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Laboratory and Field Evaluations of Biodegradable Boric Acid Hydrogel Baits for the Control of Argentine Ants (Hymenoptera: Formicidae)

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Publication Date
2022

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Laboratory and Field Evaluations of Biodegradable Boric Acid Hydrogel Baits for the Control of Argentine Ants (Hymenoptera: Formicidae)

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Entomology

by

Benning Duc-Nguyen Le

March 2022

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Acknowledgments

Thank you to my wife, parents, and family for their love and support. Thank youto Dr. Dong-Hwan Choe, Kathleen Campbell, Hoeun (Joyce) Park, Ludymar Cester, and everyone from the Choe lab for their support and help with the research. Thank you to Dr. Michael Rust and Dr. Chow-Yang Lee for being my committee members and helping me with their knowledge and wisdom. Thank you to Dr. Jessica Purcell, Kathy Redd, and Dr. Erin Wilson-Rankin for saving me from administrative catastrophes of my own design. Thank you to Dr. Peggy Mauk, Michael Cardey, and Gerardo Barnett for your critical help in the field experiment. Thank you to Dr. Darren Haver, Chris Martinez, and Tanner Bucklin from the South Coast Research and Extension Center for their help in hydrogel bead production. Thank you to Dr. Shu-Ping (Sue) Tseng for performing genetic analyses on ants collected from the tested field site. Thank you to Dr. Raju Pandey and Dr. Gregory Simmons for providing information on ACP field numbers. Thank you to the whole Entomology department for supporting and educating me, as well as departmental funding sources for providing for my tuition. Thank you to the California Department of Pesticide Regulation (CDPR) for funding this research (Pest Management Research Grant Agreement Number: 20-PMG-GR003). US Borax / Rio Tinto provided some of the boric acid used in the current research. Suterra, LLC. provided the pheromone adjuvant (BioAmp AA) for the current research.

Dedication

To my wonderful wife, McKenna.

ABSTRACT OF THE THESIS

Laboratory and Field Evaluations of Biodegradable Boric Acid Hydrogel Baits for the Control of Argentine Ants (Hymenoptera: Formicidae)

by

Benning Duc-Nguyen Le

Master of Science, Graduate Program in Entomology
University of California, Riverside, March 2022
Dr. Dong-Hwan Choe, Chairperson

Argentine ants are a majorly invasive tramp species in natural, urban, and agricultural settings all over the world. Practical and effective control strategies for their control in agricultural settings are still being sought out as the predominant form of control, chlorpyrifos, continues to be phased out in the United States. This research develops our understanding of boric acid hydrogel bait as a potential solution for Argentine ant, Linepithema humile (Mayr) control. Laboratory studies revealed 1) that this bait with a representative preservative, potassium sorbate, is effective in causing Argentine ant mortality 2) that bait is best used fresh and 3) that prior to mortality, Argentine ants exhibit a clumping behavior in response to bait consumption. A large- scale and continuous method of manufacture of the hydrogel beads was devised, allowing for large-scale field applications of the bait in a citrus grove operated by UCR Agricultural Operations. The bait proved effective in reducing ant numbers by up to 80%.

If the levels of control prove to be sufficient, then boric acid hydrogel bait utilizing calcium alginate can be considered an effective, practical, sustainable, and environmentally friendly means of Argentine ant control in citrus groves.

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Chapter I. Introduction

The Argentine ant, *Linepithema humile* (Mayr), is one of the most invasive and pestiferous ant species in the world according to the Invasive Species Specialist Group (Paul Krushelnycky and Andrew Suarez 2009). Native to South America, it has spread worldwide in areas with Mediterranean-type climates usually associated with disturbed habitats as a result of human activities (Suarez et al. 1999). The Argentine ant's proliferation as a pest in the introduced range is in part due to its reduced intraspecific aggression, resulting in polydomous and polygynous biology, its spread by budding, and its close association with honeydew-producing hemipteran insects (Silverman and Brightwell 2008). Argentine ants evolved in humid wetlands and river basins and are naturally adapted to high amounts of moisture in their environment, which is imperative for their survival (Klotz et al. 2008). Because of this reason, Argentine ants tend to be found in agricultural lands and urban landscapes, where artificial irrigation provides ample moisture, and hemipterans can supply them with honeydew (Walters and Mackay 2003).

The Argentine ant is a significant pest in natural, urban, and agricultural settings (Holway et al. 2002). Argentine ants are recognized as a majorly invasive tramp species in natural lands, displacing native ant species and disrupting native ecosystems (Suarez et al. 2000), justifying eradication programs in some areas (Rowles and O'Dowd 2007, Randall et al. 2011, Hanna et al. 2015). In urban settings, the Argentine ant is mainly considered a nuisance pest, having little impact on the health or well-being of humans (Vega and Rust 2001). However, in California and many locations in the southern United

States, it is one of the major urban pests that pest management professionals (PMPs) encounter (Greenberg et al. 2017). Argentine ants tend honeydew-producing hemipterans in agricultural settings and prevent their natural predators and parasitoids from performing biological control, consequently leading to crop damage (Tillberg et al. 2007). Thus, managing Argentine ants is an important part of the integrated pest management (IPM) of various honeydew-producing plant pests (Milosavljević et al. 2021).

Some honeydew-producing hemipterans are considered plant pests due to their capacity to vector diseases and damage plants (Buckley 2003). By inserting piercing-sucking mouthparts into the plant tissues, they feed on the phloem sap, which is rich in nutrients, especially sugars (Tillberg et al. 2007). The fluid from the phloem is so concentrated that the hemipterans must excrete some of the excess sugar in the form of honeydew (Tillberg et al. 2007). This honeydew is a preferred food source for many ants, including Argentine ants (Buckley and Gullan 1991). Agricultural issues caused by honeydew-producing hemipteran plant pests are two-fold: vectoring of diseases and honeydew buildup. In citrus, Huanglongbing (HLB), a disease attributable to several bacteria is vectored domestically by the Asian citrus psyllid (ACP), *Diaphorina citri* (Kuwayama). This disease has cost billions of dollars in damage to citrus industries worldwide (da Graça et al. 2016). It has been found that when ACP is in more densely populated areas, they are likely to disperse, and subsequent increases in HLB transmission occur (Udell et al. 2017). Therefore, while increased susceptibility of ACP by Argentine ant control (Milosavljević et al. 2021) may not eliminate the threat of ACP and HLB, it may slow the spread. Several species of mealybugs, such as *Planococcus ficus* (Signoret) and *Pseudococcus longispinus* (Targioni Tozzetti)*,* are responsible for transmitting *Grapevine leafroll-associated viruses,* which attack the leaves of grapevines (Tsai et al. 2010). Honeydew buildup becomes a problem as it provides nutrition for mold which can harm crops by defoliation, blocking photosynthesis, promoting surface infections, and making fruits unmarketable (Mansour et al. 2018).

Chlorpyrifos is an organophosphate neurotoxin with broad-spectrum insecticidal activity (Whitney et al. 1995, Michereff-Filho et al. 2004). In agricultural settings, chlorpyrifos has been commonly used as a general insecticidal spray to treat for the honeydew-producing hemipterans and any indirect pests such as Argentine ants (John and Shaike 2015). For foliage pests in citrus, such as hemipterans, the trees can be sprayed with solutions containing chlorpyrifos (Vehrs and Grafton-Cardwell 1994). This type of treatment may also impact other insect pests in the vicinity, including the Argentine ant (Michereff-Filho et al. 2004). To target ants more specifically, chlorpyrifos has been sprayed in bands on the trunk of the citrus tree to provide residual activity against trailing ants on the trunk (Moreno et al. 1987). However, the use of chlorpyrifos has been slowly phased out for issues pertaining to human health risks, predominantly concerns of disruption of neurological development of children, and environmental concerns (US EPA 2014). In May 2019, CalEPA announced that the Department of Pesticide Regulation (DPR) was initiating the cancellation of chlorpyrifos for agricultural use (CDPR 2022). While the discontinuation of large-scale chlorpyrifos use may have a net positive effect, it certainly left a large void in agricultural pest management, particularly for pest ants. As a result, there is an urgent need to find safer alternative methods to control pest ants in certain agricultural crops.

A potential alternative to general sprays is the application of bait. Ant baits do not directly target the honeydew producers. If they can effectively control Argentine ants, then the honeydew producers would become more vulnerable to predation and parasitism by their natural predators (Milosavljević et al. 2021). Important characteristics for a good ant bait include palatability (i.e., the bait is readily consumed by the ants), delayed action (i.e., the bait has time to be spread in the colony), solubility of the active ingredient (i.e., complete incorporation of the active ingredient into the bait material), and effectiveness in causing mortality even after dilution via trophallaxis (Stringer et al. 1964, Rust et al. 2004). Unlike the toxicants used in oil based red imported fire ant baits, one of the major issues is that most toxicants are not soluble in aqueous sugar water and thus difficult to formulate. Liquid baits have successfully controlled Argentine ants in urban and agricultural settings (Klotz et al. 1998, Greenberg et al. 2006, Cooper et al. 2008). This target-specific insecticide delivery method can be further improved in its specificity when other semiochemicals, such as pheromones, are added (Welzel and Choe 2016). However, the conventional liquid baiting method requires bait stations to store and dispense the bait. While traditional bait stations can permit the use of toxicants that would not otherwise be allowed into a field, the bait stations are typically expensive and require high maintenance levels (e.g., inspection, cleaning, refilling, etc.) (Nelson and Daane 2007, Rust et al. 2015, Cooper et al. 2019). These drawbacks often prevent the baiting from being widely adopted in commercial agriculture.

Hydrogel materials have been investigated as carriers of liquid bait to overcome this challenge. In the past, calcium alginate hydrogels were studied as a means for the controlled release of insecticides onto the soil where pest insects would forage (Kulkarni et al. 2000). The hydrogels would slowly release the insecticide, and insects that come into contact with the insecticide would be affected (Roy et al. 2009). The incorporation of phagostimulants and water soluble oral toxicants into these hydrogels turned them into controlled-release hydrogel baits (Boser et al. 2014, Buczkowski et al. 2014, Rust et al. 2015). Initially, the bait utilized a synthetic hydrogel, polyacrylamide, which was highly effective in absorbing and delivering insecticidal liquid baits to target ant species (Boser et al. 2014, Rust et al. 2015, Cooper et al. 2019). Unfortunately, polyacrylamide did not readily break down on the soil surface, and when it breaks down, it yields toxic acrylamide monomers (Holliman et al. 2005). This led to a search for alternative biodegradable materials. As a result, calcium alginate hydrogel was recently investigated as a potential bait matrix for liquid bait targeting the Argentine ants (Tay et al. 2017, McCalla et al. 2020, Choe et al. 2021). Alginate is a naturally occurring polysaccharide derived from brown algae, and it can be broken down by enzymatic activity of both bacterium and animals (Linhardt et al. 1987). Though many of the known organisms with alginate lyase or eliminase enzymes are of marine origins, there are examples of terrestrial bacteria with alginase enzymes (Hansen et al. 1984).

Boric acid is a well-documented insecticidal compound. Various boron compounds, including boric acid, have been used in controlling cockroaches in dust and bait formulation, as a wood preservative against fungi, and to a lesser degree, in control of ants (Cochran 1995, Zurek et al. 2003, Thévenon et al. 2010, Harper et al. 2012). Boric acid was also an active ingredient in many gel and liquid baits for various ant and cockroach pest species (Klotz et al. 1998, Gore et al. 2004). Boric acid has many merits as an oral toxicant for Argentine ant baiting in the agricultural settings. These include its delayed action, low non-target toxicity, partial solubility in water, and lack of instances of insecticide resistance (Rust et al. 2004). Boric acid hydrogel bait has been successful in urban settings, both on its own, and with barrier sprays (Choe et al. 2021).

One of the primary purposes of these baits is to replace less environmentally and people-friendly treatments, using an active ingredient that is generally regarded environmentally safe, even organic friendly, is an additional step forward (Greenberg et al. 2006).

