Evaluation of water-storing granules as a promising new baiting tool for the control of invasive ants in Hawaii.

Report of Year 1 Activities to the Hawaii Invasive Species Council

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INTRODUCTION

An absence of practical methods for controlling invasive ants that are primarily attracted to sweet liquid foods has been a persistent problem. Sugar-loving species are typically best controlled with sugar water-based baits dispensed in bait stations. In natural or agricultural landscape settings, deployment of numerous bait stations quickly becomes extremely laborious, costly, and in most cases prohibitive. Unfortunately, several of the most destructive established ant species in Hawaii belong to this sugar-loving group, including species that invade and impact natural areas, like the Argentine ant, yellow crazy ant and glaber ant, as well as urban and agricultural pests like white-footed ants and the odorous house ant. In addition, some worrisome new threats, like the tawny crazy ant currently invading the US mainland, belong to this group.

A recent advance has employed polyacrylamide crystals, or hydrogels, to convert liquid baits into an easily dispersed granular form (Buczkowski et al. 2014a,b; Boser et al. 2017). These hydrogels, used as soil amendments in horticultural and forestry applications, absorb many times their weight in water and then slowly release it as they dry. They also absorb water containing dissolved sugar and pesticides, which ants can imbibe directly from the dispersed granules. This approach is being used experimentally in attempts to eradicate Argentine ants in the California Channel Islands and yellow crazy ants at Johnston Atoll and Australia (Boser et al. 2017; Peck et al. 2017; B. Hoffmann, CSIRO Australia, pers. comm.). Textured vegetable protein (TVP) also has water-absorbing properties, but has the advantage of being biodegradable, and showed promising results in initial testing at Johnston Atoll (Peck et al. 2016, 2017). Another biodegradable water-absorbing medium based on alginate was recently developed at UC Riverside (Tay et al. 2017). These media, which I refer to collectively as water-storing granules (WSG), represent a highly promising new tool for invasive ant control in Hawaii.

However, no commercial pesticides are yet labelled for this use pattern, and a variety of questions need to be addressed to develop this as a usable approach in Hawaii. This project is conducting a series of studies to investigate some of the initial questions concerning the use of WSG as a new ant control tool, pertaining both to aspects of their effectiveness and their non-target risks. In the first year of the project, work has focused on several topics: 1) drying rate of the three WSG types under investigation (polyacrylamide, TVP, and alginate beads), which influences duration of bait attractiveness, 2) bait preference among the three WSG types for three target ant species (yellow crazy ant, Argentine ant, and little fire ant), 3) repellency of three pesticides under investigation (thiamethoxam, dinotefuran, and indoxacarb) when formulated in WSG to the three target ant species, 4) efficacy of the most promising bait and pesticide formulations for controlling Argentine ants and yellow crazy ants, and 5) non-target species attraction to WSG baits, focusing on pollinating insects and ground-foraging birds. Additional efficacy testing and non-target risk analysis is planned for year 2 of the project.

MATERIALS AND METHODS

Target ant species and study sites

As mentioned above, work on this project is focusing on three highly invasive and problematic ants species: the yellow crazy ant (YCA, *Anoplolepis gracilipes*), the Argentine ant (AA, *Linepithema humile*), and the little fire ant (LFA, *Wasmannia auropunctata*). Studies involving YCA took place at disturbed lowland grassland and shrubland at James Campbell National Wildlife Refuge (JCNWR) and adjacent county property, Oahu. Studies involving AA took place in native subalpine shrubland at Haleakala National Park (HALE), Maui. Studies involving LFA took place at several rural residential properties in the Puna District, Hawaii Island. Non-target attraction studies were conducted at some of the same sites, as well as in native coastal strand communities at Kaiwi State Scenic Shoreline (KSSS), Oahu, and Kaena Point Natural Area Reserve (KPNAR), Oahu.

Preparation of WSG formulations

The three WSG types were used to deliver a 25% sucrose solution as the bait attractant. All unspecified references to sucrose solutions in this report refer to solutions made with table sugar in tap water at a concentration of 25% (w/vol). For repellency and efficacy trials, pesticide active ingredients (AI) were mixed into the sucrose solution at the stated concentrations (w/vol) prior to absorption with WSG. WSG were allowed to absorb bait solutions for approximately 24 hours prior to use.

Miracle-Gro® Water Storing Crystals were used for the polyacrylamide WSG, at a rate of 20 g per L of bait. Bob's Red Mill® Textured Vegetable Protein was used for the TVP WSG, at a rate of 350 g per L of bait. Alginate bead WSG were manufactured for the study following the protocol developed by Tay et al. (2017). Alginate beads were mass-produced by allowing a 10 g/L sodium alginate solution (Na-Alg, Sigma-Aldrich, CAS 9005-38-3, in distilled water) to gravity drip from a 100-nozzle shower head into a 5 g/L calcium chloride solution ($CaCl_2$, Sigma-Aldrich, CAS 10043-52-4, in distilled water). Beads were allowed to cross-link in the calcium chloride solution for approximately five minutes, after which they were rinsed with distilled water, producing beads that were >98% water by weight. Finished beads were then conditioned in a bait solution for approximately 24 hours to produce the WSG for the various trials. During the conditioning period, solutes (sugar and AI, if applicable) equilibrated between the water within the beads and the conditioning solution as the beads absorbed more liquid, increasing in weight by approximately 30% in the case of mass-produced beads. The conditioning solution was typically formulated with concentrations of solutes that were twice the target concentrations obtained after equilibration. Equilibration was confirmed by measuring the final sucrose concentration of the conditioning solution with a hand-held refractometer (Eclipse model 45-03, Bellingham + Stanley Ltd.). After equilibration, excess conditioning solution was drained prior to use of the alginate WSG.

WSG drying rates

Prior work has found that WSG formulated with sucrose become less attractive to ants once approximately 50% of the water in the granules has evaporated (Rust et al. 2015, Tay et al. 2017), which can in turn reduce their efficacy (Buczkowski et al. 2014a). To estimate the rate of water loss of the three WSG types, drying trials were conducted on the roof of Gilmore Hall on the University of Hawaii campus in Honolulu (49 m elevation) to approximate lowland natural areas, and at 2070 m elevation at HALE to represent high elevation natural areas. One trial each was conducted in full sun and in full shade on the roof of Gilmore Hall. A single HALE trial was initiated in full sun, but intermittent low clouds occurred during later portions of the trial. For each trial, 10 individual granules of each of the three WSG types formulated with sucrose solution were randomly assigned to an array of 30 petri dishes (6 cm diameter). For polyacrylamide and TVP, an attempt was made to select 10 granules that spanned the majority of the range of granule sizes observed in a sample of granules; alginate beads were much more uniform in size, so 10 granules were selected haphazardly. Dishes were weighed at the start of the trial, and then approximately every hour for 5 hours after the array was placed outdoors. The low elevation, full sun trial was started at 9:41 am on 6/21/18, and the low elevation full shade trial was started at 10:23 am on 6/22/18. The high elevation trial was started at 9:26 am on 8/11/18. Air temperature and relative humidity was measured hourly for the two low elevation trials using a sensor (HOBO UX100-001, Onset Computer Corp.) placed in the shade next to the dish array. Air temperature and relative humidity was logged every three minutes during the high elevation trial, using a sensor mounted within a radiation shield (HOBO U23-002, Onset Computer Corp.) and placed next to the dish array. Final dry weights of granules were calculated by letting them air dry in the lab for at least 1 week after the trial. Percent water loss was subsequently calculated for each granule at each hourly measurement interval.

The time to reach 50% water loss (T50) was estimated for each granule from the slope of the line joining the two successive hourly measurements that spanned this percentage. T50 was then regressed against the initial saturated weight for each granule (natural log transformed) to determine the relationship between granule size and T50 for each WSG type. To estimate typical T50 values for each WSG type under each of the three trial scenarios, the regression relationships were applied to 50 individually weighed granules of each WSG type formulated with sucrose solution. The 50 granules were the first 50 encountered within approximately 15 g batches of formulated WSG, and were therefore haphazardly selected. Median estimated T50 values were compared among WSG types with box plots.

Bait preference among WSG

For each of the three target ant species, a combination of choice trials and no-choice trials were conducted to test the relative attraction to the three WSG formulated with 25% sucrose solution (no AI). In choice trials, 20 replicate stations were established along transects at each site, with stations separated by approximately 5 m or more. At each station, the three WSG were offered side by side on laminated cards (4.5 x 3.5 cm) placed on the ground (Fig. 1). Relative positions of the baits to one another at each station were assigned haphazardly. Baits were photographed at 30 min, 60 min, 120 min and 180 min after placement, and numbers of ants at each were subsequently counted in the digital images. In no-choice trials, 60 stations were established along multiple transects at each site, with stations separated by approximately 5 m or more. Each station received only one of the three WSG types, with each WSG type being randomly assigned to 20 of the 60 stations. Baits were offered on laminated cards (4.5 x 3.5 cm) placed on the ground, were photographed at 30 min, 60 min, 120 min and 180 min after placement. Bait preference tests for YCA were conducted at JCNWR on 5/8/18 and 5/15/18. Bait preference tests for AA were conducted at HALE on 6/28/18 and 6/29/18. Bait preference tests for LFA were conducted at a residence in Nanawale Estates, Puna, Hawaii Island on 6/19/18 and 6/21/18. Numbers of ants were compared among the three WSG types at each time interval using generalized linear models fit with the log link function and negative binomial distribution. Prior to analysis, data were excluded for stations in which one or more of the baits were blown away by wind or removed by rodents or chickens. For two time intervals at the HALE site where nearly all ant counts were zero, data were not analyzed statistically.

Figure 1. Example station from choice bait preference trial with AA. Top left card contains polyacrylamide, top right contains TVP, bottom contains alginate beads.

Pesticide repellency trials

Repellency towards different concentrations of the three active ingredients (AI) being tested was assessed with choice trials for each of the three target ant species. For each ant species, trials for the three AI were run sequentially on the same day for a given WSG type. In each trial, three concentrations of the AI (w/vol) formulated in 25% sucrose solution were compared with a control (sucrose solution only), at each of 20 replicate stations. Stations were established along transects at each site, and were separated by approximately 5 m or more. The concentrations of AI tested were 0.25%, 0.05%, 0.005%, and 0% (control) for indoxacarb and dinotefuran, and were 0.025%, 0.005%, 0.0005%, and 0% (control) for thiamethoxam. At each station, the four baits were offered side by side on laminated cards (4.5 x 3.5 cm) placed on the ground (Fig. 2). Relative positions of the baits to one another at each station were assigned randomly. Baits were photographed at 30 and 60 min after placement, and numbers of ants at each were subsequently counted in the digital images. Repellency trials were conducted for YCA on county land adjacent to JCNWR on 7/24/18, 7/26/18 and 7/27/18. Trials were conducted for AA at HALE on 7/31/18, 8/1/18, and 8/2/18. Trials were conducted for LFA at several sites in Puna, Hawaii Island, on 8/16/18 and 10/1/18. Because attractiveness of the WSG typically decreases with time as the granules dry (see Bait preference results) irrespective of any repellency to AIs, the repellency analysis used ants counts from only the higher of the two station counts (30 and 60 minutes post placement): the time interval with the higher total across all four cards at each station was used, to account for possible differences in discovery time and recruitment rate to different stations. Numbers of ants were compared among the three concentrations of AI and control for each AI and WSG type using generalized linear models fit with the log link function and negative binomial distribution. Prior to analysis, data were excluded for stations in which one or more of the baits were blown away by wind or removed by rodents or chickens.

Figure 2. Example station from trial testing repellency of indoxacarb formulated in alginate beads to AA. Card 9 is the control (sucrose solution only), card 10 is 0.25% indoxacarb, card 11 is 0.005% indoxacarb, and card 12 is 0.05% indoxacarb.

Efficacy testing

Nine WSG formulations were selected for the initial round of efficacy screening for AA, based on the results of the bait preference and pesticide repellency trials. These were 0.0005% thiamethoxam (w/vol) formulated in each of the three WSG types, 0.005% indoxacarb (w/vol) formulated in each of the three WSG types, and 0.05% indoxacarb (w/vol) formulated in each of the three WSG types. Each of the nine formulations was tested in one 25 x 25 m plot at HALE. In each plot, WSG were broadcast by hand at a rate of 55 L of absorbed sucrose bait (with AI) per ha, which is similar to rates found to be effective in prior studies using the WSG approach

(Rust et al. 2015; Peck et al. 2016, 2017; Boser et al. 2017). Each plot was treated twice, first on 10/31/18 and again on 11/13/18.

Twelve WSG formulations were selected for the initial round of efficacy screening for YCA, based on the results of the bait preference and pesticide repellency trials. These were 0.005% dinotefuran (w/vol) formulated in each of the three WSG types, 0.05% dinotefuran (w/vol) formulated in each of the three WSG types, 0.005% indoxacarb (w/vol) formulated in each of the three WSG types, and 0.05% indoxacarb (w/vol) formulated in each of the three WSG types. Each of the 12 formulations was tested in one 25 x 25 m plot at JC. In each plot, WSG were broadcast by hand at a rate of 55 L of absorbed sucrose bait (with AI) per ha. The nine plots testing formulations using 0.005% dinotefuran, 0.05% dinotefuran, and 0.005% indoxacarb were treated once on 6/14/19. The three plots testing 0.05% indoxacarb were also treated on 6/14/19, but were also treated a second time on 6/29/19 to test whether a second application would yield good levels of control.

