait has the potential to improve the efficacy by increasing discovery time and overall consumption of the dsRNA contained in the baiting medium. Furthermore, the chemical ecology of Argentine ant aggression has been elucidated and has provided insight into their invasive success. By understanding how Argentine ant semiochemicals play a role in agnostic

interactions, may help to predict future Argentine invasions and native ant displacements. The findings of this research highlight the importance for additional exploration into Argentine ant biology for developing novel management strategies and for understanding the mechanisms associated with their worldwide invasive success.