The overarching goal of my thesis research was the development of boric acid hydrogel bait to manage Argentine ants in agricultural settings. The first step was to investigate the effect of different parameters (e.g., different active ingredient sources) to develop the bait based on the laboratory experiments. In Chapter 2, the boric acid hydrogel bait was evaluated under laboratory conditions. Multiple variables were examined, including the source of boric acid, the effects of a preservative on bait, and the longevity of the formulated bait. Some behavioral observations of the Argentine ants were also made to document the impact of boric acid bait on their behavior (clumping within a nest). In Florida carpenter ant, *Camponotus floridanus* (Buckley), some behavioral changes resulting from boric acid consumption were associated with a disruption of water regulation (Klotz and Moss 1996). To explore this topic further, the current study quantified a clumping behavior of Argentine ants when treated with boric acid, which was previously noticed (D.-H. Choe, personal communication).

Based on the final compositions of hydrogel bait supported by the laboratory study, the field experiment was conducted. Chapter 3 describes a field study with the boric acid hydrogel bait in a citrus grove, demonstrating its potential for real-world application. Chapter 3 also describes a new method of Argentine ant monitoring, which is adapted from Cooper et al. (2019) with some modifications. For the large-scale field application, larger amounts of hydrogel bait needed to be produced. Chapter 3 reports the new method of hydrogel production, which allowed us to produce enough hydrogel baits to treat a 2.0-ha field.

Chapter II. Laboratory Investigation of the Lethal and Behavioral Effects of Boric Acid Hydrogel Baits on Argentine Ants (Formicidae: Hymenoptera)

Introduction

Argentine ants are an invasive pest in urban, agricultural, and natural environments (Vega and Rust 2001). In agricultural settings, their pest status is associated with their trophobiotic relationship with honeydew producers (Markin 1970). The honeydew producers can vector disease for the plants (Mansour et al. 2018). The buildup of honeydew on plants allows a fungus to grow on the contaminated surfaces (leaves and fruits), causing “sooty mold” (Queiroz and Oliveira 2001). In the integrated pest management (IPM) approach to control honeydew-producing hemipteran pests, natural predators and parasitoids are a critical component. However, it is impossible to maximize the impact of biological control if Argentine ants continue tending the hemipteran pests and protecting them from predators and parasitoids (Buckley 2003). Conversely, Argentine ants do not have any known natural enemies, and various biological control strategies are not effective against them (Orr et al. 2001).

Argentine ant infestations in agriculture often require some level of chemical control (Klotz et al. 2003, Silverman and Brightwell 2008). However, broad-spectrum spray insecticides can contaminate environments and impact non-target organisms such as natural predators of plant pests (John and Shaike 2015). While the use of chlorpyrifos for ants is typically spraying preventative bands around the trunk of the tree (Moreno et al. 1987, James et al. 1998), which may pose less of a risk to the environment, the effort to reduce and remove chlorpyrifos usage is broad and not specific to methodology. As an alternative to insecticide sprays, liquid baiting has been suggested to suppress Argentine ant populations in the agricultural crops (Klotz et al. 1998). However, conventional liquid baiting strategies necessitate the use of bait stations, putting a financial hinderance on liquid baiting strategies at larger scales (Daane et al. 2008). As a solution, a synthetic hydrogel was investigated as a carrier of liquid bait for Argentine ant populations, using polyacrylamide as the binding agent (Buczkowski et al. 2014). Recently, the use of biodegradable calcium alginate hydrogels as a liquid bait carrier was also developed (Tay et al. 2017).

As an active ingredient for baits, boric acid has been widely tested (Klotz et al. 1998, Gore and Schal 2004, Rust et al. 2004). Boron is found naturally in soil and waterways. Boric acid used at insecticidal levels is considered non-toxic to non-target organisms such as non-insect invertebrates and vertebrates (US EPA 1993). Boric acid also has delayed action, resulting in slow mortality in treated insects, often measured in the time to kill 50% of treated insects at a specific concentration of treatment (i.e., LT_{50} of 2.5 days for a 1% boric acid concentration) (Rust et al. 2004). Delayed action is an important characteristic to maximize the bait toxicant transfer (i.e., trophallaxis) within Argentine ant colonies (Rust et al. 2004). Boric acid has no recorded insect resistance, likely owing to its multitude of modes of action: metabolic activity, necrosis of gut lining, and neurotoxic activity (Woods 1994, Habes et al. 2006). Boric acid liquid baits have been demonstrated as an effective tool for Argentine ant management in vineyards (Cooper et al. 2008). Boric acid liquid bait absorbed in calcium alginate hydrogel was tested as a maintenance treatment option for Argentine ants in a recent field demonstration study at urban residential settings (Choe et al. 2021). However, to move this novel approach of boric acid baiting to full-scale research trials in agricultural settings, it was necessary to conduct a detailed laboratory study to determine the effective composition of boric acid liquid bait to be used with a new biodegradable alginate hydrogel.

The current study had the following objectives: First, boric acid from two different suppliers, Optibor® (>99.5%) (U.S. Borax Inc., Boron, CA) and boric acid ReagentPlus® (≥99.5%) (Sigma-Aldrich, St. Louis, MO), was tested with the alginate hydrogel. Since Sigma-Aldrich was the source of boric acid in a previous trials with calcium alginate hydrogel (Choe et al. 2021), this comparative study was necessary to ensure Optibor®, a new source of boric acid chosen for the field studies, was comparable to the boric acid from Sigma-Aldrich in its insecticidal effect.

Secondly, the current study examined whether the presence of potassium sorbate (PS), a preservative to prevent mold, has any impact on the efficacy of the boric acid baits. The addition of a preservative in the bait would be necessary for real-world applications, where the manufactured bait may need to be stored for some time before being applied in the field. Should the bait need to go unused and unrefrigerated, PS helps prevent the growth of mold, ideally without any negative impact on the effectiveness of the bait (Qin et al. 2017). Many factors can affect the overall capacity of the hydrogel beads to absorb a solution, one of which can be the concentration and physiochemical properties of the solutes, altering the degree to which the beads swell (Golmohamadi and

Wilkinson 2013). Thus, the current study also quantified how the swelling of the hydrogels was affected by the addition of PS as a representative preservative.

Thirdly, the effect that aging had on bait was tested. Former studies evaluated how aging via water loss affected bait palatability (Buczkowski et al. 2014, Rust et al. 2015, Tay et al. 2017). Instead, this study looked at the long-term effects that storing the bait at room temperature would have. With the presence of PS, no mold would grow. It is useful to know how long-term storage alters bait efficacy, as this would be practical information for users.

Lastly, in addition to these toxicological assays, the behavioral and physiological effects of boric acid on the Argentine ant colonies were examined. In previous laboratory trials of ants treated with boric acid, there were observations of ants excreting more liquid feces and spending more time on moisture sources (Klotz and Moss 1996). Similarly, anecdotal observations indicated that Argentine ants treated with boric acid tended to aggregate more densely within their colony tubing, closer to the water source (D.-H. Choe, personal communication). The current study attempted to quantify the clumping behavior of Argentine ants after ingesting boric acid bait. Studies on behavioral and physiological effects of boric acid in Argentine ants might provide an important piece of information to understand changes in ant activity in the field (e.g., foraging, trailing, etc.) after application of boric acid bait. Possible implications of the findings are discussed.

Materials and Methods

Ants. Argentine ants were collected from the field and kept in laboratory conditions within large plastic containers. Collection was carried out by digging up Argentine ant nests in citrus groves in UCR Agricultural Operations and placing them into 5-gal buckets. The buckets were taken back to a collection room and the dirt was spread evenly in bins. Plaster disks with hollow centers, soaked in tap water, were stacked in the middle of the bin with twigs serving as bridges from the dirt to the disks. A small weigh boat with sugar water-soaked cotton was placed atop the disk stack to attract the ants. As the dirt dried, the ants moved into the plaster disks in search of moisture. This process took 1-5 d, depending on air temperature, humidity, soil moisture, and quantity of soil and ants. Once the ants had aggregated in the plaster disks, workers, queens, and brood were shaken off the plaster into plastic containers (transfer bins) which have base dimensions of 32 by 14 cm. Inner walls of the transfer bins were coated with a fluoropolymer resin (Insect-A-Slip - Fluon®; Bioquip Products, Inc. Rancho Dominguez, CA) to prevent ants from escaping. The ants were placed into a larger colony box (31 by 77.5 cm) with plastic Petri dishes filled with plaster. The sides of the colony box were coated with fluoropolymer resin. Water, pieces of chicken breast, and 25% (wt/vol) sucrose water were provided to sustain a laboratory stock colony. The stock colonies were kept at 23°C and 30% relative humidity (RH) in laboratory conditions.

Experimental colonies. Clear 473-mL (16 fl. oz.) polypropylene deli cups (bottom diam. 8.9 cm, top diam. of 11.6 cm, and height 7.6 cm, PK16S-C; Fabri-Kal, Kalamazoo, MI) were used as experimental colony containers (Fig. 1a). Within the experimental colony container, a piece of tubing was provided. The tubing was comprised of a 7-cm section of clear vinyl tubing (Tygon®, 8 mm ID, 11 mm OD) (Saint-Gobains Performance Plastics, Akron, OH) fitted to a 2-mL glass vial (Agilent, Santa Clara, CA) filled with deionized water and plugged with a piece of 2.5-cm cotton wick (Absorbal, Wheat Ridge, CO) (Fig. 1b). A small section of cotton wick exposed within the tubing provided moisture inside of the tubing. The tubing was marked every 5 mm with a marker to measure the distribution of the ants within the tubing based on visual observation (Fig. 1b). The tubing unit was affixed to the interior of the cup by attaching the bottom of the vial to the inner surface with hot glue. The location of attachment (e.g., between a third and half of the height of the cup) and angle of the vial were carefully chosen to ensure that the open end of the nest tubing contacted the bottom corner of the cup, making it readily accessible for the ants (Fig. 1b). The top portion of the inner surface of the cup (top 3-4 cm, above the line of tubing attachment) was treated with a fluoropolymer resin to prevent the ants from escaping from the cup (Fig. 1a).

Argentine ants from the stock laboratory colony boxes were transferred in an empty plastic box (32 by 14 cm) with the inner sides coated with a fluoropolymer resin to prevent them from escaping. Several sections of 5-cm vinyl tubing (“transfer tubing”) (Tygon®, 8 mm ID, 11mm OD) were provided on the bottom of the box. Once the ants were placed into the box, they quickly formed dense aggregations within the tubing sections by bringing many queens, and brood into them. Once all the transfer tubing sections were full (30-60 min after introducing ants), the transfer tubing sections were quickly picked up, held over the cups, and shaken vigorously to transfer the ants into the cups. The ants moved into the nest tubing with their brood soon (30-45 min.). This pooling process minimized any possible colony differences (e.g., presence or absence of brood) resulting in relatively similar-sized colonies with brood, workers, and queens (Fig. 1c). For example, the average worker count was 473 ± 10 (mean ± SEM, n = 89). Upon initial transfer of the ants, a visual inspection was done to ensure that each cup had at least one queen. Once the experimental colonies were set up, 50 µL of 25 % sucrose water was provided per colony cup, typically completely consumed overnight. No sugar water was provided for the next two days to ensure that all the experimental colonies were similarly starved at the time of the experiment. The experimental bait was provided on day 3 after the experimental colony set up.

Bait preparation. To examine if different ingredients affect the overall insecticidal effect of the bait, following baits were prepared and tested using the experimental colonies. Boric acid from two sources was tested to determine any differences in the bait performance against Argentine ants. The first boric acid was Technical Powder Optibor® (>99.5%) (U.S. Borax Inc., Boron, CA) [abbreviated as BA(O)], and the second boric acid was Boric Acid ReagentPlus® (≥99.5%) (Sigma-Aldrich, St. Louis, MO) [abbreviated as BA(S)]. A representative preservative, potassium sorbate ReagentPlus® (99%) (abbreviated as PS) (Sigma-Aldrich), was also included in the experimental design to determine if it had any effect on bait performance (i.e., any interaction with boric acid). For controls, only sucrose (abbreviated as C, either with or without PS) was included in the bait without boric acid. Overall, the treatments tested were BA(O), BA(O)+PS, BA(S), and BA(S)+PS, and the controls were C+PS and C.

All hydrogel baits were prepared using the same stock hydrogel beads produced via methods explained in more detail in the next chapter. In brief, a 1% sodium alginate solution in deionized water was prepared by mixing thoroughly. The hydrogel beads were produced by introducing the sodium alginate solution dropwise into a 0.5% calcium chloride crosslinking solution. After a brief crosslinking process (1.5-2 min), the hydrogel beads were rinsed with tap water to arrest crosslinking, and strained (Tay et al. 2017). The hydrogel beads were stored in a refrigerator (4°C) to preserve freshness. To prepare the hydrogel bait, the stock hydrogel beads needed to be conditioned in a bait solution containing sugar, insecticide, or preservative. For each treatment, 100 mL of conditioning solution was prepared to condition 5 g of hydrogel beads. Following concentrations were used for each of the ingredients in conditioning solution. 25% for sucrose, 1% for BA, and 0.25% for PS. The hydrogel beads were left in the refrigerator to condition for 3 d before testing with the ant colonies.