Results of the bait applications were assessed by comparing numbers of ants attracted to baited monitoring cards in each plot and in an untreated control plot at each site. Monitoring was conducted two days before the application, then on two, four, and six days after each application. On each monitoring date, 12 monitoring cards baited with a blend of tuna and corn syrup were placed on the ground within 5 m of the center of each plot, and numbers of ants were counted after 60 to 75 minutes for AA and after 30 to 40 minutes for YCA. Percent reduction in ant numbers relative to pretreatment numbers was calculated for each plot on each monitoring date, pooling the 12 monitoring cards on each date. Mean percent reduction was calculated after each bait application by averaging the percent reductions on each of the three monitoring days (two, four and six days) post treatment for each plot. Trends in percent reduction across plots at each site following each application were analyzed with a two-factor ANOVA, in which the mean percent reduction for each plot was the response, and granule type and AI formulation were the factors in the model. For the second application in the YCA plots, mean reductions following the second application in the three 0.05% indoxacarb plots were compared to means following the first application for the remaining plots, which were not treated a second time. Significant differences among levels of factors in the model were assessed with Tukey HSD pairwise comparisons (at $\alpha = 0.05$).

<u>Non-target species attraction: video observations of pollinators</u>

Attraction of pollinators and other insects to WSG was assessed by filming small clumps of WSG formulated with sucrose (no AI) that were placed either on the ground or near flowers at several sites. Observations were conducted only on sunny days between approximately 10 am and 3 pm, at sites known to support abundant populations of *Hylaeus* bees (Hymenoptera: Colletidae), other bees, and/or other pollinating insects. For ground observations, approximately one spoonful of WSG was placed on the ground, near the base of vegetation around which pollinating insects were observed to be active. For flower observations, as much WSG as was practical, up to approximately one spoonful, was perched on or near individual flowers or flowering panicles, depending on the plant species. During each observation event, nine video cameras (Sony HDR-CX405) were used to film three replicates of each of the three WSG types, usually for a duration of four to five hours. Replicates were separated by approximately 1 m or more (Fig. 3). Videos were subsequently viewed and all non-ant visitors that made contact with the WSG were noted. For flower observations, visits to the adjacent flowers (>2 sec duration)

Fig. 3. Examples of non-target species attraction video observation. Top: three of nine cameras at Kaena Point Natural Area Reserve filming small clumps of WSG placed on the ground near the base of vegetation that was actively visited by insect pollinators. Bottom: four of nine cameras at James Campbell National Wildlife Refuge filming small clumps of WSG placed on or near flowers of *Heliotropium foertherianum* and *Scaevola taccada*.

were also noted. Length of each visit, time of day of each visit, and identity of each visitor to the lowest taxonomic level recognizable was tabulated for each replicate observation. Total number of visiting taxa, and total number of individual visits was also calculated for each replicate observation, with the latter defined as the total number of visits that were separated from previous visits by at least one minute.

Ground observations were conducted at JCNWR on 5/15/18 and 6/14/18 (n = 6 replicates per WSG type), at KPNAR on 5/10/18, 5/11/18, and 5/17/18 (n = 9 replicates per WSG type), at KSSS on 5/24/18 and 6/8/18 (n = 6 replicates per WSG type), and at HALE on 5/30/18, 5/31/18, and 6/1/18 (n = 9 replicates per WSG type). Only videos with at least 210 minutes of usable footage were included in analyses, resulting in 29 replicate ground observations for polyacrylamide and TVP granules, and 28 replicate observations for alginate beads. These comprised 120 to 125+ hours of video footage for each WSG type.

Figure 4. Examples of polyacrylamide WSG granules placed next to flowers of *S. taccada* (top left), *H. foertherianum* (top right), *G. cuneatum* (bottom left), and *S. haleakalae* (bottom right), to test for pollinator attraction. Location of granules indicated with red arrows.

Flower observations were conducted at JCNWR on 8/31/18 and 9/19/18 (n = 6 replicates per WSG type), at KSSS on 9/21/18, 10/25/18, and 1/9/19 (n = 9 replicates per WSG type), and at HALE on 6/26/18, 6/27/18, and 6/29/18 (n = 9 replicates per WSG type). Only videos with at least 210 minutes of valid footage were included in analyses, resulting in 22, 21, and 23 replicate flower observations for alginate, polyacrylamide, and TVP granules, respectively (comprising 91 to 100+ hours of video footage for each). Plant species used for flower observations were *Scaevola taccada* and *Heliotropium foertherianum* at JCNWR and KSSS, and *Geranium cuneatum* and *Santalum haleakalae* at HALE (Fig. 4). For each observation event, two replicates of each WSG type were assigned to one of the focal plant species at the site, and the third replicate of each WSG type was assigned to the second focal plant species at the site. The total number of replicate flower observations that were analyzed for each plant species were as follows: *S. taccada* (19), *H. foertherianum* (21), *G. cuneatum* (17), and *S. haleakalae* (9).

Numbers of visitors per observation event, number of taxa per observation event, and duration of visits were compared among WSG types for both ground and flower observations with generalized linear models, using the log link function and negative binomial distribution. For flower observations, number of visits and number of visiting taxa to both flowers and adjacent granules were also compared among plant species (pooling granule types), with generalized linear models using the log link function and negative binomial distribution.

Non-target species attraction: video observations of birds

An attempt was made to assess bird attraction to WSG using two video filming methods. In the first method, three replicate clumps of each WSG type (formulated with sucrose and no AI) were placed on the ground at JCNWR in an area where shorebirds were active, and cameras were set up to film each clump at a distance of approximately 10 to 20 m. This method was attempted on 9/25/18 and 10/23/18. In both events, the act of setting up the baits and cameras caused the birds to leave the area, and they did not return for the duration of filming (approximately four hours). In the second method, three 20 x 20 m plots were established at JCNWR near the northeast corner of the refuge, in coastal flats inland of the coastal dune system, an area where shorebirds and other birds were commonly active. On each of three dates, WSG were broadcast by hand in the plots at an application rate of 55 L/ha of absorbed sucrose bait (with no AI). One WSG type was allocated to each plot. WSG were broadcast at 11:30 am on 2/13/19, at 7:45 am on 2/28/19, and at 1:45 pm on 3/4/19. After broadcast, cameras positioned unobtrusively 30-50 m away near the coastal dune vegetation recorded bird activity in the plots. On the first two dates, two cameras were used for each plot, one set up with a wide angle to capture the entire plot, and the second focused on an area several meters wide that was provisioned with a high density of WSG. On the third date, three cameras were used per plot, with all three focused on different areas within the plot. At the same time, two observers monitored the plots with binoculars in an effort to observe bird feeding behavior. Across the three observation dates, a total of over 9 hours of observation was performed for each plot.

Non-target species bait consumption: protein marking and detection

A bait marking and detection approach was used to test consumption of sucrose bait in broadcast WSG. A common approach uses mammalian IgG as a marking protein, whose presence in the gut of a target insect can be screened after exposure using ELISA (Hagler 1997, DeGrandi-Hoffman and Hagler 2000, Buczkowski and Bennett 2006). However, the large quantity of bait required when broadcasting WSG in test plots necessitated the use of a cheaper marking method. Consultation with James Hagler, an expert in the field, suggested that rabbit serum would be an effective way of delivering IgG much more cheaply than using standard, purified IgG. To confirm this, concentrations of rabbit serum (Sigma Aldrich R4505) ranging from 0.5% to 20% in sucrose solution were fed to individual honey bees (*Apis mellifera*, Hymenoptera: Apidae) and white-footed ants (*Technomyrmex difficilis*, Hymenoptera: Formicidae) in the lab in preliminary trials, and these insects were screened with the ELISA procedure (see below). This determined that insects feeding on sucrose solutions containing rabbit serum at or above 2% concentration were consistently and strongly marked. All subsequent tests used 2% rabbit serum in sucrose solution as bait.

To confirm that pollinating insects would be marked when feeding on sucrose bait absorbed in WSG, honey bees and non-native solitary bees were fed in the lab using polyacrylamide WSG formulated with 2% rabbit serum in sucrose solution (no AI). Wild *A. mellifera* were captured on the UH campus, were individually restrained in harnesses, and fed from a polyacrylamide granule by eliciting the proboscis extension reflex. Only bees that fed for at least 30 seconds were retained for analysis (n = 30). Wild solitary bees (belonging to *Ceratina smaragdula* (Hymenoptera: Apidae), *Hylaeus strenuus* (Hymenoptera: Colletidae) and *Lasioglossum microlepoides* (Hymenoptera: Halictidae)) were captured at various coastal locations and were placed in individual cages in the lab for 48 hrs. Each cage was provisioned with a single artificial flower that contained a central receptacle holding a polyacrylamide granule, and bees were allowed to feed naturally and self-mark in the process. Only bees that survived the 48 hr period were retained for analysis (n = 30).

To test consumption of WSG bait by pollinating and other insects under natural conditions, WSG formulated with 2% rabbit serum in sucrose solution (and no AI) were broadcast in a total of 18 10 x 10 m plots, 6 plots for each of the three WSG types. Three plots each were treated on 5/11/18 and 5/17/18 at KPNAR, both sunny days with ample flying insect activity. The six plots were located in dune habitat dominated by *Scaevola taccada*, *Sesbania tomentosa* and *Euphorbia degeneri*. Three plots each were treated on 5/30/18, 5/31/18, 6/26/18, and 6/27/18 at HALE, also on sunny days with ample flying insect activity. The 12 plots were located in native shrubland habitat dominated by *Leptecophylla tameiameiae*, *Sophora chrysophylla, Santalum haleakalae, Dubautia menziesii, Geranium cuneatum, Dodonea viscosa and Coprosma montana*. On each treatment date, granules were broadcast in the three plots by hand between 9:30 and 10:30 am at a rate of 55 L of absorbed liquid bait per ha, with one plot allocated to each WSG type. Beginning 60 to 75 minutes after WSG broadcast, flying insects were sampled during four 10 minute periods in each plot, rotating between plots for each successive sampling period. During each 10 minute period, as many flying insects as possible were collected with a sweep net, focusing on bees and other common flower-visiting insects. A total of 441 insects were collected across the 18 plots. To confirm that the protein marker remained active within WSG under field conditions, 25 to 28 foraging ants (*Anoplolepis gracilipes, Ochetellus glaber* and *Paratrechina longicornis*) were also collected within each of the three plots at KPNAR on 5/17/18, focusing on ants observed near broadcast granules.

Because the sweep net sometimes came into contact with WSG lodged in vegetation during sampling, it was possible that bait absorbed by the net could externally mark captured insects that did not feed on WSG, resulting in false positive detections. To test for this type of net contamination, flying insects were captured with the same sweep net at a location approximately 1 km from the test plots after normal sampling was completed on 5/31/18 (n = 36 insects) and 6/27/18 (n = 17 insects) at HALE.

All insects were stored at -20° C until they were screened for the presence of the protein marker with double-antibody sandwich ELISA, using the following procedure. All wells of 96-well microplates were coated with 100 µL of goat anti-rabbit IgG (Sigma Aldrich AP132) diluted 1:500 in distilled water and incubated overnight at 4° C. Primary antibodies were then discarded and 310 µL of 1% non-fat dry milk in distilled water was added to each well to block remaining non-specific binding sites. After incubation for 30 minutes at 26° C, the milk was discarded and plates were rinsed five times with phosphate buffered saline (PBS) Tween 20

(0.05%). Insect samples were individually homogenized in PBS; 200 µL of PBS was used for small insects (e.g. ants), 0.5 ml was used for medium insects (e.g. solitary bees), and 1.0 ml was used for large insects (e.g. honey bees). Each well then received 100 µL aliquots of a sample: 84 wells on each plate received test samples (insects exposed to bait treatments), eight wells received negative controls (insects never exposed to bait treatments), and four wells received positive controls (100 µL of the 2% rabbit serum in sucrose solution bait). Plates were incubated for 2 hrs at 26° C, after which samples were discarded and plates were rinsed five times with PBS Tween 20. Next, 100 µL of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Aldrich A3687) diluted 1:5000 with 1% non-fat dry milk in distilled water was added to each well and incubated for 2 hrs at 26° C, after which antibodies were discarded and plates were again rinsed five times with PBS Tween 20. Finally, 100 µL of phosphatase substrate (Sigma Aldrich, CAS 333338-18-4) was added to each well, and after 30 minutes plates were read on a Biotek Epoch Microplate Spectrophotometer set at 405 nm.

Samples were scored as positive for the presence of the protein marker if their optical density reading exceeded the mean negative control reading by three standard deviations (SD) (Hagler 1997, Hagler et al. 2014). Because some of the net contamination samples scored positive according to this threshold despite not being exposed to bait (see Results), a second threshold was used for the samples collected in the field test plots: the mean + 3 SD of the net contamination sample readings. A chi-square test of association was used to compare incidences of marked to unmarked individuals among the three WSG types and among the main orders of insects sampled.

RESULTS

WSG drying rates

Granules of all three WSG types dried fairly quickly, reaching 50% water loss, on average, in under 2 hours in all three scenarios (Fig. 5). The mean drying rate among the 10 selected granules of each WSG type was substantially lower for polyacrylamide compared to alginate beads and TVP, and drying rates were lower in the shade at low elevation and at high elevation (Fig. 5). Air temperature and relative humidity averaged 31.5° C and 37.7% during the low elevation full sun trial, 30.0° C and 45.2% during the low elevation full shade trial, and 22.1° C and 72.6% during the high elevation full sun trial. However, the air temperature/relative humidity sensor was shaded in all three trials, so did not accurately capture the temperature differences between the low elevation full sun and full shade trials.