Swelling analysis. The degree of swelling was estimated by weighing 5 sets of 5 hydrogel beads before conditioning (blank hydrogel) and a set of 5 beads of each treatment after conditioning (hydrogel bait). The degree of swelling was calculated in the difference between post- and pre-conditioned gel weights (Degree of swelling (%) = (Ws-Wd)/Wd*100 [Wd = weight of blank hydrogel; Ws = weight of swollen hydrogel].

Bait evaluation. The effectiveness of hydrogel baits from BA(O), BA(O)+PS, BA(S), BA(S)+PS, C, and C+PS were determined against the small experimental colonies of Argentine ants previously described. Immediately before treatments, any dead ants in the colony cups were removed to prevent them from being counted as post-treatment mortality. The hydrogel bait beads (2-3 beads) were provided in the small cup (lids from 1.5mL centrifuge tubes) placed at the bottom of colony cup. The amount of bait provided per colony was sufficient to satiate the colony initially and support the colony for 14-d experimental period (in controls). As these hydrogel beads are primarily water and have a high surface area to volume ratio, they dry out quickly. To maintain palatability to ants, the beads were rehydrated every other day until the end of the trial with ≈0.1mL (two pipet drops) of deionized water. The number of replicates varied with treatment, but the lowest number of replicates was 11.

To determine the longevity of the hydrogel bait, insecticidal effects of fresh and aged baits (2-month-old) were compared. One combination of bait ingredients [BA(O) + PS] was used for this experiment. The aged bait was tested against its fresh counterpart to evaluate how effective it would remain after aging for 2 months. Aged bait was prepared by keeping the bait at 24°C in a 5-gal bucket with a lid. There were 12 replicates each of aged and fresh bait treated colonies.

With the boric acid bait treatment, Argentine ant mortality occurs relatively slowly (Rust et al. 2004), and many ants succumb and die within the nest. By day 14, the surviving ants would have been removed most of their dead from the nest tubing and placing them on the bottom of the colony cup, allowing the full extent of the mortality to be measured. If any dead ants remained in the nest tubing, the tubing was slightly manipulated to distinguish between live and dead ants inside of the tubing. After taking final mortality on day 14, all ants in the experimental colony were anesthetized with CO_2 and subsequently frozen for their total count.

Clumping behavior. Clumping behavior was measured by counting the number of dead ants on day 2 across all replicates used for the bait evaluation trials and looking at the amount of available tubing section the live ants occupied (Fig. 1). On day 2, mortality was relatively low, and the tubing was typically clear of dead ants. The number of dead was subtracted from the number of total ants after day 14, to obtain the number of live ants occupying the colony tubing. Considering from the number of live ants and the length of tubing occupied (Fig. 1a and Fig. 1d), shown by marks made on the tubing every 5 mm, three-dimensional volumetric number densities were calculated (ants/cm^3). Since the hypothesis was specifically about boric acid, only treatments BA(O), BA(S), and C were included in the clumping behavior study, removing potassium sorbate (PS) preservative from being a factor.

Statistical analyses. All statistics were run on Statistix 10 (Analytical Software 2017). For both mortality and density data, Kruskal-Wallis tests were run to determine if there were statistically significant differences between treatments. Dunn's All-Pairwise Comparison test was performed to determine which groups are statistically different or similar. A Wilcoxon-Rank-Sum test was used to compare day 14 mortality values between aged and fresh baits.

Results

Swelling analysis. Hydrogel beads conditioned with PS were less than half the mass of beads conditioned without PS (Table 1). Without the presence of PS, boric acid appeared to reduce the degree of swelling of the beads (Table 1). However, when PS was present (C+PS vs boric acid treatments with PS), boric acid seemed to have no effect on the degree of swelling (Table 1). Relative to their respective controls, both PS and boric acid affect swelling, the PS beads typically being 1/3 the size of control, and boric acid beads being roughly ¾ the size of the average control bead.

Bait evaluation. Ants fed on all treatments. No quantitative measurements of feeding behavior were taken, and no subjective visual differences were noticed between initial feeding behavior on the baits and controls. Ant mortalities became noticeable on day 2 and gradually increased over time. One replication from a C treatment is omitted from both mortality and clumping data due to ants escaping during the trial, making it impossible to determine how many ants were initially in the cup. The median number of dead ants at day 14 was 368, 445, 425, 353, 9, and 15, for BA(O), BA(O)+PS, BA(S), BA(S)+PS, C, and C+PS, respectively. A Kruskal-Wallis test revealed a significant difference among treatments ($H = 51.03$, df = 5, $P<0.0001$). A Dunn's All-Pairwise Comparisons test revealed two distinct groups; namely, BA(O), BA(O)+PS, BA(S), and BA(S)+PS, and C and C+PS (Fig. 2).

With BA(O)+PS, median mortality values in fresh and aged bait treatments were 445 and 162 ants, respectively. The distributions in the two groups differed significantly

(Mann–Whitney $U = 5$, $n_1 = 11$, $n_2 = 12$, $P < 0.0001$ two-tailed), indicating there is a significant difference in insecticide effectiveness between fresh and 2-month aged hydrogel baits (Fig. 3).

Clumping behavior. For the clumping behavior study, two additional replications, both from BA(O), were removed from analysis as the accumulation of dead ants in the nest tubing made it impossible to accurately determine the number of dead/live ants, and the space that live ants were occupying within the nest tubing. The average three-dimensional volumetric number density (ants/cm^3) at day 2, were 328.29, 182.73, 295.92, with an average of 462, 479, 435 living ants for treatments BA(O), BA(S), and C, respectively. A Kruskal-Wallis test revealed a significant difference among treatments for clumping density ($H = 20.99$, df = 2, $P < 0.0001$). A Dunn's All-Pairwise Comparisons Test placed treatments BA(O) and BA(S) in one group, signifying no significant difference between the two boric acid treatments, and treatment C in another group, signifying a significant difference from the other two treatments with boric acid (Fig. 4).

Discussion

Laboratory experiments indicated that the hydrogel bait conditioned with 1.0% boric acid and 25% sucrose water killed Argentine ants, resulting in significantly higher mortality in the treatment colonies than the controls (Fig. 2). Hydrogel baits prepared with Optibor® boric acid with PS and Sigma Aldrich boric acid with PS provided on average 410 and 371 dead ants by day 14, respectively, indicating they are similar in their effectiveness against laboratory colonies of Argentine ants when incorporated in the alginate hydrogel (Fig. 2). The addition of potassium sorbate as a representative preservative (0.25%) did not influence the insecticidal activity of boric acid baits delivered with the hydrogel (Fig. 2). Previous studies have shown potassium sorbate to be non-repellant in liquid baits for ants and useful in extending shelf-life (Qin et al. 2017). The former is reaffirmed by the non-significant difference between boric acid treatments with and without PS.

The laboratory mortality data supports the use of Optibor® and potassium sorbate to manufacture the boric acid hydrogel bait for the large-scale field experiment. In any replicate, 100% mortality was never reached by any treatment, unlike similar formulations of liquid boric acid bait in other laboratory evaluations (Klotz et al. 1996). Two main differences between Klotz et al. (1996) and the current study are the length of trials and the ant numbers in experimental colony. For example, Klotz et al. (1996) allowed the trial to continue for 10 weeks, averaging 250-300 ants per colony (Klotz et al. 1996). In the current study, the assay was ran for 2 wk, with a mean worker count of 473 ± 10 (mean ± SEM) per colony similar to Rust et al. (2004). Also, Klotz et al. (1996)

provided the liquid bait constantly, unlike the current study where the bait was provided once at the beginning and only rehydrated throughout the trial.

Experiment with 2-month aged hydrogel bait (with Optibor® and potassium sorbate) indicated that the aged bait was significantly less effective than the fresh bait (Fig. 3). With the storage conditions used in the current study (e.g., room temperature, closed container, in the bait solution), the 2-month-old hydrogel bait was about half as effective as the fresh hydrogel bait. The aged bait was still consumed by the ants and caused substantial mortality but was significantly less effective that fresh bait. The reason why the aged bait is less effective than fresh bait is unknown. Perhaps the aged bait might be simply less palatable than fresh bait; it is difficult to say as most literature that investigated aged bait are considering the water loss of the bait, not the long term effects of sealed storage (Buczkowski et al. 2014, Rust et al. 2015, Cabrera et al. 2021). It is also possible that over time the boric acid begins to form complexes with the sucrose, potassium sorbate, or calcium alginate, that puts the boron in forms that are either less toxic or less available (Klotz et al. 2002). For example, Klotz et al. (2002) reported that the addition of certain sugar alcohols, such as sorbitol, in the bait could reduce the efficacy of a boric acid bait (Klotz et al. 2002). Boric acid readily forms esters with the hydroxyl groups of sugars (Woods 1994). Alginate has a large number of hydroxyl groups (PubChem 2022a). Some research has been done on using calcium alginate hydrogels to adsorb boron from aqueous solutions to remove boron from water (Demey-Cedeño et al. 2014). At certain conditions, it was found that boric acid readily forms complexes with the diols of alginate (Demey-Cedeño et al. 2014). Indeed, the duration of storage is also likely to affect these chemical reactions. Further research is warranted to understand the chemical interaction between calcium alginate and boric acid, and its impact on the bait efficacy over time.

Behavioral observation and quantification of clumping indicated that Argentine ants treated with boric acid tended to aggregate in significantly more dense groups in their colony tubing when compared to the untreated control group (Fig. 1c and 1d). For example, based on the three-dimensional volumetric number density, the treated colonies aggregated 1.67 times more densely than did the untreated ants inside their nest tubing. Since total numbers of live ants were comparable between treatments and control (479, 435, and 462, for C, BA(S), and BA(O) respectively) at the time of evaluation, the difference in the number density was not due to the difference in total number of live ants.

It is not clear what is responsible for the observed clumping behavior in Argentine ants. The exact mode of action of boric acid, which results in mortality, is ambiguous. There are several recorded modes of action across insect species: neurotoxicity, metabolic disruption, and gut lining cell death leading to starvation (Cochran 1995, Habes et al. 2006, Sumida et al. 2010). However, seemingly no studies have been conducted to investigate if boric acid affects the osmoregulation of insects, despite some observations indicating water regulation issues in ants after boric acid consumption (Klotz et al. 1996). Considering boric acid has shown signs of alimentary canal cell destruction (Cochran 1995, Sumida et al. 2010), the damage may extend beyond the midgut to the hindgut. The hindgut is where the final absorption of water from the alimentary canal occurs (Mullens 1982). Therefore, if the hindgut is severely damaged, the insect would lose its ability to osmoregulate. While the reason remains unknown, the current data justify further exploration.

While the current study successfully quantified one of the behavioral impacts of boric acid baiting on Argentine ants (i.e., clumping), the implications of that behavioral change are still only speculative. Traditionally, it has been considered that foraging workers are the ones most impacted by an initial bait treatment. Still, this initial reduction of ant activity is typically recovered by other new foragers or ants from neighboring colonies readily repopulating the treated areas (McCalla et al. 2020). However, the current behavioral observation indicates that what has been seen in the field after the boric acid application might be a combination of population reduction via direct mortality and suppression of foraging activity via a larger version of the “clumping” behavior.

In summary, this laboratory study confirmed that boric acid hydrogel bait (1% boric acid) was effective in killing Argentine ants. The addition of potassium sorbate in the hydrogel bait as a preservative did not have any deleterious effect on the bait’s efficacy. To further evaluate the boric acid hydrogel bait in controlling Argentine ants, a field study needs to be conducted. The final bait ingredients selected for the field study was BA(O)+PS. Also, the bait will need to be made fresh and used within a few days of production to prevent any age-related issues, with a new batch made for each subsequent treatment. Next chapter describes the field experiment in a citrus field.

Table 1. Swelling of the alginate hydrogel beads (n = 5) when conditioned in different bait solutions with different combinations of boric acid (BA) and potassium sorbate (PS) preservative. Boric acid, obtained from two different sources [US Borax Optibor®, BA(O); Sigma Aldrich, BA(S)], were included in the study. Sugar only and sugar + potassium sorbate served as controls (C and C+PS, respectively). Concentrations for the compounds are provided in the text.

Treatment	Initial wt (g)	Final wt (g)	Degree of swelling (%)	Percentage of C mass
BA(O)	0.31	1.11	258.06	72.55
BA(O)+PS	0.31	0.58	87.10	37.91
BA(S)	0.31	1.22	293.55	79.74
BA(S)+PS	0.31	0.58	87.10	37.91
C	0.31	1.53	393.55	NA
C+PS	0.31	0.56	80.65	36.60

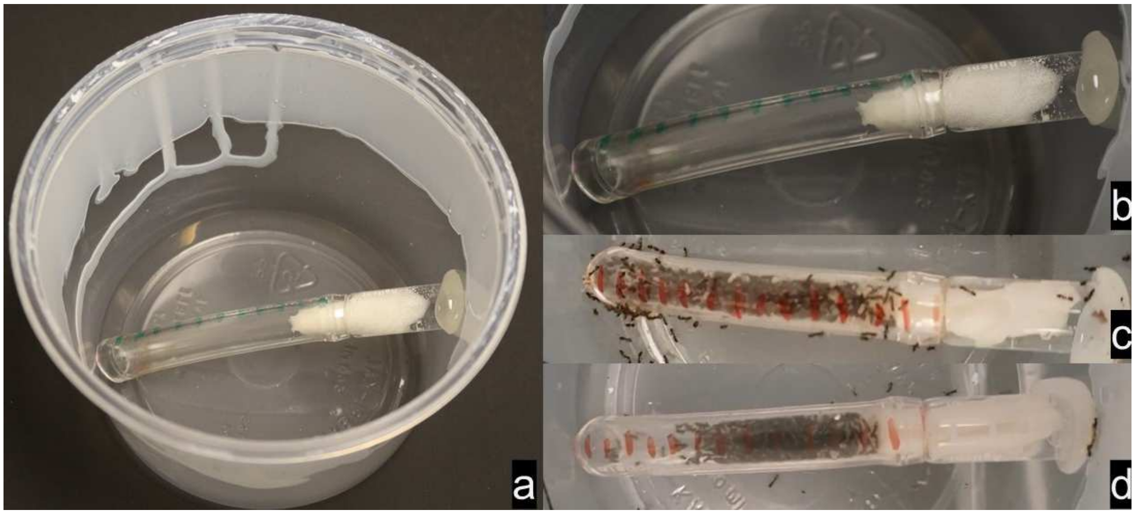


Fig. 1. Experimental colony set up. (a) An experimental colony container. (b) Close-up image of the colony tubing. (c) Picture of colony tubing with ants inside (control colony). Marker lines on the colony tubing were used to determine the location of the ants within the tubing (5 mm between marker lines). (d) Picture of colony tubing with ants clumping inside (baited colony).

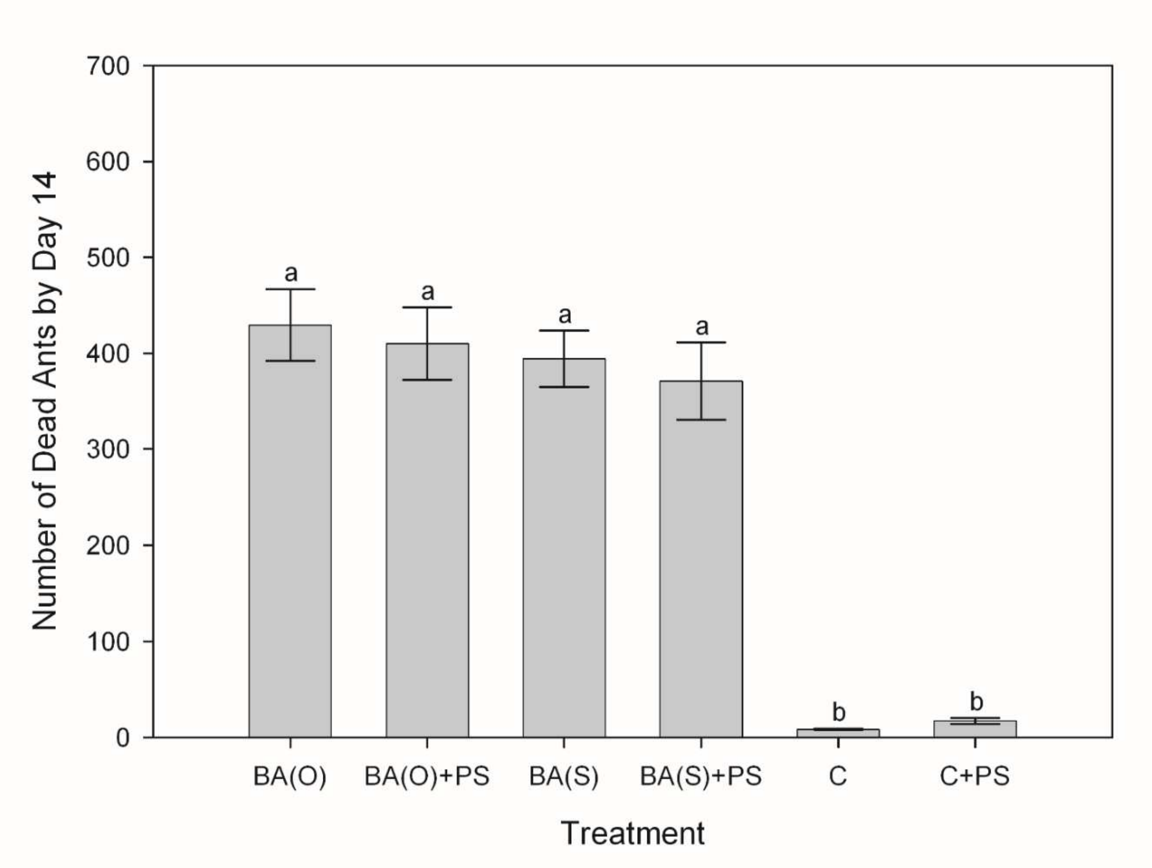


Fig. 2. Comparison of average number of dead ants per cup for each treatment. Error bar indicates standard error of means (SEM). Different letters above bars represent groups with significant differences (Kruskal-Wallis test with Dunn's All-Pairwise Comparisons test; $\alpha = 0.05$).

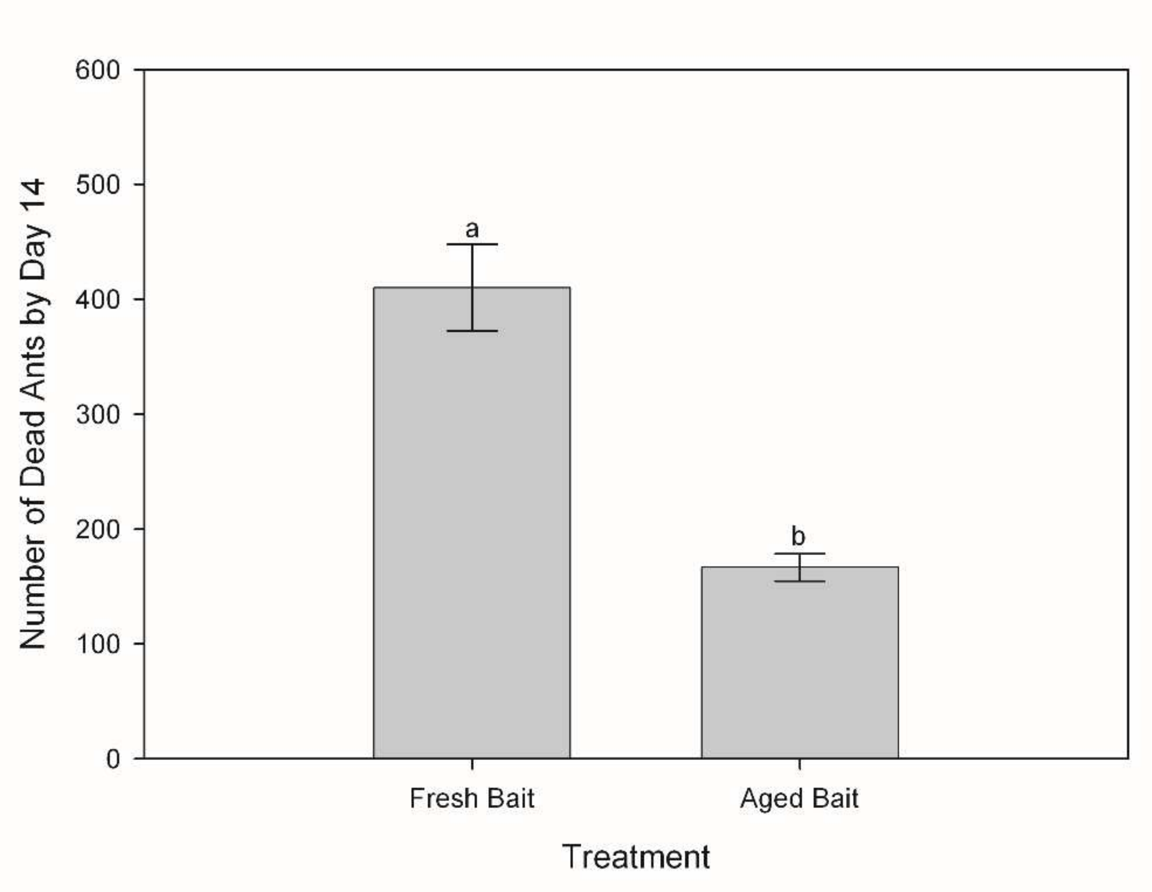


Fig. 3. Comparison of average number of dead ants per cup at day 14 between fresh and aged bait [BA(O)+PS]. Error bar represents standard error of means (SEM). Different letters above bars denote significant differences (Wilcoxon Rank Sum test; α = 0.05).

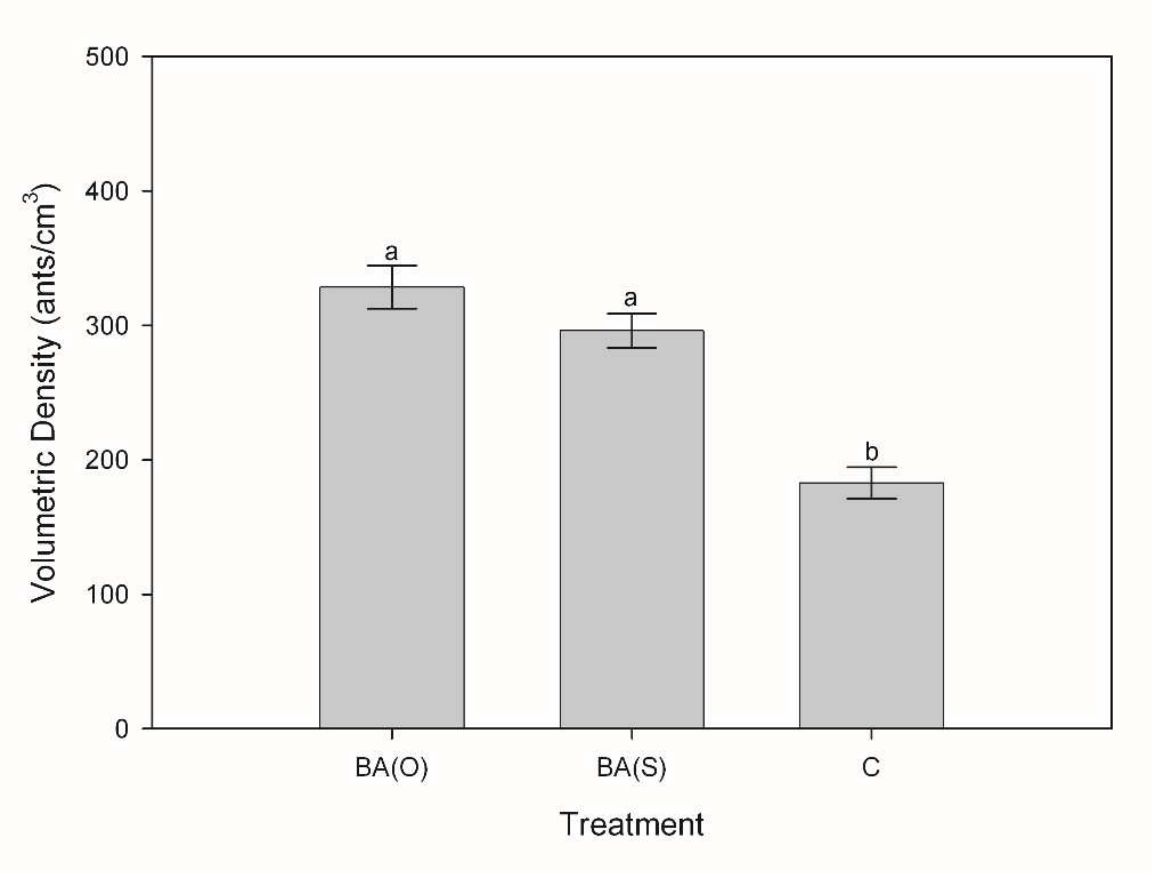


Fig. 4. Comparison of clumping behavior between treatment and control groups. Error bar indicates standard error of means (SEM). Different letters above bars represent groups with significant differences (Kruskal-Wallis test with Dunn's All-Pairwise Comparisons test; $\alpha = 0.05$).