For polyacrylamide and TVP granules, there were strong and highly statistically significant positive linear relationships between granule mass (ln-tranformed) and T50 in all three scenarios (Fig. 6) (polyacrylamide low elevation, full sun: $r^2 = 0.947$, $p < 0.001$; polyacrylamide low elevation, full shade: $r^2 = 0.986$, $p < 0.001$; polyacrylamide high elevation, full sun: $r^2 = 0.961$, $p < 0.001$; TVP low elevation, full sun: $r^2 = 0.844$, $p < 0.001$; TVP low elevation, full shade: $r^2 = 0.813$, $p < 0.001$; TVP high elevation, full sun: $r^2 = 0.818$, $p < 0.001$). There were also positive linear relationships between granule mass and T50 for alginate beads, but these were generally weaker and less consistent, likely because of the much smaller size range of the highly uniform beads (Fig. 6) (alginate low elevation, full sun: $r^2 = 0.184$, $p = 0.215$;

alginate low elevation, full shade: $r^2 = 0.864$, $p < 0.001$; alginate high elevation, full sun: $r^2 = 0.496$, $p = 0.023$). In all three scenarios, polyacrylamide had higher T50 values than TVP for all but the smallest granule sizes, with the difference increasing as granules increase in size (Fig. 6). This indicates that water evaporates from polyacrylamide granules more slowly than from TVP granules, even after adjusting for granule size. The equivalent comparisons with alginate beads are more difficult because of their smaller size and small size range, but where alginate beads overlap in size with polyacrylamide granules, the data suggest that they retain water similarly (e.g. Fig. 6, middle panel).

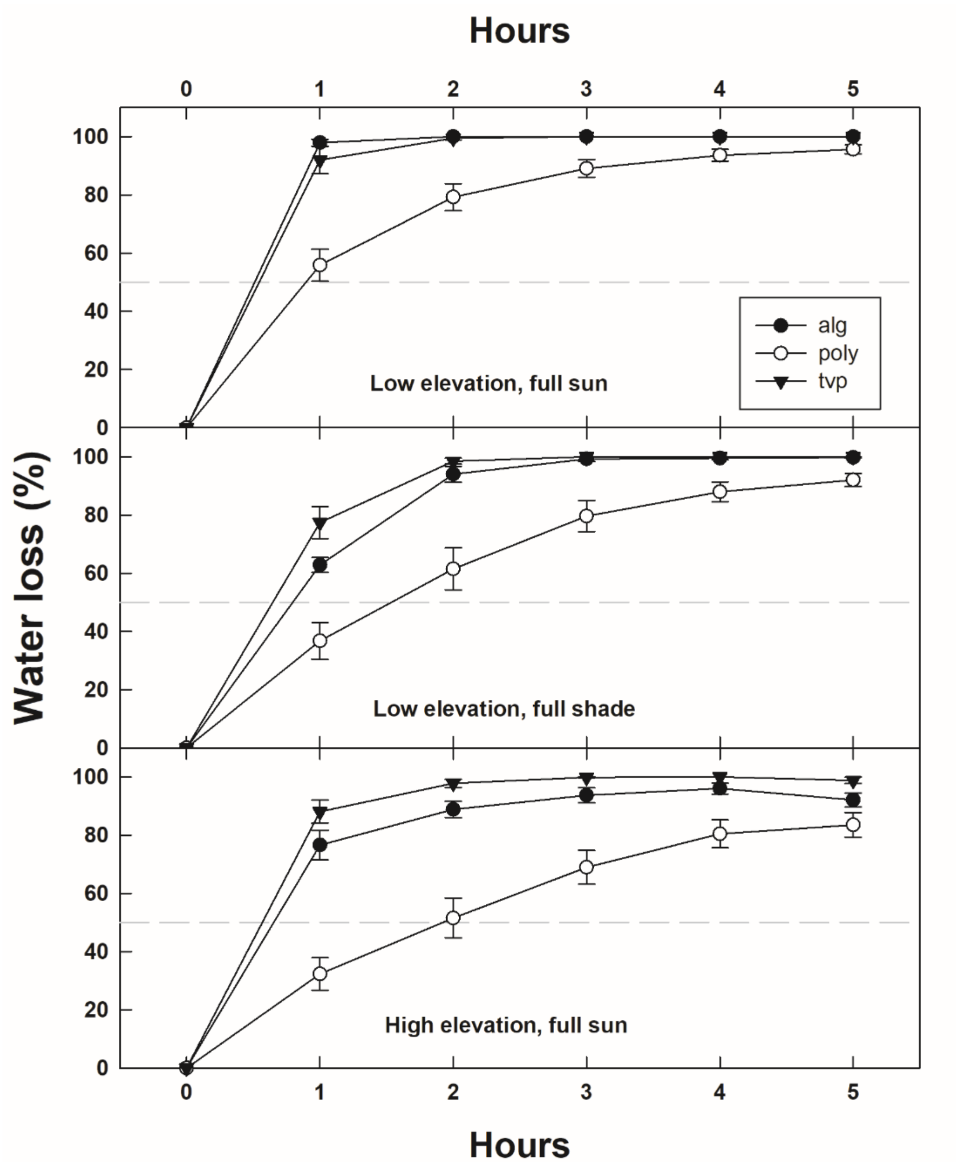

Figure 5. Mean percent water loss (± SE) over time for individual granules of each WSG type for three scenarios: low elevation in full sun (top panel), low elevation in full shade (middle panel), and high elevation in full sun (bottom panel). 50% water loss indicated with grey dashed line.

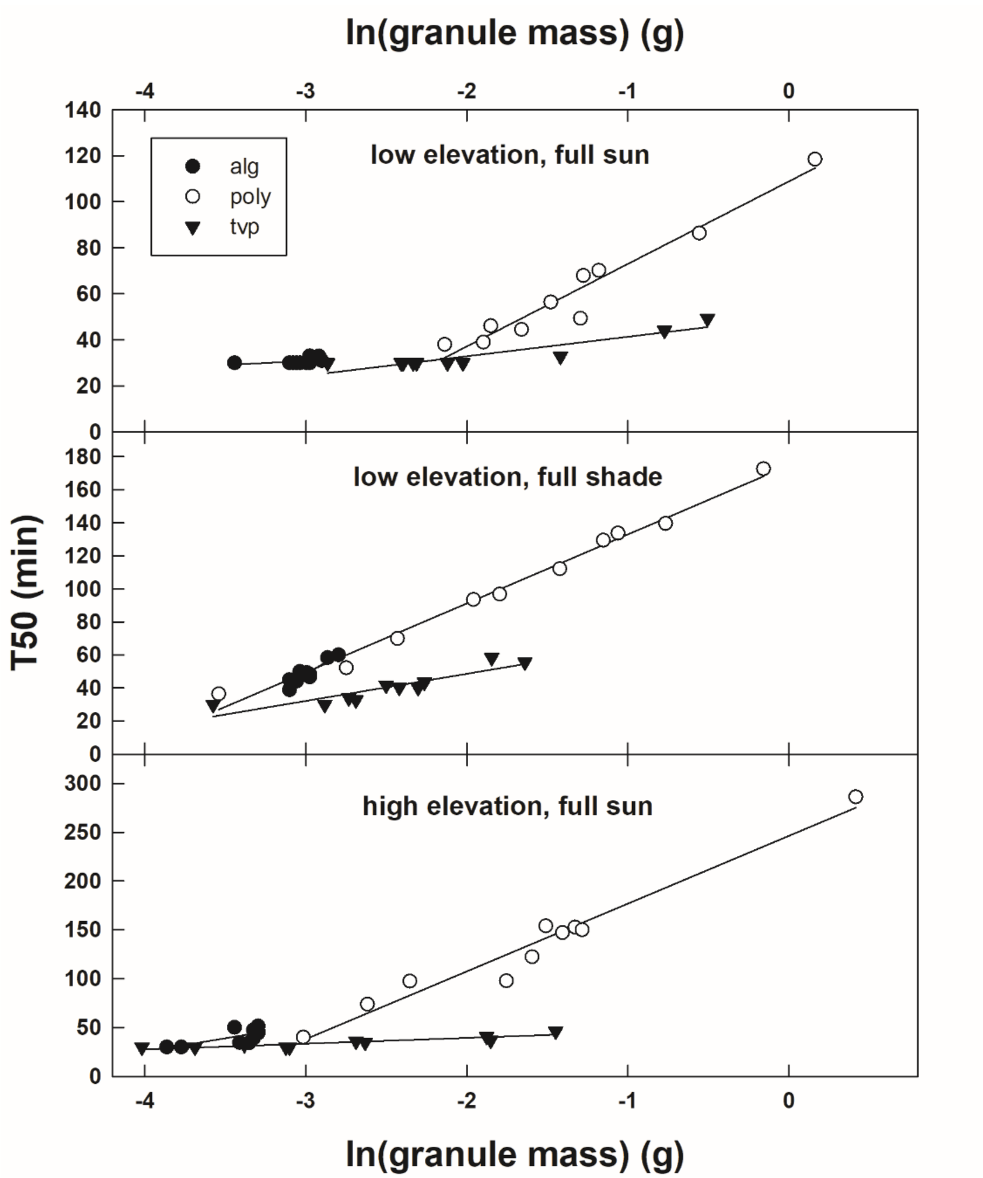

Figure 6. Relationships between granule mass (ln transformed) and the time to reach 50% water loss (T50) for the three WSG types under three scenarios. All linear relationships are statistically significant ($\alpha = 0.05$) except for alginate beads at low elevation in full sun (see text).

Estimated typical T50 values differed substantially among the WSG types and environmental scenarios (Fig. 7). These estimates, based on the regression relationships in Figure 6, were calculated for 50 haphazardly selected granules, and should approximate performance in a typical batch of formulated WSG of each type. The median time for granules to lose 50% of their water, and therefore to decline in attractiveness to ants, was substantially longer for polyacrylamide than for alginate or TVP (Fig. 7). The range of values was also much larger for polyacrylamide, indicating that many granules should stay attractive for considerably longer than the median time. In comparison, the ranges of T50 values were much smaller for alginate and TVP (Fig. 7).

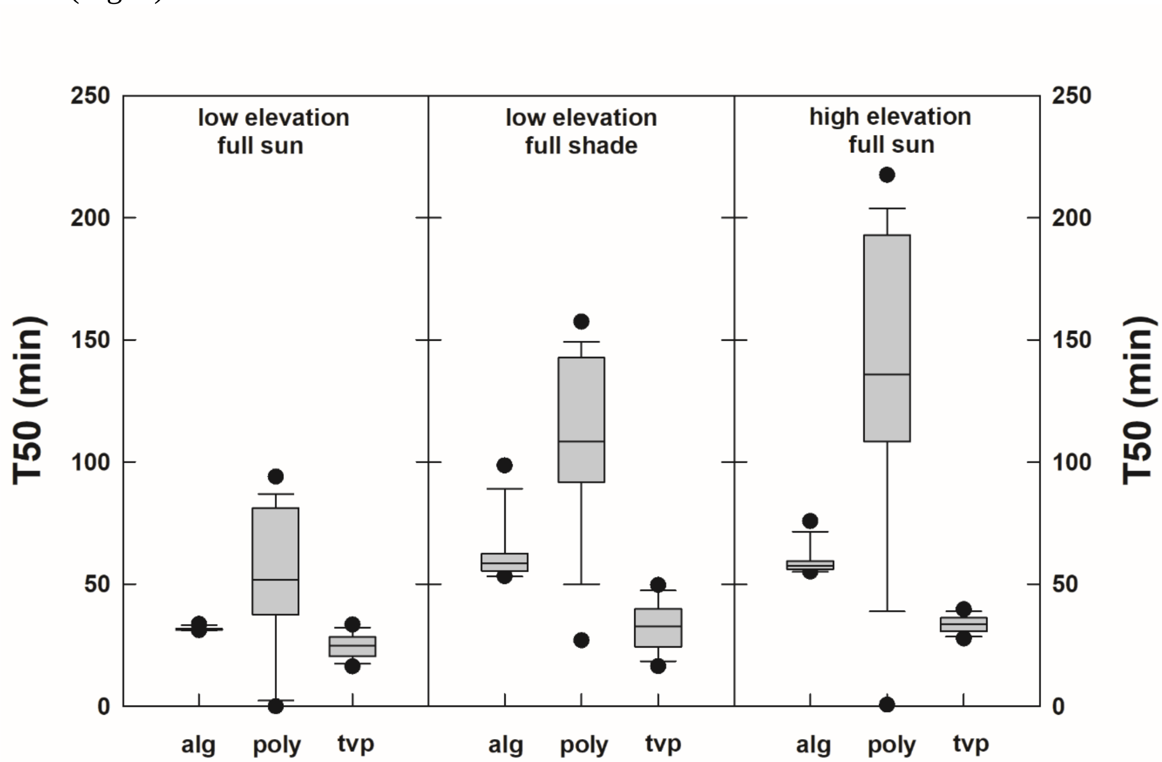

Figure 7. Box plots of estimated T50 values for each of 50 typical granules of each WSG type, under three environmental scenarios. Boxes span 25th to 75th percentiles with median indicated with horizontal line; whiskers indicate 10th and 90th percentiles, and outliers up to 5th and 95th percentiles are shown with dots.