Chapter III: Use of Biodegradable Boric Acid Hydrogel Bait to Manage Argentine Ants in Citrus Groves

Introduction

In agricultural settings, the Argentine ant negatively impacts the biological control of honeydew producers by tending them (Vega and Rust 2001). This is an important issue as many of these honeydew-producing hemipterans vector plant diseases, weaken the plant, and cause a buildup of honeydew that can lead to sooty mold (Mansour et al. 2018). The significance of the trophobiotic relationships between Argentine ant and honey-producing hemipterans is especially relevant in citrus systems today. For example, Asian citrus psyllid (ACP), *Diaphorina citri*, vectors Huanglongbing, a bacterial disease that has cost billions of dollars in citrus damage, domestically and abroad, and now threatens California's citrus industry (da Graça et al. 2016). Argentine ants tend ACP and ward off their natural predators and parasitoids, increasing survivorship of ACP and subsequent risk of disease transmission by them (Milosavljević et al. 2021). Argentine ants are challenging to control due to the astounding rate at which they can invade an area, expand their territory, and even repopulate an area after a control effort (Silverman and Brightwell 2008). While broad-spectrum insecticide sprays have been applied for Argentine ant management in agricultural systems, other alternative strategies, such as liquid baiting, have been considered (Rust et al. 2000, Gore and Schal 2004, Daane et al. 2006, Cooper et al. 2008). The use of baits may reduce environmental contamination by insecticides while achieving higher target specificity, and possibly a colony wide impact, as bait can be effectively transferred between colony members via trophallaxis (Rust et al. 2004). This is an important change since the use of those broad-spectrum sprays, such as chlorpyrifos, has been restricted or phased out over time (CDPR 2022).

The type of bait predominantly tested for Argentine ants is a liquid bait containing carbohydrates, often sucrose, as the phagostimulant. One of the shortcomings of conventional liquid baiting is the need for bait stations to contain and dispense the bait. Baiting a sizable agricultural field often requires many bait stations which can be costly to purchase and maintain (Daane et al. 2008). This shortcoming has been overcome with the advent of hydrogel bait systems (Buczkowski et al. 2014, Rust et al. 2015). The use of hydrogels as a carrier makes the liquid baiting possible without bait stations, allowing application of the bait directly on soil surfaces where ants are foraging (Tay et al. 2020).

Different hydrogel compounds have been tested for ant baiting, initially synthetic polyacrylamide (Rust et al. 2015), and more recently, biodegradable calcium alginate (Tay et al. 2017). Many hydrogel bait studies, both polyacrylamide and calcium alginate hydrogel beads, have used thiamethoxam as the toxicant (Boser et al. 2014, Buczkowski et al. 2014, Rust et al. 2015, Tay et al. 2017, McCalla et al. 2020). Fewer studies have used boric acid (Cooper et al. 2019, Choe et al. 2021). This paper will be a novel test of using calcium alginate hydrogel beads with boric acid in citrus orchards. With the combination of calcium alginate hydrogel beads and boric acid, a highly sustainable and environmentally friendly bait is formed.

Boric acid is considered non-toxic to non-targets (US EPA 1993), occurs naturally in the environment (US EPA 1993), and yet still causes mortalities in liquid baits for many insect pests (Klotz et al. 1998, Gore et al. 2004, Habes et al. 2006). Boric acid hydrogel baits provided >80% Argentine activity reduction in residential environments (Choe et al. 2021), so their use in agriculture is the next step. The target specificity of bait can be increased by utilizing attractants specific to the pest species, in this case, the trail pheromone of Argentine ants, proven to work in increasing liquid bait efficacy (Welzel and Choe 2016, Choe et al. 2021).

This study tests boric acid hydrogel bait on a larger agricultural scale. While boric acid liquid baits (Greenberg et al. 2006) and hydrogel baits (Cooper et al. 2019) have successfully been used to control Argentine ants in agriculture, the combination of calcium alginate hydrogel beads and boric acid is novel. Compared to earlier experiments with alginate hydrogel, a much larger amount of hydrogel bait was necessary for the field evaluation at a 2.02-ha (5-ac) citrus grove. Production methods previously described were not adequate for producing the bait at scale for the agricultural applications (Tay et al. 2017, Choe et al. 2021). Thus, the current study developed and utilized a continuous method (in opposed to a batch production method in the previous experiments) for alginate hydrogel production. The costs of production of the baits and their application in the field will be discussed.

Materials and Methods

Study site. The experimental plot was located within Agricultural Operations, University of California, Riverside. The plot consisted of nine blocks of Valencia orange trees with a dirt road between each adjacent block (Fig. 1). Each block was about 0.67 ha (total 6.1

ha) with around 186 trees in each block, divided into 31 rows of ≤ 6 trees. The site was divided into three identically-sized zones, a baited zone on the west, a control zone on the east, and a buffer zone between the first two. This led to there being 2 ha for baiting.

During the period of the current experiment, no insecticide treatment was made at the plot except one application of Admire® Pro (active ingredient: imidacloprid) (Bayer CropScience LP, Research Triangle Park, NC) made on September 8, 2021 through the irrigation system at the rate of 981 g per ha. This systemic insecticide treatment was required for Asian citrus psyllid (ACP) control.

Monitoring. Nine rows of trees were chosen from each block to monitor foraging activity of Argentine ants. The third or fourth tree from the road in the designated rows (4, 7, 10, 13, 16, 19, 22, 25, and 28) were selected to achieve an even spread and some symmetry (Fig. 1). Monitoring started between 8 and 9 AM. The foraging activity of Argentine ants on trees was monitored using a method adapted from the technique used by Cooper et al. (2019). First, cotton squares (Walgreens Quilted Cotton Squares, item code: 269812, 5x5cm) were soaked in 25% sucrose water with the Argentine ant pheromone adjuvant (BioAmp AA, Suterra, LLC., Bend, OR) (1 µl/ml). By pouring 50 ml of the sucrose water into a plastic bag containing ten cotton squares, each cotton square was saturated with ≈ 5ml of sucrose water. This amount of sucrose solution per cotton square was ideal for yielding fully saturated squares without any excess liquid dripping from them. Nine of these bags were prepared, one for each of the columns at the study site. A single cotton square monitor was placed per tree between large branches stemming from the main trunk, avoiding irrigation water from sprinklers (Fig. 2). In most cases, ant trails were already present at that location. When ant trails were not immediately present, the volatile pheromone added to the cotton square monitors was expected to attract foraging ants from nearby trails on tree trunks. In the beginning of the season, June 15 – July 30, 2021, each monitoring square was left to be foraged on by ants for 2 h before being picked up. As the season progressed, extremely heavy ant trails were observed on tree trunks and the monitoring squares seemed to be drier upon being picked up. If the cotton square monitors lose most of sugar water and moisture (either by weather condition or heavy foraging activity by ants), the foraging ants would not stay on them, consequently impacting the accuracy of the monitoring data (Cooper et al. 2019). For this reason, from August 6, 2021 and onward, monitor squares were left out for only 1 h instead. In any case, all of the cotton square monitors were left on the tree for the same amount of time within each monitoring visit.

Each cotton square was collected and placed in a clean, labeled plastic bag, in the same order as they were placed. During collection, a pair of tweezers was used to handle the squares gently, so no ants were lost. In the laboratory, each bag was briefly flooded with CO_2 to knock out the ants; then the cotton square was removed, leaving all ants behind in the bag. After recording the weight of bag with the ants, the ants were removed into a temporary holding bin, and the empty bag was weighed. The weight of ants was the difference between these two weight values. The process was repeated for all 81 bags. For columns B and H, samples were collected for genetic analyses after weighing. The samples were placed in 95% ethanol in labeled 10mL centrifuge vials and placed in the freezer until they were genetically analyzed. After all bags were emptied into the holding bin, a small subsample of ants (between 20-40 ants) was weighed to acquire an average weight of a worker ant. With an average ant weight and a total weight of ants in each bag, the number of ants collected from a tree could be estimated (total weight of ants/average ant weight = # of ants).

Monitoring began on June 15, 2021, and was carried out weekly until October 1, 2021, except for the weeks when the bait applications were made. After that, the monitoring was carried out on a roughly monthly basis. The last monitoring was carried out on Jan 21, 2022. Monitoring was performed twice before initial baiting, once 9 days before baiting and once 7 days before baiting.

Hydrogel bait production. Sodium alginate powder (Nalgin HG, Ingredients Solutions, Inc., Waldo, ME) was mixed with warm deionized water on a ratio of 1 Kg of powder per liter of solution (1 %) in large (22.7 L) buckets. The solution was mixed thoroughly using immersion blenders (Waring Commercial, Torrington, CT). Once the sodium alginate solution was homogenous (after ~15 min of stirring), it was left in the bucket for an additional ~1-2 hours with occasional stirring until it cooled to room temperature and had released most of the air bubbles. The sodium alginate solution was poured into dripping devices manufactured with affixed multi-nozzle shower heads (Glacier Bay, part number: 43237-2) to plastic cylinders (15 cm in diameter and 22.8 cm in height). The drippers introduced the alginate solution dropwise into a crosslinking solution of 0.5% (wt/vol) calcium chloride ($CaCl_2$) in deionized water contained in the water tank of a running conveyor belt (KKI Conveyor with Water Tank, EMI Corporation, Jackson Center, OH) (Fig. 3). The conveyor belt had a flat section through the water tank with an incline at one end that led up and out of the water tank (Fig. 3). At the end of the incline, a water shower was used to collect the resulting alginate hydrogel beads into a rinsing bin. In the rinsing bin, the collected hydrogel beads were further rinsed to arrest the crosslinking process (Fig. 3).

The hydrogel beads were conditioned in a solution of 1.0% boric acid (H_3BO_3, Optibor®, US Borax Inc., Boron, CA), .25% potassium sorbate (Sigma-Aldrich, St. Louis, MO), and 25% sucrose. The initial conditioning solution was prepared in a more concentrated form so that dilution by water from blank hydrogel (i.e., 99% water) would be offset for the final bait product. For example, if 100 mL of conditioning solution were used to condition 100 g of hydrogel (99% water), the total volume of water in the mixture (blank hydrogel + conditioning solution) was 199 mL. Boric acid, potassium sorbate, and sucrose were used in amounts that would reach the target concentrations in a final 199-mL solution. The blank hydrogels were soaked in the bait solution overnight. Once the conditioning process was complete, the final baits were portioned into plastic 5-gal buckets with excess conditioning solution to keep the bait hydrated. A total of 1263.75 kg of bait was made for the entire project.

Hydrogel bait application. Based on Cooper et al. (2019), in which a boric acid liquid bait absorbed in polyacrylamide hydrogel was tested, an application rate of 94 L per ha was chosen for the current study. To make bait application efficient, the amount of hydrogel bait needed to treat each row (6.3 kg) was contained in a 5-gal bucket, necessitating a total of 31 buckets. As a result, 189 L of hydrogel bait was divided between 31 buckets, coming out to ~6.1 L of conditioned hydrogel bait per bucket. To keep the bait moist and fresh, 3 L of excess conditioning solution was added to each bucket until used.