Bait preference among WSG

At 30 minutes after bait placement YCA recruited significantly fewer workers to alginate formulated with sucrose compared to both polyacrylamide and TVP in the choice trial, but attraction was not significantly different among the WSG types in the no-choice trial (Fig. 8). Attraction of ants to the baits generally declined after 30 minutes post-placement, likely owing to drying of the baits. This decline was especially pronounced for TVP, which attracted significantly fewer YCA after 60 minutes post-placement than the other two WSG in both trials, with this pattern persisting through 180 minutes post bait placement (Fig. 8). TVP granules likely dried more quickly than the other WSG, probably both from evaporation and from consumption of sucrose by ants, given that YCA recruited the highest mean number of ants to TVP at 30 minutes. By 180 minutes post placement, attraction was attraction was low for all three WSG.

Attraction of AA at 30 minutes after placement was not significantly different among the three WSG types in both the choice trial and the no-choice trial (Fig. 9). As in the YCA trial, numbers of ants attracted to all three WSG subsequently decreased, with this decline again especially strong for TVP, at least in the choice trial. Numbers of ants were low at all three baits by 120 minutes post placement in both trials, and there were almost no ants attracted to the baits by 180 minutes post placement (Fig. 9).

In contrast to YCA and AA, LFA recruitment to the WSG baits increased over time (Fig. 10). This difference may have resulted from slower recruitment and less rapid consumption of sucrose due to the smaller size of LFA workers, combined with more humid conditions at the site of the trials in Puna, Hawaii. Although TVP and alginate beads generally attracted more LFA than polyacrylamide in both choice and no-choice trials, these differences were usually not statistically significant.

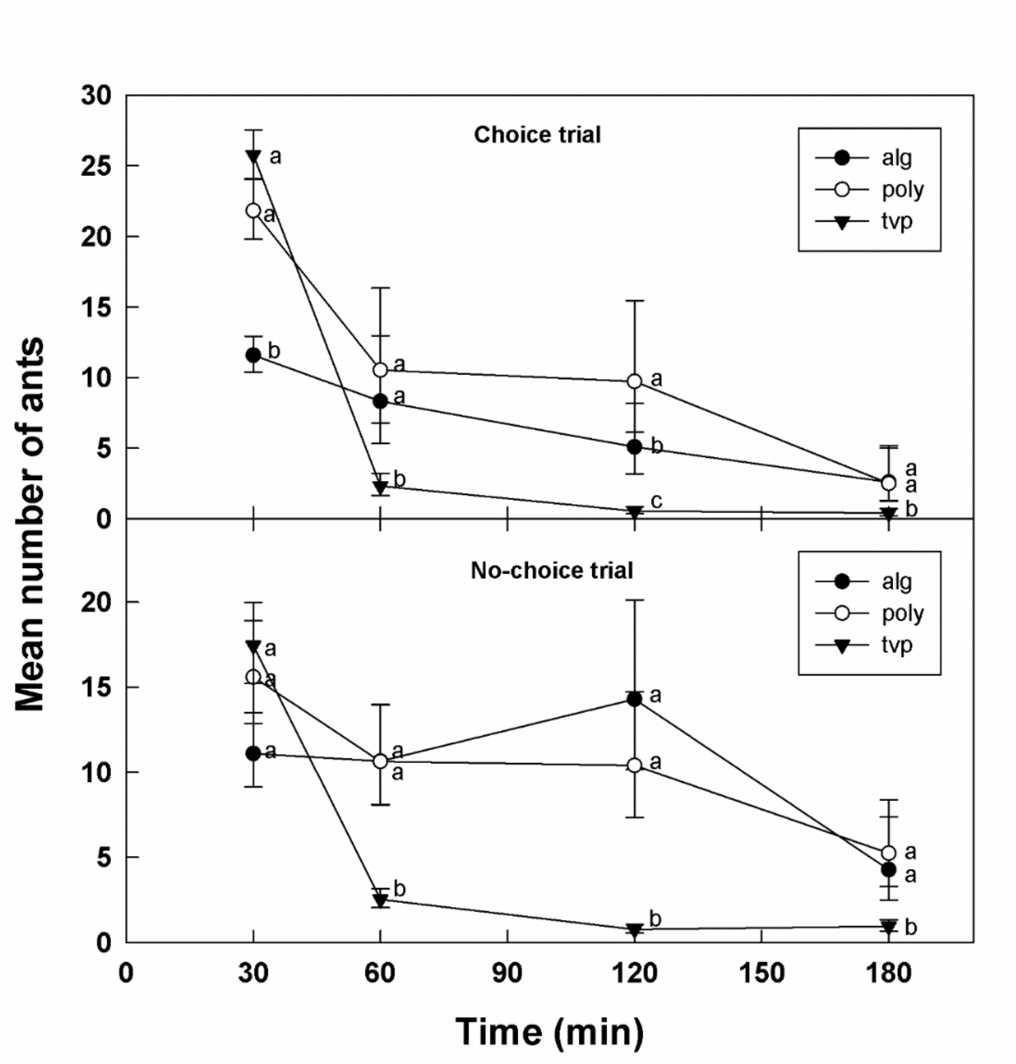

Figure 8. Bait preference trials with YCA. Back-transformed mean number of ants (±SE) attracted to the three WSG types over time shown for the choice trial (top panel) and no-choice trial (bottom panel). Means sharing the same letter within each time interval are not significantly different (at $\alpha = 0.05$).

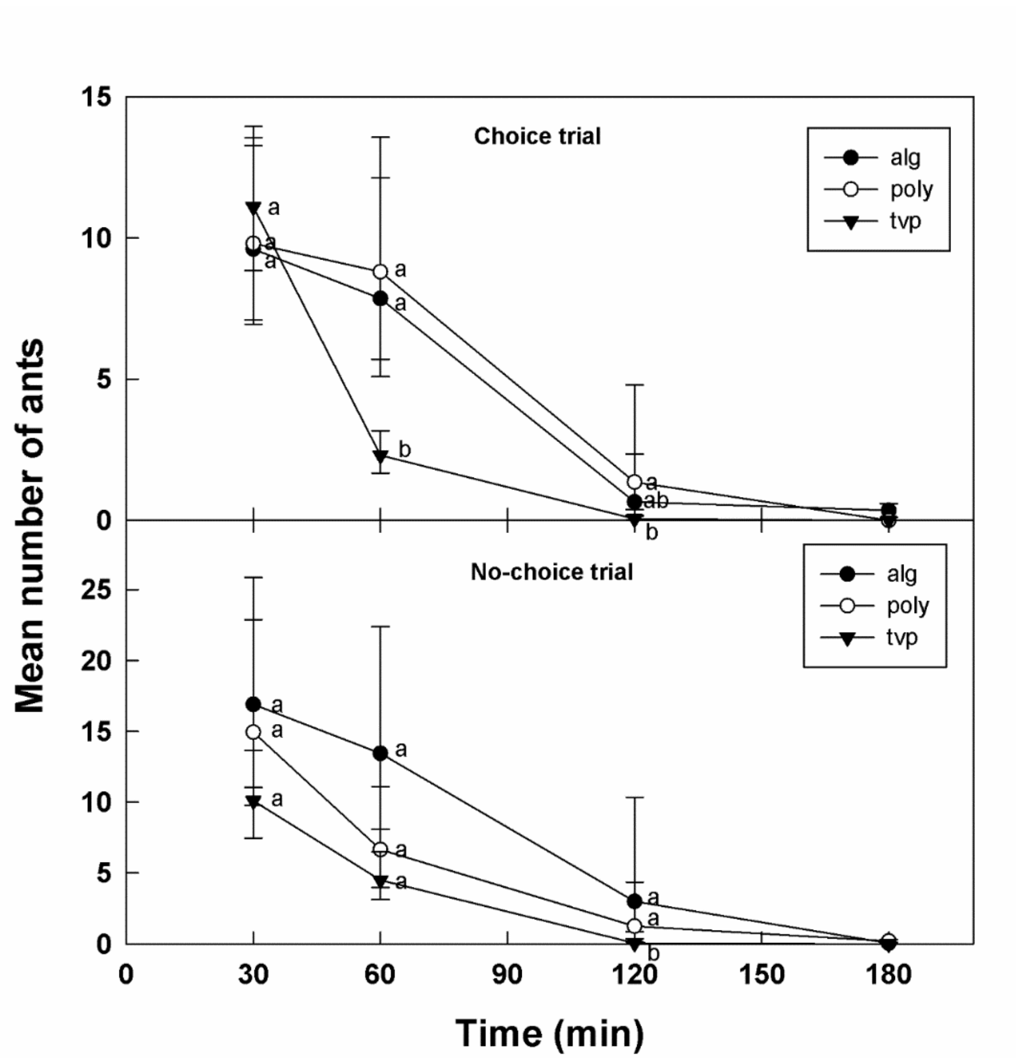

Figure 9. Bait preference trials with AA. Back-transformed mean number of ants (±SE) attracted to the three WSG types over time shown for the choice trial (top panel) and no-choice trial (bottom panel). Means sharing the same letter within each time interval are not significantly different (at $\alpha = 0.05$).

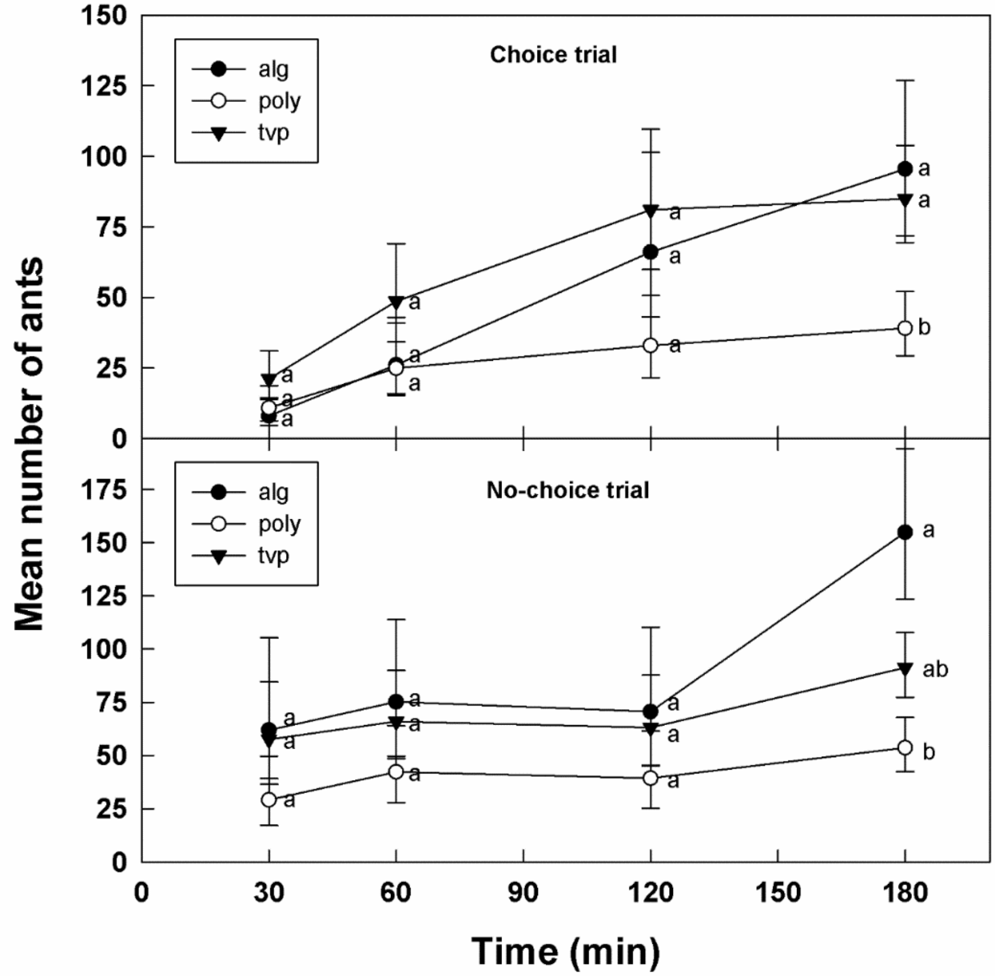

Figure 10. Bait preference trials with LFA. Back-transformed mean number of ants (±SE) attracted to the three WSG types over time shown for the choice trial (top panel) and no-choice trial (bottom panel). Means sharing the same letter within each time interval are not significantly different (at $\alpha = 0.05$).

Pesticide repellency trials

YCA exhibited little repellency to both dinotefuran and indoxacarb, with the possible exception of the highest concentrations tested (0.25%) in some formulations (Fig. 11). In contrast, YCA exhibited clear repellency to thiamethoxam at concentrations at or above 0.005% (Fig. 11). The patterns of repellency were quite consistent across all three WSG types.

AA exhibited little repellency to indoxacarb and thiamethoxam below the highest concentrations tested (0.25% and 0.025%, respectively) (Fig. 12). However, there was evidence of repellency to dinotefuran at even the lowest concentration tested (0.005%) with alginate beads, as well as above 0.05% concentration with polyacrylamide. The patterns of repellency were again fairly consistent across the WSG types.

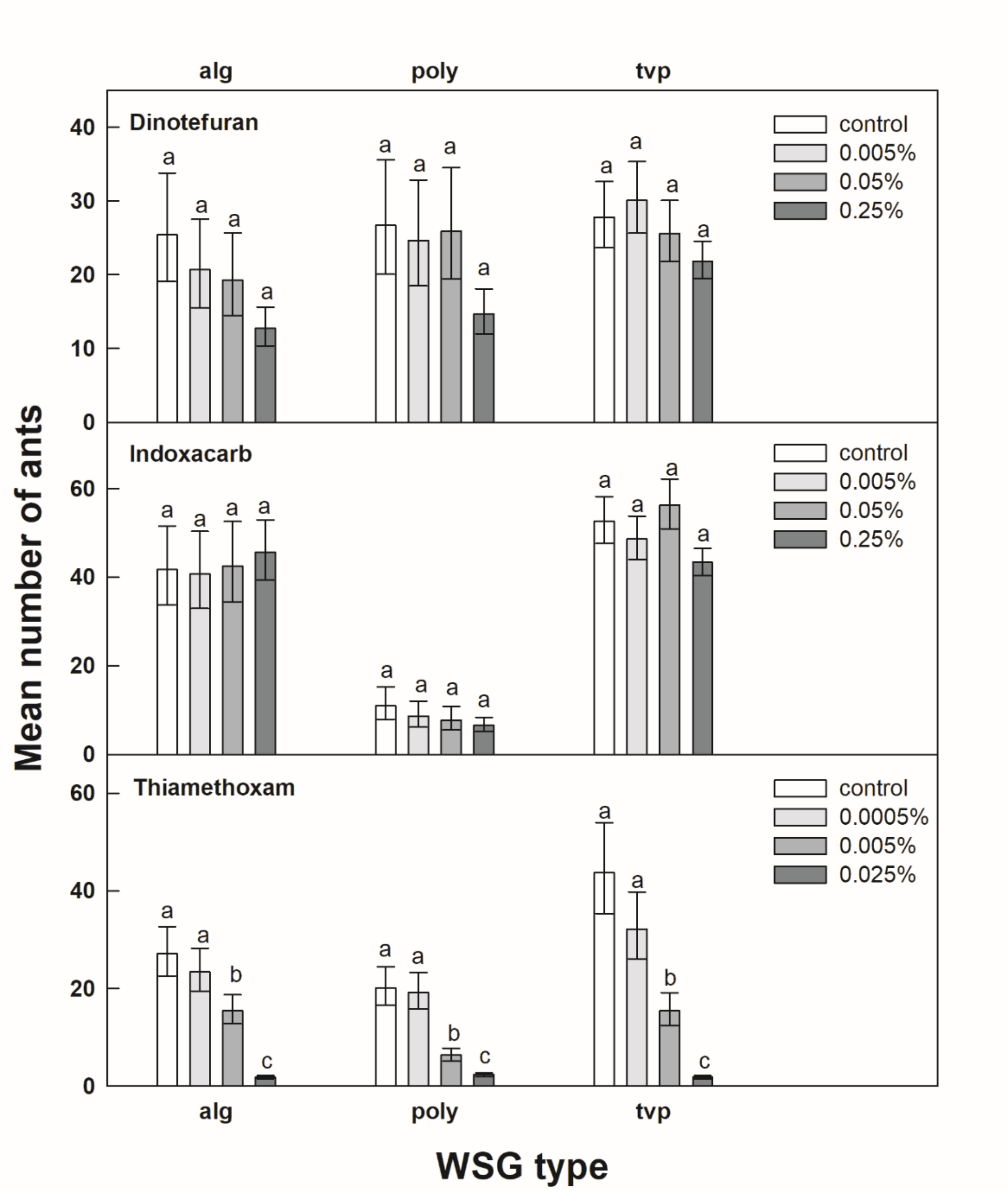

Figure 11. Pesticide repellency trials with YCA. Back-transformed mean number of ants (±SE) attracted to different concentrations of each AI are shown in separate panels, and grouped by WSG type. Means sharing the same letter within each WSG grouping are not significantly different (at $\alpha = 0.05$). Several comparisons were marginally significantly different: 0.25% dinotefuran vs. control formulated in alginate beads ($p = 0.081$), and 0.25% vs. 0.05% indoxacarb formulated in TVP ($p = 0.052$).

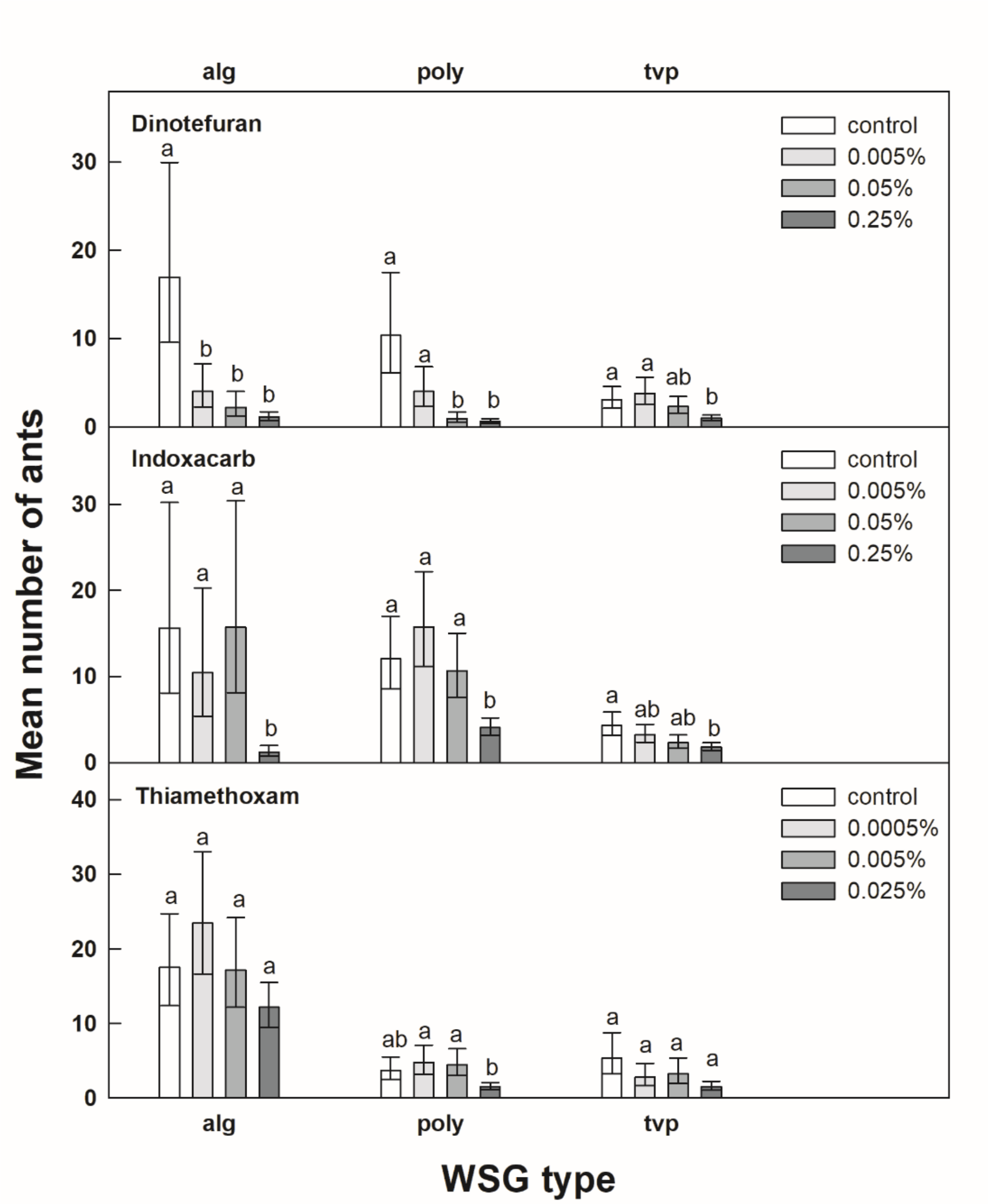

Figure 12. Pesticide repellency trials with AA. Back-transformed mean number of ants (±SE) attracted to different concentrations of each AI are shown in separate panels, and grouped by WSG type. Means sharing the same letter within each WSG grouping are not significantly different (at $\alpha = 0.05$). One comparison was marginally significantly different: 0.025% thiamethoxam vs. control formulated in TVP ($p = 0.067$).

For LFA, indoxacarb appears to be non-repellant at concentrations below 0.25% (Fig. 13). In contrast, thiamethoxam appears to be repellant to LFA at concentrations at or above 0.005%, and even exhibited signs of repellency at concentrations of 0.0005% (Fig. 13). Repellency to dinotefuran was less clear, in part because of low overall recruitment rates in two of the trials (Fig. 13). In the trial using polyacrylamide, recruitment was highest to the formulation with the highest concentration of dinotefuran (0.25%), suggesting that this AI is not repellent to LFA. However, recruitment to intermediate concentrations of dinotefuran in the polyacrylamide trial was very low (Fig. 13), which is difficult to explain. The latter may have been a spurious result.

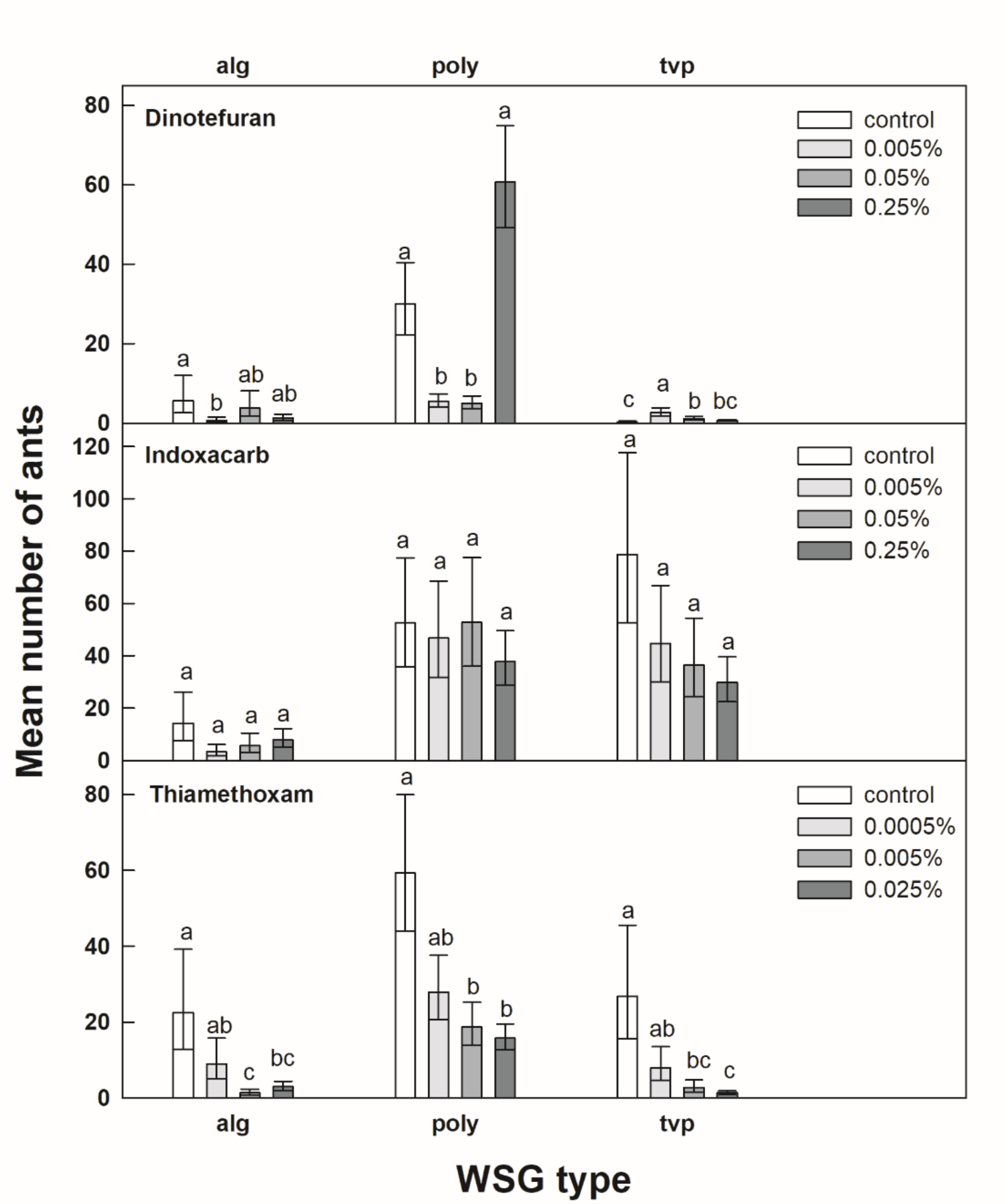

Figure 13. Pesticide repellency trials with LFA. Back-transformed mean number of ants (±SE) attracted to different concentrations of each AI are shown in separate panels, and grouped by WSG type. Means sharing the same letter within each WSG grouping are not significantly different (at $\alpha = 0.05$). Several comparisons were marginally significantly different: 0.25% dinotefuran vs. control formulated in polyacrylamide ($p = 0.095$), 0.005% indoxacarb vs. control formulated in alginate beads ($p = 0.098$), 0.25% indoxacarb vs. control formulated in TVP ($p = 0.082$), 0.0005% thiamethoxam vs. control formulated in polyacrylamide ($p = 0.061$), and 0.0005% thiamethoxam vs. control formulated in TVP ($p = 0.080$).

For all three species, differences in overall recruitment rates (i.e., maximum numbers) to different WSG types should not be taken to indicate differences in attraction to those granule types, as each WSG-specific repellency trial was conducted on a different transect and in some cases at different sites. Differences in local densities of ants are therefore likely responsible for these differences in recruitment.