Immediately before application, the Argentine ant pheromone adjuvant (BioAmp AA, Suterra, LLC.) (1 µl/ml) was added to the hydrogel bait. The hydrogel bait was applied using an automated spreader (Kubota V5003 spreader, Kubota Corp., Osaka, Japan) connected to a utility vehicle (Kubota RTV, Kubota Corp.) (Fig. 4). The hydrogel bait was dispensed at an even rate of about 6.1 L of bait per row, throughout the baited zone. The roads between blocks were not treated.

The baited zone was treated four times, occurring between monitoring trips 2 and 3, 5 and 6, 8 and 9, and 11 and 12, on June 25, July 23, August 20, and September 17, respectively. The field was irrigated (micro-sprinkler) the day before applications but not during the bait application to allow the ants to forage on the ground and find the hydrogel baits. The baited zone was treated with a total of approximately 765 kg of bait (not all bait made was used) across all four treatments.

Statistical analysis. Data from the experimental plot (ant numbers monitored) were visualized using a heat map (Babicki et al. 2016). Data were statistically analyzed using R v. 1.4.1717 (RStudio Team, 2021). Two-Way Mixed Design ANOVA was used to test if two zones (baited and control) differed over time in Argentine ant number. The dependent variable was “ant count” repeatedly measured over time. The between-subject factor was “zone”. The within-subject factor was “monitoring trip”. Post hoc comparisons were made using the least squares means (lsmeans) test using the emmeans package (Lenth et al. 2021). The buffer zone was monitored, but not analyzed by the Two-Way Mixed Design ANOVA.

Weather data collection. Weather data from the California Irrigation Management Information System (CIMIS) station 44 was analyzed to compare with monitoring trends.

Results

Immediately after the first application of boric acid hydrogel bait (within 1-2 h) Argentine ants were observed on the ground, actively foraging on the baits (Fig. 5). Treatment of the baited zone took 3-4 hours. There was no evaluation of how long the bait was actively foraged upon. Based on former studies, it could be assumed that the bait was moist enough to be palpable for a few hours (Tay et al. 2017) and may have been rehydrated in subsequent irrigation of the field (McCalla et al. 2018), but likely was no palatable regardless of rehydration beyond a few days. Based on accumulated average ant weight values, a weight of .000519 g was used as a standard across monitoring trips for an average weight of individual ants.

The heat maps were effective in visualizing some trends in ant foraging activity over time. The heat map visualization indicated that all zones started with similar levels of ant activity before the treatment. After the first bait application, the baited zone had a rapid reduction of ant activity. The numbers subsequently rebounded. There was a general downward trend of ant numbers across both baited and control zones after trip 7 (later in the season). However, the control zone experienced a large increase of ant activity around late July and early August while the ant activity in the baited zone remained low. Thereafter, the difference between baited and control zones remained consistent. While the buffer zone was not included in the subsequently statistical analysis, the heat map visualization suggested that the buffer zone showed an intermediate level of ant activity in comparison to the baited and control zones (Fig. 6).

Formal statistical analyses supported the initial visual interpretations based on the heat maps. Before the first baiting (trip 2) the average ant number monitored in baited trees was around 1,058 ants, similar to control tree averages at 1,025 ants (Table 2). After the second baiting, there was a significant difference in the average of number of ants on trees in the baited zone (1,005 ± 143) and the average number of ants on control zone trees (2,129 ± 233) (Table 2). The difference in ant numbers between baited and control zones was maintained throughout subsequent monitoring trips (Table 1). Ants in the baited zone were never eradicated but held at levels that were significantly lower than the control zone between August and October (Fig. 7 and Table 1). A two-way mixed ANOVA indicated that the ant numbers between baited and control zones were significantly different (two-way mixed ANOVA, $F = 63.1$, df = 1, 52; $P < 0.0001$). A significant interaction occurred between zone and monitoring visits, indicating that control and baited zones were significantly different in their ant numbers throughout the monitoring period (two-way mixed ANOVA, $F = 13.37$, df = 15, 780; $P < 0.0001$).

Discussion

The boric acid hydrogel bait was well accepted by Argentine ants in the citrus grove. The hydrogel bait significantly suppressed the Argentine ant activity in the baited zone over the span of 3 months (Table 1, Fig. 7). Even though Argentine ants were still found in the baited zone, the monitoring data clearly indicated that the ant population in the baited zone was substantially suppressed compared to the control zone (e.g., 47-81% reduction between August and October). Starting in late October, there was a downward trend in both the baited and control zones.

This sustainable boric acid hydrogel bait was comparable to Cooper et al. (2019) in that it successfully suppressed the activity of Argentine ants. However, while Cooper et al. (2019) seemingly brought ant numbers to zero for some time, the initial numbers as well as control numbers seemed to be much lower than the numbers in this study. This could be due to several factors, with many differences between studies. Most notably the two studies had different monitoring methods and different field sites, this one's being a citrus grove and Cooper et al. (2019) being a vineyard study. Another major difference between the studies is the polymer used to produce the hydrogel beads. Cooper et al. (2019) used polyacrylamide, whereas this study utilized calcium alginate for hydrogel production.

Immediately after the first treatment, the ant activity level in the baited zone plummeted dramatically (week 1 after treatment), but it quickly recovered by the second post-treatment monitoring. Cooper et al. (2019) also reported a relatively quick (i.e., within 15-20 d) reduction in the Argentine ant activity after the application of boric acid bait incorporated in polyacrylamide hydrogels. However, the quick recovery of ant activity was not observed in that study. A field experiment by Klotz et al. (1998) using a liquid boric acid bait showed a substantial increase of ant foraging activity after a brief period of apparent reduction in ant activity at the beginning of baiting. However, this trend was also observed in the control areas where boric acid bait was not applied (about 30 m away from the closest boric acid bait station) (Klotz et al. 1998). The unique pattern in ant foraging activity observed in the early part of baiting may be due to several factors, including quick impact on the existing populations, and repopulation and recovery by incoming ants from the buffer zone. Perhaps another factor is larger-scale clumping behavior seen in the chapter 2 laboratory studies. For example, large portion of foragers that are exposed to small amounts of boric acid may start the clumping behavior toward a high moisture source (e.g., within nests) while reducing (or even completely halting) their foraging activity only for a relatively short amount of time. If they recover, these ants will be able to start foraging again, quickly providing the overall recovery in their foraging activity level. It would be interesting to see if there are similar results in treating the entirety of a locally isolated colony of Argentine ants. A discrepancy with this idea is the lack of similar plunges and rebounds in ant activity with subsequent treatments. Perhaps with already reduced foraging, the impact of a bait was also reduced. No definitive answer can be determined without additional research.

Based on the post-treatment monitoring immediately after the first bait application, both control and baited zones had a reduction in ant numbers compared to their pre-treatment data (Fig. 6). Even though the buffer data are not plotted in Fig. 7, it also showed a similar change. While the boric acid hydrogel bait was applied only in the baited zone, all three zones were located within the same citrus grove. Seemingly all three zones were impacted by the bait, to varying degrees, despite attempts at utilizing the buffer zone to prevent the impact on the control zone. There are several possible explanations for slight reduction of ant activity in the control zone alongside the buffer and baited zones. The buffer zone is within the accepted foraging ranges of Argentine ants from the baited zone. Close boundary of the buffer zone was only 6 m from the boundary of the baited zone, and the entire buffer zone was within 124 m from the close boundary of the baited zone. Estimated foraging ranges of Argentine ants vary between experiments, likely due to abiotic factors such as foliage, moisture, and temperature (Burford et al. 2018, Clifton et al. 2020). In vineyards, for example, an estimated foraging distance was around 36 m (Hogg et al. 2018). In a wooded plot, foraging distances were closer to 50 m (Heller et al. 2008). We could not exclude the possibility that the bait applied in the baited zone in our experiments reached deeper into the buffer zone via extended trophallaxis and linkages made between nests. However, it is unlikely that the ants in the control zone also got impacted by the baits applied in the baited zone with over 127 m of separation. It is also possible that as Argentine ants in a super colony are believed to move freely among nests (Markin 1968), as nests in the baited zone become low in number, they are refilled by ants from the buffer zone, and a similar process occurs between the buffer and control zones, causing a sort of diffusion of ants across the field from high concentrations to low. Genetic analysis concurrently performed by other researchers with this study found that the ants sampled from the field were genetically indistinguishable, belonging to a single super colony (personal communication, S.-P. Tseng and C.-Y. Lee). At present, the exact reason for the seemingly simultaneous reduction in ant activity for control and baited zones after the first baiting is not known.

Boric acid hydrogel bait is designed to be efficacious in Argentine ant control and environmentally friendly. In the weeks after final treatments little to no gel was seen remaining on the ground. Occasionally, dried remains of hydrogel baits were found on ground surface a few days after the bait application, but even those disappeared over time. This is in stark contrast to the bait stations that would need to be cleaned and maintained after baiting (Daane et al. 2008). In terms of boric acid exposure in the soil, the entire 2 ha was treated with approximately 7.564kg of boric acid in the bait. There was additional conditioning solution in each treatment bucket, coming out to a rough total of 11.41 kg of boric acid across all treatments. Boron being the element of concern in boric acid, is 17.48% of boric acid by mass. Therefore, 1.97kg of boron was applied to the field. The average amount of boron in US soil is 33mg of boron per kg of soil, with a range of 30-300 mg/kg (Harper et al. 2012). While the beads were applied to the surface of the field, watering and the solubility of boric acid would likely cause the boric acid to seep deeper into the soil. At 16 cm of depth (topsoil), an acre has roughly 907,000 kg of soil. For 5 ac, that is 4.535 million kg of soil. That means around 150 kg of boron may already be present in the topsoil of the treated area. This treatment added a minimal amount of an already present chemical to the soil, increasing a little over 1% in concentration.

One of the most interesting aspects of the results is the rapid uptick in control numbers at the beginning of August, which made control and baited zones clearly separated in terms of Argentine ant activity (Fig. 7). In southern California, August-September has been a typical timing when Argentine ant activity levels reaches its peak in the citrus groves (Markin 1970, Rust et al. 2000). Gradual increase of ant activity between April and August has been previously reported (Markin 1970, Rust et al. 2000). However, the increase of ant activity in our control plot was somewhat dramatic between the end of July and the beginning of August. Even though the exact cause of this rapid uptick cannot be determined, a potential explanation could be a change in the monitoring duration (i.e., the length of time from the placement of the monitors on the tree branches and their pickup). Until July 30 (monitoring trip 6), the monitoring squares were left on the tree for 2 h until pickup. Due to increasing numbers of ants drinking the cotton squares towards the end of July (especially for the control zone), the cotton squares were getting dry upon their retrieval. Our visual observation of foraging ants on trees and average temperature data for the monitoring days clearly indicate that the higher foraging activity was the major reason for dryer cotton square monitors, not the higher amount of evaporative loss due to increased temperature (Fig. 8). If the cotton squares were getting dry, fewer foraging ants would stay on them upon pickup. Accordingly, the amount of time monitoring squares was left out on the tree was reduced to 1 h for August 6 (monitoring trip 7) and beyond. For these reasons, it is possible that some of the monitoring data in July (e.g., monitoring trips 4, 5, and 6) may have slightly underestimated the ant foraging activity in the control plot.

After the initial uptick in control zone ants, there is a general trend across the field of decreasing ant numbers, likely due to seasonal trends seen in past experiments. The two trends in foraging that could be affecting our monitoring numbers are changes in daytime temperatures and changes in food preference due to colony demands (Rust et al.

2000). Like many other ectotherms, Argentine ants' metabolism and activity, are affected by environmental temperatures. This causes them to be slower and less active as temperatures drop, ultimately lowering the number of ants monitored in a given period (Markin 1970). As holometabolous insects, Argentine ants have various life stages with particular nutritional requirements, at times demanding carbohydrates, and at other times proteins (Abril et al. 2007). If it were the case that there was a higher proportion of queens and larvae in the nests later in the season, then there would be a shift in foraging activity emphasizing protein sources and neglecting our carbohydrate-based monitoring methods.

While the difference between control and baited zones is statistically significant, it does not directly answer whether the reduction in Argentine ant activity is substantial enough to allow proper level of biological control of the honeydew producers. Little to no information is available on the levels of Argentine ant control that would be necessary to prevent their damage through tending the honeydew producers. Daane et al. (2007) demonstrated how the presence of Argentine ants drastically influences honeydew producer populations. However, exact dynamics of the relationship between hemipteran pests and Argentine ant populations are not well understood. More research would be necessary to determine the economic threshold of Argentine ants and reliable method to quantify them; whether by monitoring the honeydew producer populations or by evaluating crop quality/damage with concurrent treatment of Argentine ants (Silverman and Brightwell 2008).