Efficacy testing

Trends in ant numbers over time for the AA efficacy test plots are shown in Figure 14. Ant numbers at monitoring cards were reduced by over 98% after the first application of WSG baits in all plots except two of the plots treated with 0.005% indoxacarb (Table 1). However, across all plots, differences in mean percent reduction after the first application were not significantly associated with either granule type ($F = 1.226$, $p = 0.384$) or AI formulation ($F = 3.381$, $p = 0.138$). Least squares means for granule types and AI formulations are shown in Table 2. Results after the second application were similar, except that ant numbers dropped substantially further in at least one of the plots treated with 0.005% indoxacarb (Figure 14, Table 1). Differences in percent reduction were again not significantly associated with either granule type ($F = 0.779$, $p = 0.518$) or AI formulation ($F = 1.773$, $p = 0.281$) after the second application (Table 2).

Table 1. Mean percent reduction in ant numbers in each of the AA plots after each bait application. Reduction in each plot averaged across the three monitoring dates (two, four and six days) after each application.

AI	**Granule**	**Mean % reduction after 1st application**	**Mean % reduction after 2nd application**
0.005% indoxacarb	alginate	83.9	98.8
0.005% indoxacarb	polyacrylamide	100	96.4
0.005% indoxacarb	tvp	77.4	86.9
0.05% indoxacarb	alginate	98.1	100
0.05% indoxacarb	polyacrylamide	99.9	100
0.05% indoxacarb	tvp	98.7	99.9
0.0005% thiamethoxam	alginate	99.6	100
0.0005% thiamethoxam	polyacrylamide	99.1	95.6
0.0005% thiamethoxam	tvp	98.9	99.6
control	none	13.4	44.4

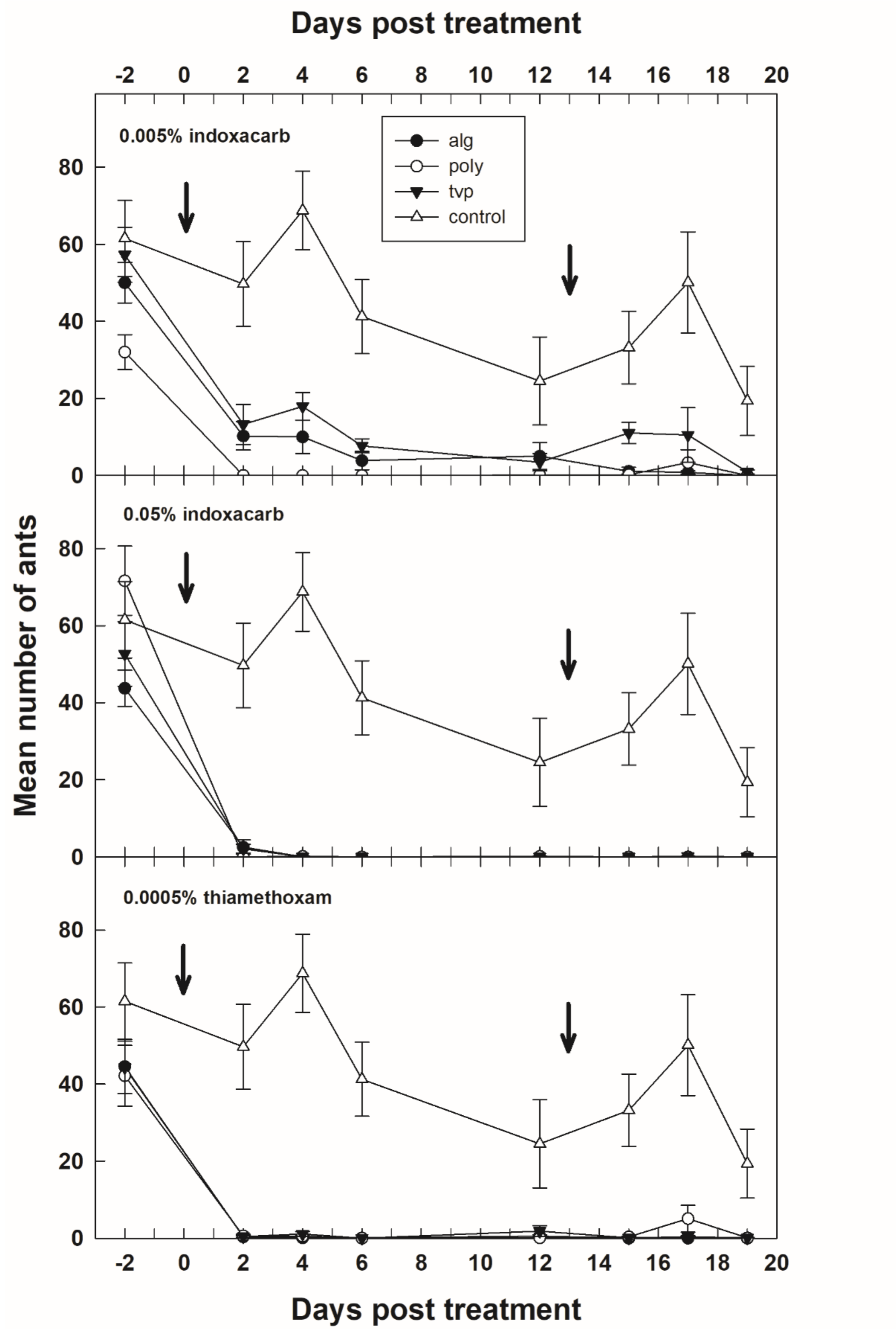

Figure 14. Efficacy test with AA. Mean numbers of ants (± SE) in treatment and control plots are shown over time, grouped by AI formulation in separate panels. Timing of the two WSG bait applications shown with arrows.

Table 2. Least squares means of percent reduction in AA numbers following each application, for the two factors included in the ANOVA model.

Factor	Mean (±SE) % reduction, 1st application[1]	Mean (±SE) % reduction, 2nd application[1]
Granule type		
alginate	93.9 (±3.8) a	99.6 (±2.3) a
polyacrylamide	99.7 (±3.8) a	97.3 (±2.3) a
tvp	91.6 (±3.8) a	95.5 (±2.3) a
AI formulation		
0.005% indoxacarb	87.1 (±3.8) a	94.0 (±2.3) a
0.05% indoxacarb	98.9 (±3.8) a	99.9 (±2.3) a
0.0005% thiamethoxam	99.2 (±3.8) a	98.4 (±2.3) a

[1]Means sharing the same letter within each factor and application are not significantly different according to Tukey HSD pairwise comparisons (α = 0.05).

Trends in ant numbers over time for the YCA efficacy test plots are shown in Figure 15. Ant numbers at monitoring cards were reduced by over 90% after the first application of WSG baits in all plots treated with either concentration of dinotefuran (Table 3). In contrast, reductions in ant numbers were substantially lower in plots treated with indoxacarb, especially those treated at the lower concentration of 0.005% (Table 3). Ant numbers increased to some degree in most plots at six days after the first application (Figure 15), which may have resulted in part from reinvasion of the plots from the periphery. YCA densities are very high in the study area, and this very active ant may be capable of recolonizing the plots more quickly than AA. Across all plots, differences in mean percent reduction after the first application were not significantly associated with granule type ($F = 1.733$, $p = 0.255$), but were highly significantly associated with AI formulation ($F = 13.262$, $p = 0.005$). Least squares means for granule types and AI formulations are shown in Table 4, and indicate that reductions in dinotefuran plots were significantly higher than those in the 0.005% indoxacarb plots, with reductions in 0.05% indoxacarb plots being intermediate.

The level of control achieved with the first application of 0.005% indoxacarb formulations was deemed insufficiently effective to pursue further. However, a second application of the 0.05% indoxacarb formulations was conducted to determine whether two treatments at this concentration could achieve control similar to a single treatment with dinotefuran. The second application achieved strong immediate reductions of foraging ant numbers in each of the three plots, but ant numbers rebounded four to six days after the second application, especially in the plot using alginate beads (Figure 15). Levels of control averaged over the three monitoring dates following the second application were similar to those achieved after the first, with the exception of the TVP plot that had somewhat better control after the second application (Table 3). When analyzed across all plots, however, results were very similar to those achieved after the first application: differences in mean percent reduction were not significantly associated with granule type ($F = 0.869$, $p = 0.466$), but were again highly significantly associated with AI formulation ($F = 10.690$, $p = 0.008$). Least squares means for granule types and AI formulations are shown in Table 4, and indicate that reductions in dinotefuran plots were significantly higher than those in the 0.005% indoxacarb plots, with reductions in 0.05% indoxacarb plots again being intermediate.

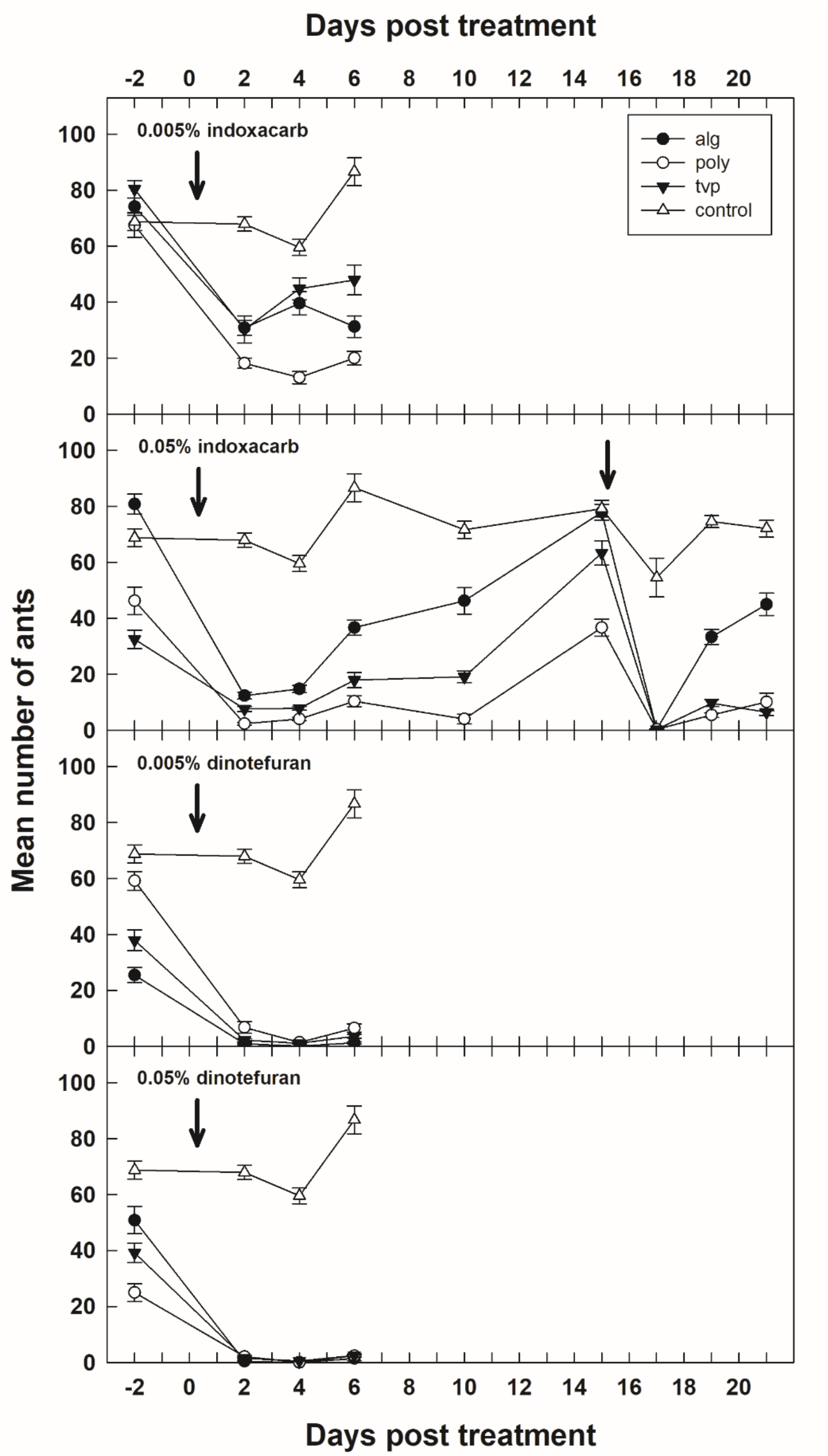

Figure 15. Efficacy test with YCA. Mean numbers of ants (± SE) in treatment and control plots are shown over time, grouped by AI formulation in separate panels. Timing of the WSG bait applications shown with arrows.

Table 3. Mean percent reduction in ant numbers in each of the YCA plots after each bait application. Reduction in each plot averaged across the three monitoring dates (two, four and six days) after each application. Only 0.05% indoxacarb plots received a second application.

AI	**Granule**	**Mean % reduction after 1st application**	**Mean % reduction after 2nd application**
0.005% indoxacarb	alginate	54.3	
0.005% indoxacarb	polyacrylamide	74.7	
0.005% indoxacarb	tvp	49.0	
0.05% indoxacarb	alginate	73.7	67.6
0.05% indoxacarb	polyacrylamide	88.0	88.6
0.05% indoxacarb	tvp	65.8	83.2
0.005% dinotefuran	alginate	97.0	
0.005% dinotefuran	polyacrylamide	91.7	
0.005% dinotefuran	tvp	94.0	
0.05% dinotefuran	alginate	98.8	
0.05% dinotefuran	polyacrylamide	94.0	
0.05% dinotefuran	tvp	96.2	
control	none	-3.8	2.4

Table 4. Least squares means of percent reduction in YCA numbers following each application, for the two factors included in the ANOVA model. Model for second application uses data for first application for all plots treated only once.

Factor	**Mean (±SE) % reduction, 1st application[1]**	**Mean (±SE) % reduction, 2nd application[1]**
Granule type		
alginate	81.0 (±4.1) a	79.4 (±4.5) a
polyacrylamide	87.1 (±4.1) a	87.2 (±4.5) a
tvp	76.2 (±4.1) a	80.6 (±4.5) a
AI formulation		
0.005% indoxacarb	59.3 (±4.8) b	59.3 (±5.2) b
0.05% indoxacarb	75.8 (±4.8) ab	79.8 (±5.2) ab
0.005% dinotefuran	94.2 (±4.8) a	94.2 (±5.2) a
0.05% dinotefuran	96.3 (±4.8) a	96.3 (±5.2) a

[1]Means sharing the same letter within each factor and application are not significantly different according to Tukey HSD pairwise comparisons ($\alpha = 0.05$).

Non-target species attraction: video observations of pollinators

Videos of WSG placed on the ground in the vicinity of active pollinators recorded 132 non-ant visitors to the 90 replicate bait piles observed for a collective >384 hours (Table 5). The most common visitors were overwhelmingly flies (Diptera) of various types (85), followed by parasitic Hymenoptera (17) and a variety of taxa with seven or fewer visits each. Among common pollinating insects, hover flies (Diptera: Syrphidae) made 5 visits to WSG baits, a single native *Hylaeus volatilis* bee visited alginate beads, and four visits were made by the non-native solitary bee *Ceratina dentipes*.

Table 5. Total number of visitors to WSG placed on the ground during video observation events (n = 30 per WSG type).

Taxon	**Alginate**	**Poly.**	**TVP**	**Total**
Acari Total	5	2	0	7
Araneae Total	0	4	1	5
Chilopoda Total	0	0	1	1
Isopoda Total	0	1	0	1
Collembola Total	1	0	3	4
Diptera Total	9	21	55	85
Sarcophagidae	1	11	30	42
Syrphidae	3	2	0	5
Other/unknown	5	8	25	38
Hymenoptera Total	6	3	14	23
Bees Total (Apidae or Colletidae)	2	0	3	5
Ceratina dentipes	1	0	3	4
Hylaeus volatilis	1	0	0	1
Sphecidae Total	0	0	1	1
Tachysphex apicalis	0	0	1	1
Parasitoids Total	4	3	10	17
Unknown Total	2	2	2	6
Grand Total	23	33	76	132

Most visits to ground baits occurred during the first two hours after placement (Fig. 16). TVP baits attracted the most visitors and the greatest diversity of taxa per observation event, although differences among WSG types in these two metrics were statistically significant only between TVP and alginate beads (Fig. 17). Duration of visits were not significantly different among any of the WSG types (Fig. 17).

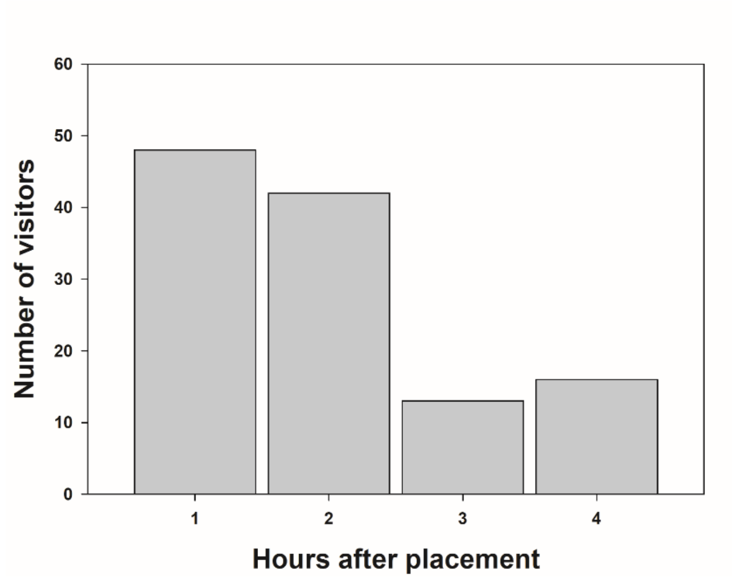

Figure 16. Histogram showing frequency of visitation during each hour after bait placement for WSG placed on the ground. Number of visitors shown for each 60 minute period ending in the hour indicated.

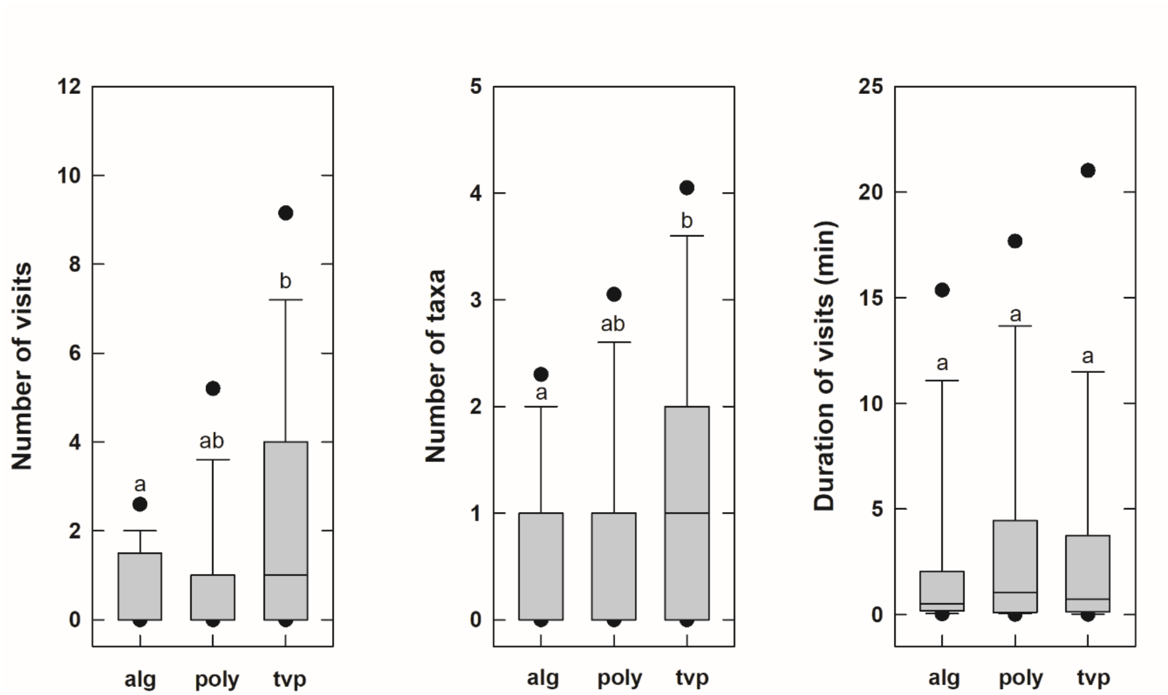

Figure 17. Box plots showing the number of non-ant visits (left panel), the number of non-ant taxa (middle panel), and the duration of non-ant visits (right panel) to WSG of each type placed on the ground in the vicinity of active pollinators. Boxes, whiskers and outliers as in Fig. 7. In all cases where median line is not visible, the median is 0. WSG types sharing the same letter within each panel are not significantly different (generalized linear models, $\alpha = 0.05$). Number of visits were marginally significantly different between polyacrylamide and TVP ($p = 0.092$).

Videos of WSG placed adjacent to flowers recorded many more non-ant visitors compared to those of granules placed on the ground: 394 visits to the 72 replicate bait piles occurred during the collective >307 hours of observation (Table 6). These visits were dominated by Hymenoptera, and to a lesser extent, Lepidoptera. Among Hymenoptera, bees were the most common visitors, predominantly honey bees (*Apis mellifera*), but also native and non-native *Hylaeus* yellow-faced bees. Vespid wasps were also observed visiting granules. Lepidoptera visiting granules were represented by several unidentified species of crambid moths occurring only in the trials conducted at HALE, and are quite possibly native species. Surprisingly few visits were made by syrphid flies.

Table 6. Total number of visitors to WSG placed near flowers during video observation events (n = 24 per WSG type).

Taxon	**Alginate**	**Poly.**	**TVP**	**Total**
Araneae Total	0	4	1	5
Diptera Total	8	3	6	17
Syrphidae	5	0	2	7
Other/unknown	3	3	4	10
Hemiptera Total	0	0	2	2
Miridae	0	0	2	2
Hymenoptera Total	113	115	68	296
Bees Total (Apidae or Colletidae)	98	111	61	270
Apis mellifera	73	83	45	201
Hylaeus spp. (native)	3	12	6	21
Hylaeus strenuus (non-native)	22	16	10	48
Vespidae Total	14	4	7	25
Pachodynerus nasidens	1	2	0	3
Polistes aurifer	13	2	7	22
Parasitoids Total	1	0	0	1
Lepidoptera Total	8	32	33	73
Crambidae	8	32	33	73
Orthoptera Total	0	1	0	1
Tettigoniidae Total	0	1	0	1
Elimaea punctifera	0	1	0	1
Grand Total	129	155	110	394

Unlike baits placed on the ground, baits placed near flowers attracted similar numbers of visitors throughout each of the first four hours after placement (Fig. 18). Among the four plant species investigated, numbers of visitors to WSG granules was highest on *S. haleakalae* and *H. foertherianum*, intermediate on *G. cuneatum*, and lowest on *S. taccada* (Fig. 19). This pattern largely followed rates of visitation to the flowers of these species: floral visitation was high for *S. haleakalae*, *H. foertherianum* and *G. cuneatum*, and much lower for *S. taccada* (Fig. 19). The lower visitation rate to WSG granules near *G. cuneatum* flowers, compared to those near *S. haleakalae* flowers, likely resulted from the fact floral morphology required placement of granules further from flowers for *G. cuneatum*, whereas granules could be perched immediately adjacent to *S. haleakalae* flowers (and *H. foertherianum* flowers, Fig. 4). The number of taxa visiting WSG granules placed near flowers followed similar patterns among plant species as the number of visitors, with more taxa visiting granules placed near flowers of *S. haleakalae*, *G. cuneatum* and *H. foertherianum*, and fewer taxa visiting granules place near flowers of *S. taccada* (Fig. 20). This pattern again generally followed the number of taxa visiting the flowers of those plant species (Fig. 20).

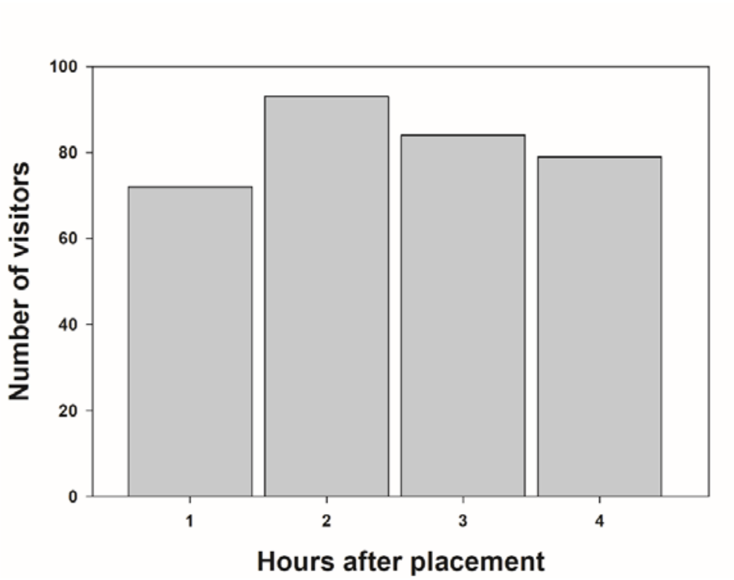

Figure 18. Histogram showing frequency of visitation during each hour after bait placement for WSG placed near flowers. Number of visitors shown for each 60 minute period ending in the hour indicated.

When visitation to WSG granules was examined across the four plant species, there were no significant differences in the number of visits per observation event, the number of visiting taxa per event, or the duration of visits among the three WSG types (Fig. 21). (To facilitate comparison of the majority of visit durations, 9 visits (out of 385 analyzed) that lasted longer than 15 minutes each were excluded from this analysis. These were all made by small crambid moths or in one case a katydid.) The lack of differences in visitation among WSG types supports the inference that differences in visitation to WSG on different plant species (Figs. 19, 20) is driven by differences in attraction to the flowers of those species, rather than differences in attraction to the WSG types.

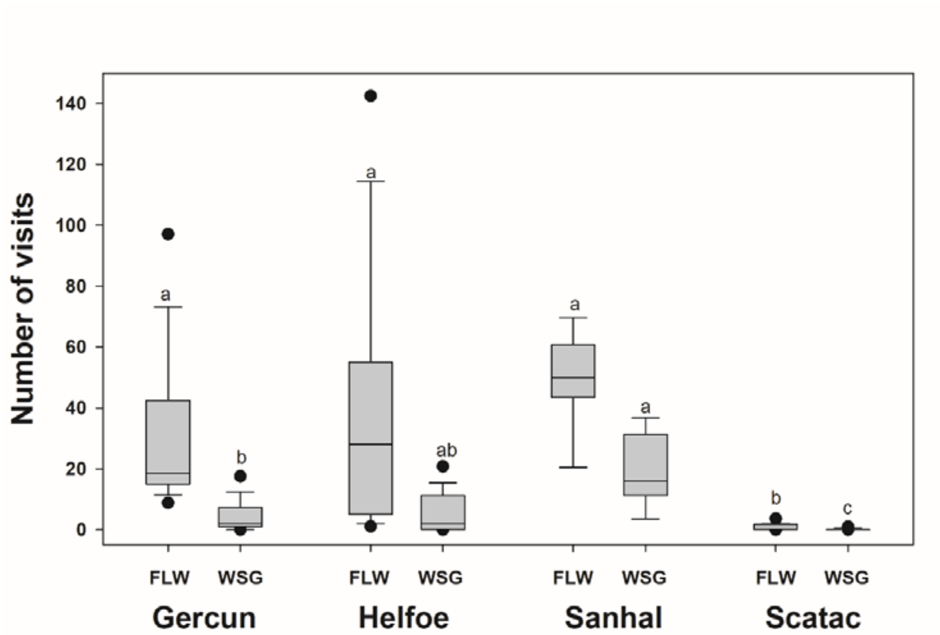

Figure 19. Box plots showing the number of non-ant visits to flowers and to WSG granules placed adjacent to flowers for each of four plant species. Boxes, whiskers and outliers as in Fig. 7. Boxes sharing the same letter within each substrate type (flower or WSG) are not significantly different (generalized linear models, α = 0.05). Number of visits to WSG granules on Sanhal and Helfoe were marginally significantly different (p = 0.057).