Table 1. Least square means (LSMEANS) test for the average ant numbers between control and baited zones. Instances of statistical significance are shown in bold. SEM means standard error of means.

Trip - Date	Difference (mean ± SEM)	*t*-ratio	df	*P*
1 – Jun-15	-224.5 ± 138	-1.627	745	0.1041
2 – Jun-18	-32.5 ± 138	-0.236	745	0.8137
Bait application #1 (June 25)				
3 – Jul-2	**442.7 ± 138**	**3.209**	**745**	**0.0014**
4 – Jul-9	-131.6 ± 138	-0.955	745	0.3397
5 – Jul-16	103.9 ± 138	0.753	745	0.4516
Bait application #2 (July 23)				
6 – Jul-30	-122.5 ± 138	-0.888	745	0.3748
7 – Aug-6	**1124.8 ± 138**	**8.153**	**745**	**<0.0001**
8 – Aug-13	**1193.8 ± 138**	**8.653**	**745**	**<0.0001**
Bait application #3 (August 20)				
9 – Aug-27	**992.9 ± 138**	**7.197**	**745**	**<0.0001**
10 – Sept-6	**1038.8 ± 138**	**7.529**	**745**	**<0.0001**
11 – Sept-10	**438.7 ± 138**	**3.180**	**745**	**0.0015**
Bait application #4 (September 17)				
12 – Sept-24	**853.8 ± 138**	**6.196**	**745**	**<0.0001**
13 – Oct-1	**378.9 ± 138**	**2.747**	**745**	**0.0062**
14 – Oct-29	**328.9 ± 138**	**2.384**	**745**	**0.0174**
15 – Dec-3	90.7 ± 138	0.658	745	0.5109
16 – Jan-21	202.2 ± 138	1.466	745	0.1431

Table 2. Number (mean ± SEM) of Argentine ants per monitor for control and baited zones. Percent reduction value was calculated by [(control zone average) – (baited zone average)] / control zone average. Negative % reduction values represent when the baited zone has greater numbers than the control zone and positive % reduction values represent when the baited zone has lower numbers than the control zone.

Trip - Date	Control zone	Baited zone	% Reduction
1 – Jun-15	654 ± 129	878 ± 109	-34.3
2 – Jun-18	1,025 ± 98	1,058 ± 138	-3.2
Bait application #1 (June 25)			
3 – Jul-2	551 ± 63	108 ± 22	80.4
4 – Jul-9	595 ± 82	727 ± 87	-22.1
5 – Jul-16	814 ± 85	710 ± 82	12.8
Bait application #2 (July 23)			
6 – Jul-30	849 ± 114	971 ± 101	-14.4
7 – Aug-6	2,130 ± 233	1,005 ± 143	52.8
8 – Aug-13	1,845 ± 206	651 ± 106	64.7
Bait application #3 (August 20)			
9 – Aug-27	1,450 ± 134	457 ± 76	68.5
10 – Sept-6	1,370 ± 90	331 ± 32	75.9
11 – Sept-10	938 ± 87	499 ± 46	46.8
Bait application #4 (September 17)			
12 – Sept-24	1,052 ± 78	197 ± 35	81.3
13 – Oct-1	672 ± 49	293 ± 48	56.4
14 – Oct-29	580 ± 55	251 ± 39	56.7
15 – Dec-3	293 ± 34	202 ± 31	31.0
16 – Jan-21	226 ± 43	24 ± 6	89.3

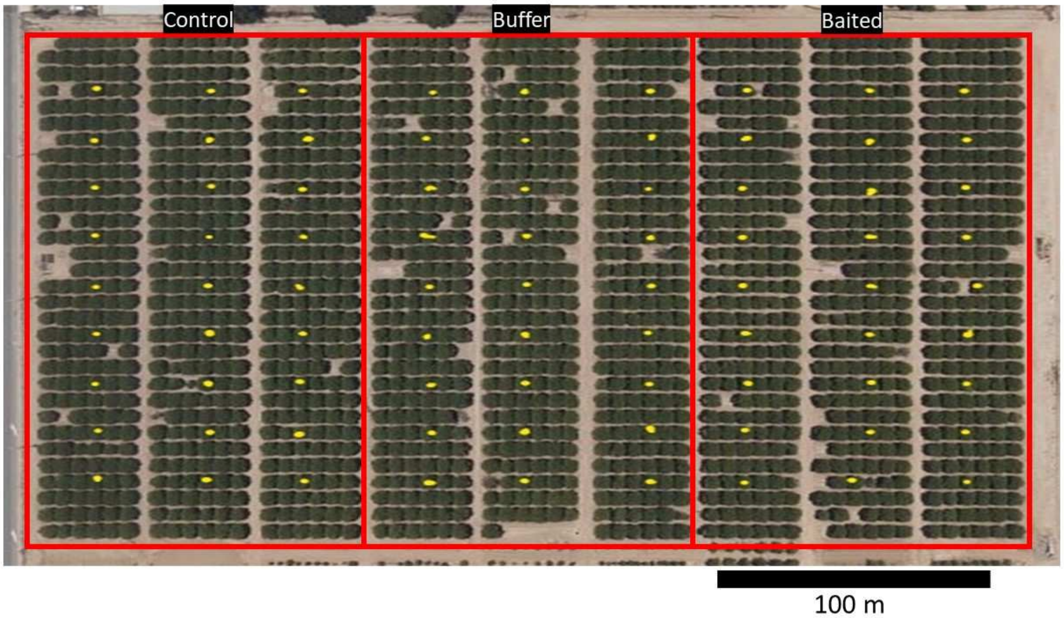


Fig. 1. Field site for the experiment. Each "block" is 6 trees wide and 31 trees long. Left to right, the first three blocks were the control zone, the middle three blocks were the buffer zone, and the final three blocks were the baited zone. The yellow dots indicate trees that were monitored for ant activity.

Fig. 2. Cotton squares used for monitoring Argentine ant foragers on trees. (a) A tree in the baited zone. (b) A tree in the control zone

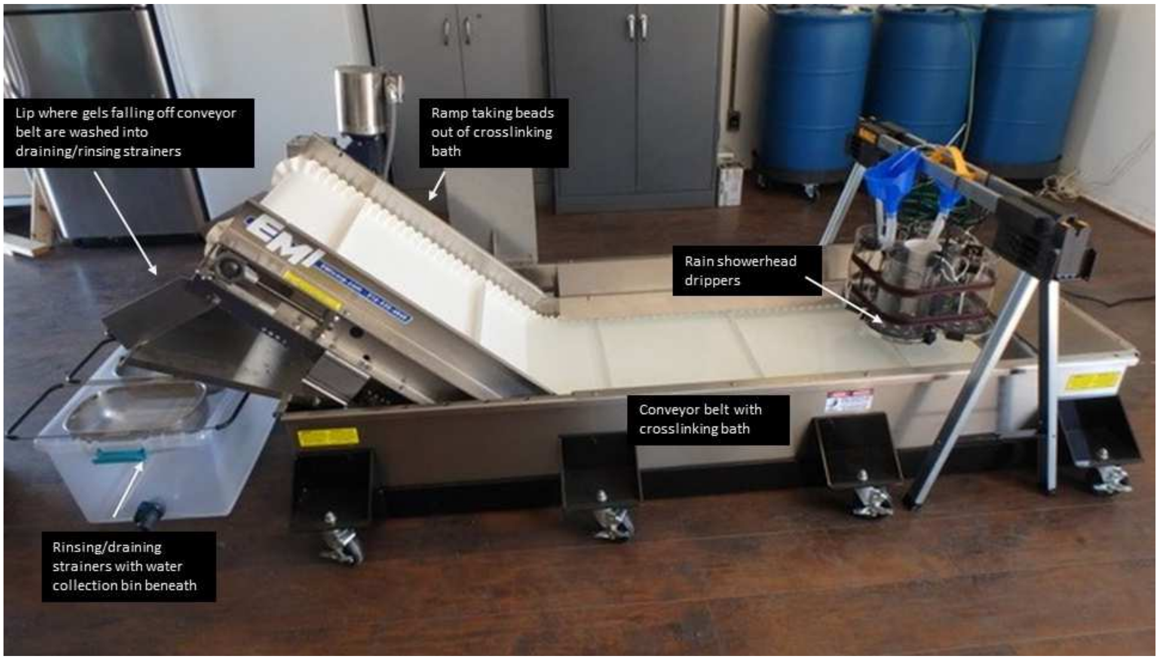
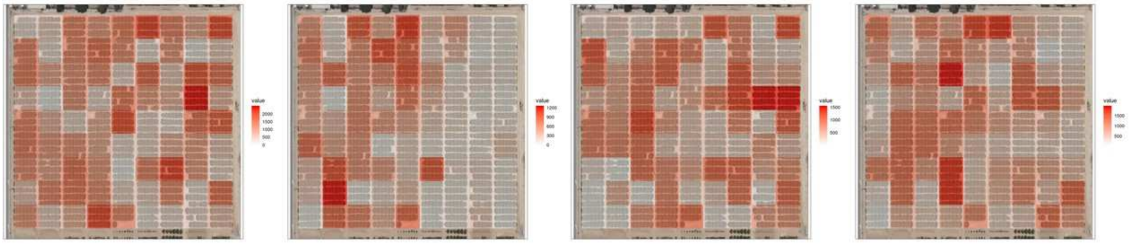


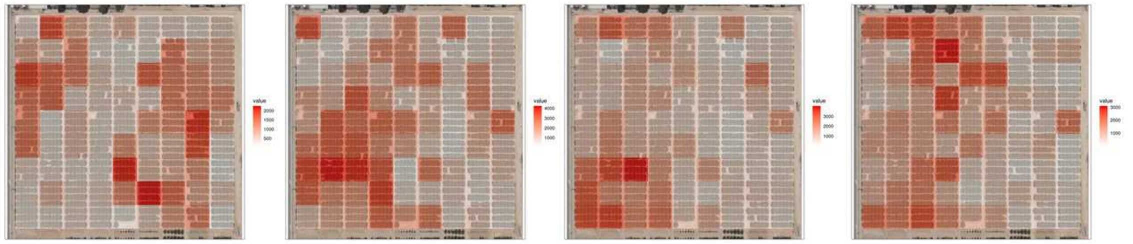
Fig. 3. The conveyor belt setup for hydrogel bead production.

Fig. 4. A utility vehicle with a spreader attached for bait application at the field site.

Fig. 5. Argentine ants feeding on hydrogel bait soon after application.



Jun. 18 (pre-baiting 1) Jul. (1 wk post-baiting 1) Jul. (2 wk post-baiting 1) Jul. 16 (3 wk post-baiting 1)



Jul. 30 (1 wk post-baiting 2) Aug. 6 (2 wk post-baiting 2) Aug. 13 (3 wk post-baiting 2) Aug. 27 (1 wk post-baiting 3)

Fig. 6. Heat maps of test site, the gradient of red to white implies higher numbers to lower numbers of ants monitored, respectively. Gradients across heat maps are not identical, each map has a maximum value which varies between monitoring trips.

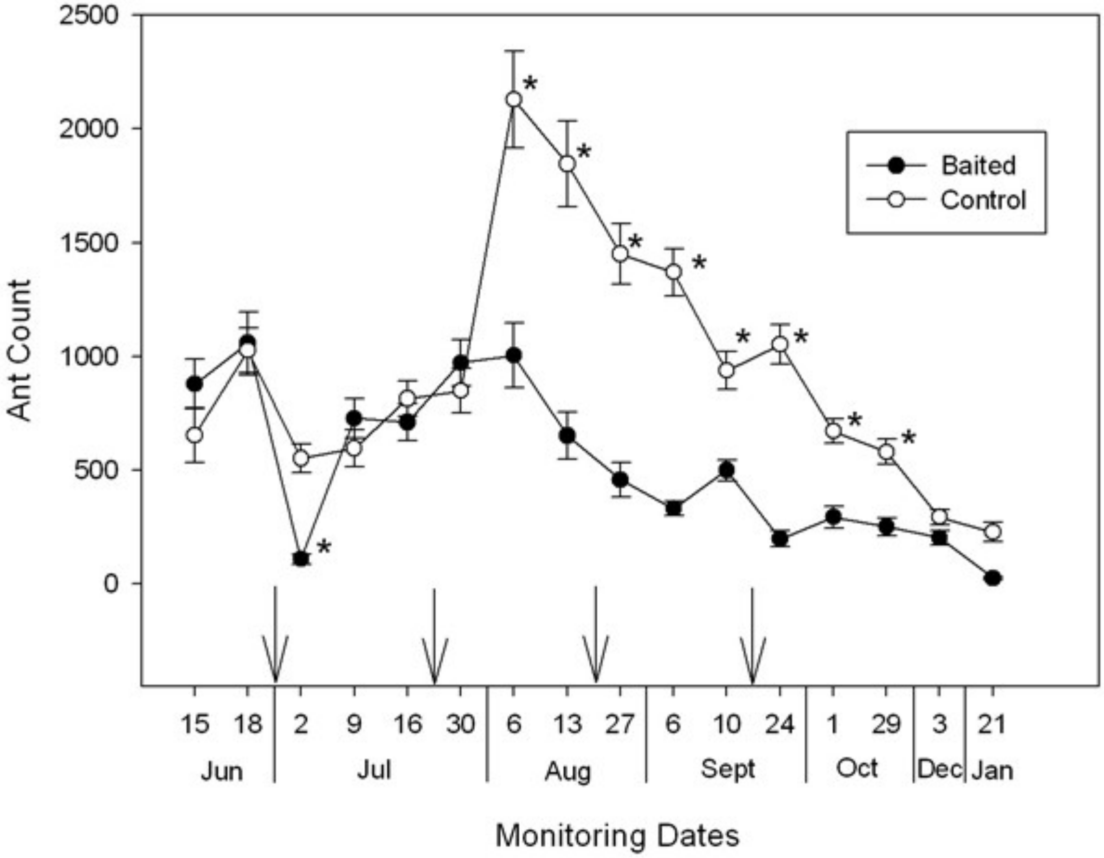


Fig. 7. Average number of ants per monitor for each monitoring period. Error bars represent the standard error of means (SEM). Arrows indicate the timing of four bait applications. Asterisks denote significant difference between control zone and baited zone data (Two-Way Mixed Design ANOVA; $\alpha = 0.05$)

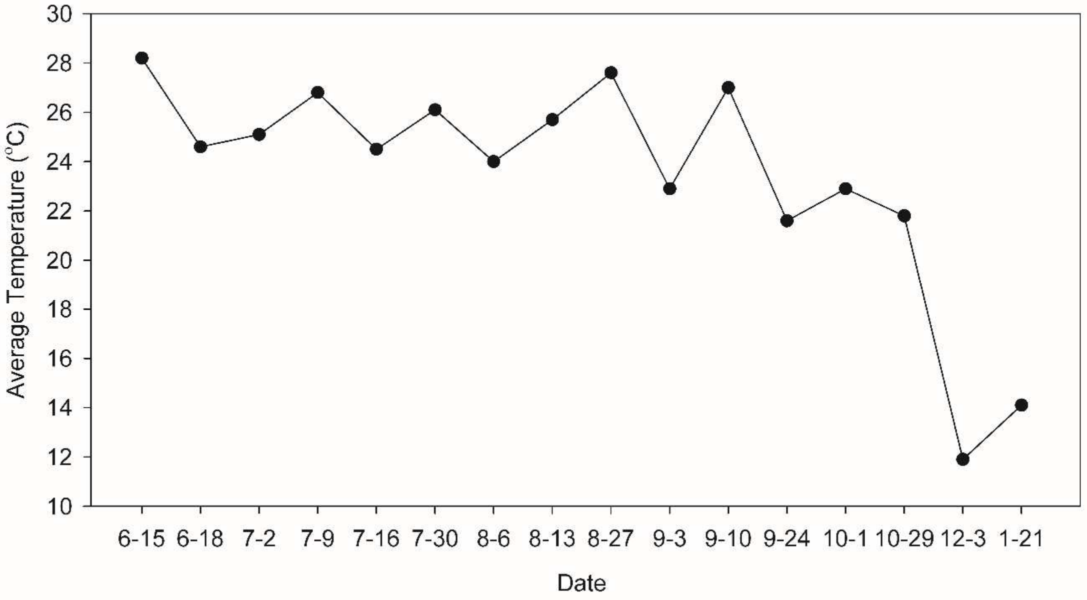


Fig. 8. Average temperatures on days when monitoring took place. Temperature recorded by UCR Agricultural Operation weather station [California Irrigation Management Information System (CIMIS) station 44].

Chapter IV. Conclusions

Both laboratory and field studies demonstrated that boric acid hydrogel bait effectively controls Argentine ants. One percent boric acid incorporated in alginate hydrogel bait caused 82% mortality in laboratory colonies. Field application of the same bait at a rate of 94 L/ha provided up to an 80% reduction of ant foraging activity on citrus trees compared to the untreated control zone. Findings from these experiments suggested that complete colony elimination was never achieved in the field. Still, the worker population (or its foraging activity) was significantly reduced and suppressed by the bait application. Testing with the aged bait showed that long-term storage of the boric acid hydrogel bait negatively impacted its insecticidal efficacy.

Further research is necessary to determine whether the reduced insecticidal potency of the aged baits was due to chemical reactions in the bait, loss of palatability, or some other unknown factors. In any case, the current finding suggests that the bait (as it is presently formulated) should be used fresh rather than allowed to age. Laboratory behavioral assays successfully demonstrated that treatment with boric acid bait caused the ants to be more likely to form dense aggregations (i.e., clumping) within their colony before significant mortality started to occur. Even though definitive explanations remain to be found for this behavioral impact of boric acid bait on Argentine ants, documentation of this change might be a step forward in understanding the impact of boric acid on insects.

Similarly, total colony mortality was never achieved in laboratory and field assessments. In the case of the field study, while trophallaxis does allow the bait to be transferred to non-foraging members of the colony, such as queens and brood, bait does not become the sole food for the colony. Alternative food sources likely dilute the bait distributed to colony members who did not directly feed on the bait. As a result, not all members will die, and the colony will be able to continue functioning even after the initial exposure to the bait toxicant. This differs from the laboratory study, where for 14 d, their only food source is the provided bait. It is likely that if allowed to run for an extended period, the laboratory colonies would reach total mortality, as seen in (Klotz et al. 1996). There seems to have been a trend post-baiting in the field study where ant numbers would drop for 1-2 wk, only to jump back up afterward. In the laboratory study, two weeks was how long the investigation was allowed to run, but test colonies seemed to be rebounding in the final days. While no quantitative data was taken for this, workers had cleared dead ants out of the colony tubing and were usually moving about the cup arena. This is, in contrast, to immediately after baiting when ants would stay condensed in the tubing, and ants that did walk about the arena would fall over, walk in circles, and generally seemed in poor health. Perhaps this is the general timeline for the Argentine ants exposed to a non-lethal dose of the boric acid bait to recover and contributes in part to rebounds of ant populations in the field trials. Still, some papers show a good deal of connectivity between nests less than 50m apart in field colonies (Heller et al. 2008), making spillover and intracolonial immigration from nearby nests also possible explanations for the rebound.

Not including labor, the cost of boric acid hydrogel bait with the pheromone adjuvant was estimated to be $1.1 per liter (Choe et al. 2021). With 6.1 L used per row, and 31 rows in this experiment, the cost per treatment is approximately $190 for 2 ha, labor not included. The usage of chlorpyrifos sprays for ground coverage and ant activity suppression in citrus is 7.4 kg per ha (Grafton-Cardwell et al. 2017). At $4.19 per kg (Chlorpyrifos 15G by Drexel) ("Agricultural Chemical Solutions, Inc. - Pesticide Prices" 2022), it would cost about $63 to treat 2 ha of citrus. Thus, the boric acid hydrogel bait option is three times more expensive than the chlorpyrifos sprays under current material costs. The bait may never outcompete general insecticide treatments, in this case a granular application, in terms of price. As regulatory changes prohibit the use of chlorpyrifos, a reduced impact on the environment, beneficial insects, and people, may be deemed an acceptable trade-off.

The degree of contribution of boron to the soil by boric acid hydrogel bait is not a cause for concern, introducing 1.97 kg of boron to the estimated 150 kg of boron already present in the topsoil of the baited zone. Similarly, the other ingredients included in the bait are likely not a threat to the agricultural crop, environment, or people. Sucrose, also known as table sugar, is a simple disaccharide of glucose and fructose. There is no environmental concern with putting sucrose into soil or waterways. Calcium alginate is made from algae, designed to be biodegradable, and could even serve as food for the microbiota of field soil (Roy et al. 2014). Finally, potassium sorbate, the preservative used in the bait, is considered a food-safe additive (Mohammadzadeh-Aghdash et al. 2018). It is a naturally occurring but often synthetically manufactured potassium salt with an unsaturated fatty acid (sorbic acid) (PubChem 2022b). Potassium sorbate has fungicidal activity, making it useful for mold prevention (Mutasa et al. 1990). Potassium sorbate was assessed for risks by the European Union as readily biodegradable indicating safe use in the environment (European Union, Germany 2015). At low levels, potassium sorbate is deemed to be safe for human consumption (Mohammadzadeh-Aghdash et al. 2018). As the baits are not directly sprayed or placed on the fruits of the citrus trees, it is unlikely that a meaningful amount of potassium sorbate is allowed to gather on them. Therefore, potassium sorbate likely has little to no food safety impact when used as a bait preservative.

A major question is whether the level of Argentine ant reductions achieved in the current study would be sufficient to increase the susceptibility of hemipteran honeydew producers to their natural enemies. This field study baited four times throughout a summer season when Argentine ants are most active (Markin 1970). This was done to see if eradication could ever be achieved. Still, if suppression is all that is possible, it would be valuable to know the minimal treatment necessary to achieve this level (up to 80%) of suppression. Understanding acceptable management levels of Argentine ants will inform future tests of how often and when baiting should take place. Unfortunately, the citrus system has established no economic injury level/threshold for Argentine ants. Various reports have shown that the presence of aggressive ants, such as Argentine ants, typically increases the populations of these damaging hemipterans (Buckley and Gullan 1991, Daane et al. 2007, Milosavljević et al. 2021). Daane et al. (2008) concluded that incomplete Argentine ant suppression could suppress mealybug densities in vineyard systems based on multi-year field project data. Daane et al. (2008) also speculated that the effect of Argentine ant control (or suppression) might be reflected in the hemipteran population with some delays. While these reports are helpful, the remaining question is how much reduction in Argentine ant activity is sufficient to help with hemipteran population levels in citrus. Whether by evaluating pest levels or crop damage, establishing economic injury levels for Argentine ants would be an important step for developing ant IPM practices.

Using yellow sticky traps, additional monitoring was done in the field site to examine insect species in the canopy (about 150 cm height from ground), including Asian citrus psyllid (ACP) (personal communication, R. Pandey and G. Simmons). This trapping was not a part of this project, and it was independently conducted by an entomologist from Citrus Research Board (Riverside, CA). A series of surveys (4 surveys covering 8 weeks between September 17 to November 12) did find that numbers of ACP and Argentine ants caught on sticky traps were consistently lower in the baited zone than in the control zone, closely resembling our ant monitoring data.

There are many research projects that could stem from these findings. Testing the bait on different crops and ornamentals could be of interest and expand the scope of the boric acid hydrogel bait. The production of the bait itself could be examined. If unconditioned hydrogel beads could be dehydrated for storage and then rehydrated with conditioning solution, the shelf life could be extended. Despite the many merits of boric acid as an active ingredient, further experimentation with other active ingredients (e.g., Spinosad) could be of some value, as it is possible that the other active ingredients might be more efficient in achieving a sufficient level of control based on a calculated economic injury level of the Argentine ant.

In conclusion, boric acid hydrogel bait is a promising method of controlling Argentine ant populations. The environmental friendliness of boric acid hydrogel bait makes it a valuable tool in modern pest ant management in agricultural settings. With success in preliminary urban and agricultural field studies, further testing is warranted. While boric acid can be considered a safe yet effective insecticide, it is important to fully understand the chemicals we put into the environment. With that, the behavioral and physiological effects of boric acid on target insects and its modes of action should be further explored. Suppose it is found that treatment with boric acid hydrogel bait sufficiently suppresses Argentine ants to allow biological control of hemipteran pests. In that case, its adoption for Argentine ant control should be widespread.

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