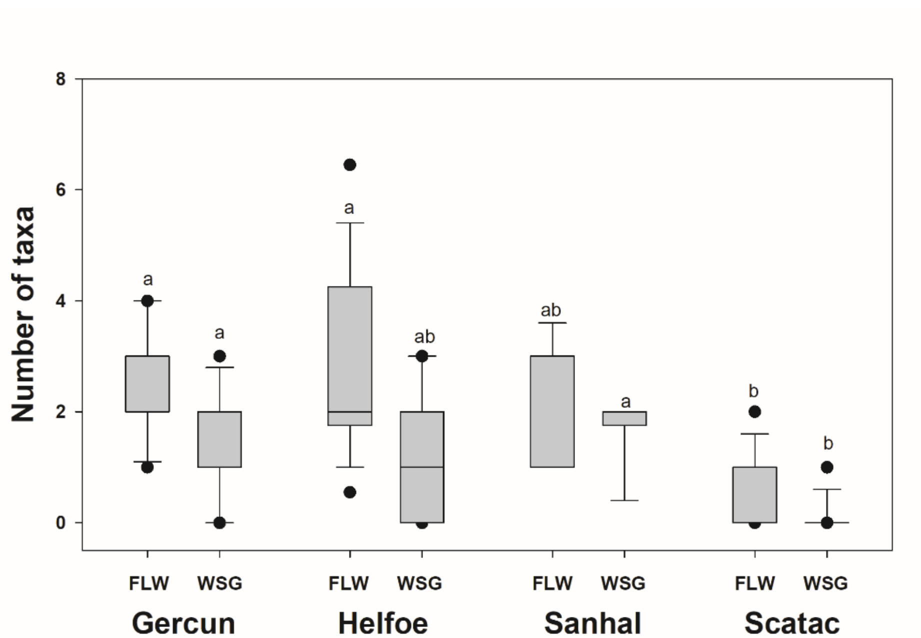

Figure 20. Box plots showing the number of non-ant taxa visiting flowers and WSG granules placed adjacent to flowers for each of four plant species. Boxes, whiskers and outliers as in Fig. 7. Boxes sharing the same letter within each substrate type (flower or WSG) are not significantly different (generalized linear models, α = 0.05). Number of visits to WSG granules on Helfoe and Scatac were marginally significantly different (p = 0.066).

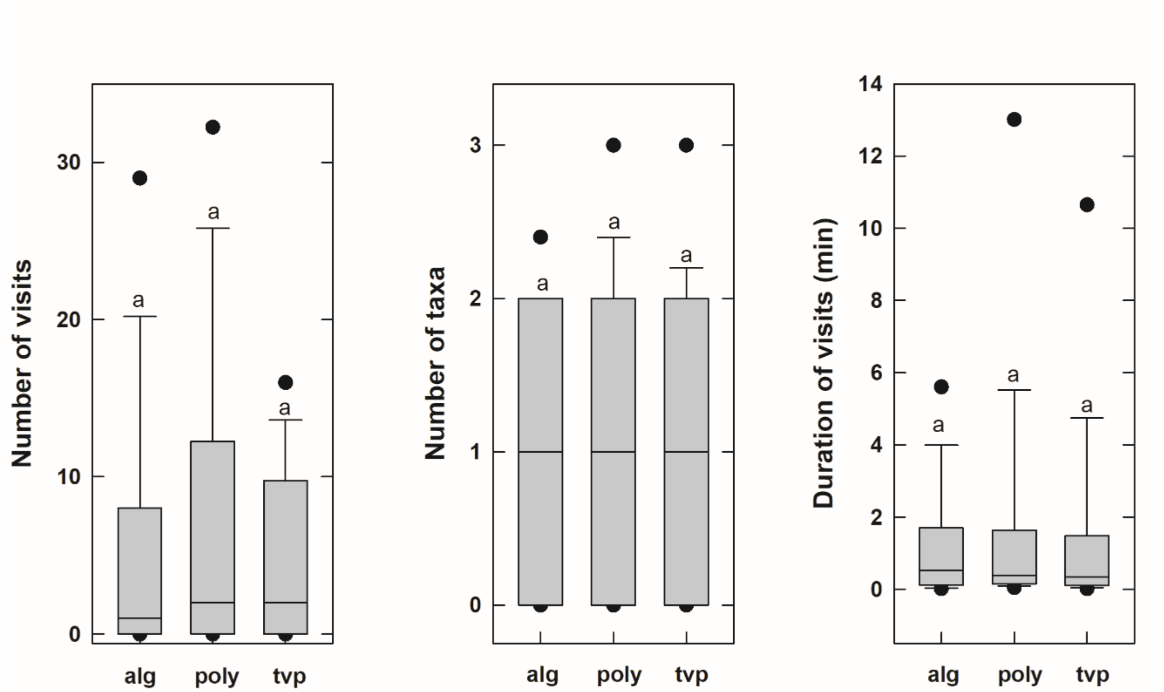

Figure 21. Box plots showing the number of non-ant visits (left panel), the number of non-ant taxa (middle panel), and the duration of non-ant visits (right panel) to WSG of each type placed near flowers. Boxes, whiskers and outliers as in Fig. 7. WSG types sharing the same letter within each panel are not significantly different (generalized linear models, $\alpha = 0.05$).

Non-target species attraction: video observations of birds

Attempts to observe attraction of birds to WSG baits were largely unsuccessful. In the two attempts to film individual piles of granules, birds fled the area and failed to return during the filming period. During the three events in which WSG were broadcast in 20 x 20 m plots, some birds did return to the area and did in fact enter the plots. These included Ruddy turnstones, Pacific golden plovers, Mynahs, Mourning doves and Common waxbills. However, it was not possible to discern the targets of foraging in either the video footage or when observing from a distance with binoculars. Behavior of the birds did not suggest unusual attraction to the baits, as birds did not appear to linger within the plot boundaries, unusual numbers of birds did not congregate in the plots, and foraging behavior (e.g., rate or nature of ground pecking) did not appear to differ when birds were within plot boundaries as compared to when they exited the plots. It is nevertheless not possible to state whether birds did or did not consume WSG bait while foraging within the plots.

Non-target species bait consumption: protein marking and detection

Among honey bees fed with polyacrylamide granules for at least 30 seconds via the proboscis extension reflex, 100% (30 of 30) were clearly marked relative to the negative control-based threshold (mean + 3SD). Among the solitary bees that self-fed on polyacrylamide in lab cages, 70% (21 of 30) tested positive for the protein marker. The rate of marking varied strongly among species: 100% (4 of 4) of *H. strenuus* bees were marked, 86% (12 of 14) of *L. microlepoides* bees were marked, and 42% (5 of 12) of *C. smaragdula* bees were marked. The relatively low marking rate among *C. smaragdula* likely resulted from lower tolerance of the lab protocol (the mortality rate among additional, un-analyzed individuals was noticeably higher for this species), which probably also impacted their likelihood of feeding. Notwithstanding, the combined results indicate that both honey bees and solitary bees are clearly marked when they feed on WSG formulated with 2% rabbit serum.

Ants collected in three of the test plots treated with WSG at KPNAR also demonstrated moderate to high rates of marking relative to the negative control-based threshold. In the plot treated with alginate bead WSG, 100% of ants were marked (28 of 28 *A. gracilipes*); in the plot treated with polyacrylamide, 96% of ants were marked (8 of 8 *A. gracilipes*, and 19 of 20 *O. glaber*); in the plot treated with TVP WSG, 54% of ants were marked (10 of 15 *O. glaber*, and 5 of 13 *P. longicornis*). These results indicate that the rabbit serum marker remains highly active in WSG broadcast in field conditions.

Insects that were collected to test for the possibility of external contamination from the sweep net used during sampling had optical density readings ranging from 0.071 to 0.866. The mean reading was 0.117, and the mean + 3SD equaled 0.507. According to the standard negative control-based threshold, 4 of these insects (out of 53) tested positive for the marker. These were one *Hylaeus nivicola* (Hymenoptera: Colletidae) and three individuals of an unidentified *Hyposmocoma* species (Lepidoptera: Cosmopterigidae). This indicates that some insects can become externally marked when captured in the net, without feeding on the bait. A higher marking threshold based on the mean + 3SD of the net contamination readings may therefore be more reasonable when evaluating test samples collected with the sweep net method. Although this higher threshold still results in one individual being scored as positively marked among the net contamination samples, it reduces the incidence of false positive detections among the treatment samples.

When using the higher net contamination-based threshold, 9.3% of the 441 flying insects sampled across the 18 plots treated with WSG baits were marked (Table 7), suggesting that they fed on or at least came into contact with the baits. When using the lower negative control-based threshold, the percentage increased slightly to 14.0% (not shown). Using the net contamination-based threshold, the incidence of marked vs. unmarked individuals of all taxa combined was significantly associated with the type of WSG used (Pearson Chi-Square = 14.23, $p = 0.001$), with marking rates higher than expected for alginate beads, lower than expected for polyacrylamide, and similar to expected for TVP. Similarly, the incidence of marked vs. unmarked individuals across all WSG types was significantly associated with taxonomic order (Pearson Chi-Square = 15.11, $p = 0.001$), with marking rates higher than expected for Hymenoptera, lower than expected for Lepidoptera, and similar to expected for Diptera (the single Hemiptera individual was excluded from analysis).

Most taxa were either consistently unmarked or exhibited low rates of marking, while several taxa had higher rates of marking (Table 7). Among the latter, the non-native sphecid wasp *Bembecinus* sp. showed the most consistent evidence of feeding on the baits, with 46.9% of the 49 captured individuals being marked. Interestingly, none of the five individuals of the native sphecid wasp *Ectemnius nesiotes* were marked. Marking rates were also high among several species of vespid wasps, including the native *Nesodynerus molokaiensis*, but sample sizes were very low for these taxa so the reported rates should be viewed with caution. Among bees, marking rates were generally low, the highest being for *A. mellifera* (6.7%). However, one of the three marked honey bees captured had an optical density reading of only 0.856 (compared to 3.499 and 3.691 for the remaining two), which is lower than the highest reading among the net contamination samples (0.866). It is therefore possible that this bee may have been externally contaminated in the net. The same may be true for the single native *Hylaeus* bee that was marked, out of 70 captured, as it had an optical density of only 0.678. Other native insects that exhibited at least some incidence of marking were the tephritid fruit fly *Trupanea cratericola* (21.4% marking rate) and an abundant but unidentified case-making moth in the genus *Hyposmocoma* (5.0% marking rate).

Table 7. Percent of individuals that were positively marked (and number of individuals captured) among taxa sampled in the WSG treatment plots. Percents and sample sizes tabulated for each WSG type and for all plots combined. Native taxa denoted with asterisk.

Taxon	**Alginate % marked (n)**	**Poly. % marked (n)**	**TVP % marked (n)**	**All WSG % marked (n)**
Diptera Total	14.3 (14)	5.9 (17)	15.4 (13)	11.4 (44)
Calliphoridae Total	0 (1)	0 (1)	0 (1)	0 (3)
Eucalliphora latifrons	0 (1)	0 (1)		0 (2)
Gonia longipulvilli			0 (1)	0 (1)
Muscidae Total		0 (1)	0 (1)	0 (2)
Muscidae sp.		0 (1)	0 (1)	0 (2)
Pterophoridae Total	0 (3)	0 (3)	0 (4)	0 (10)
Stenoptilodes littoralis rhynchophora	0 (3)	0 (3)	0 (4)	0 (10)
Sarcophagidae Total	33.3 (3)	0 (2)	0 (1)	16.7 (6)
Blaesoxipha plinthopyga	50.0 (2)	0 (1)	0 (1)	25.0 (4)
Ravinia anandra		0 (1)		0 (1)
Sarcophaga albiceps	0 (1)			0 (1)
Syrphidae Total	0 (3)	0 (3)	0 (2)	0 (8)
Allograpta exotica	0 (2)	0 (1)	0 (1)	0 (4)
Simosyrphus grandicornis	0 (1)	0 (2)	0 (1)	0 (4)
Tephritidae Total	25.0 (4)	14.3 (7)	50.0 (4)	26.7 (15)
Bactrocera dorsalis			100 (1)	100 (1)
**Trupanea cratericola*	25.0 (4)	14.3 (7)	33.3 (3)	21.4 (14)
Hemiptera Total		0 (1)		0 (1)
Lygaeidae Total		0 (1)		0 (1)
**Nysius* sp.nr. *abnormis*		0 (1)		0 (1)
Hymenoptera Total	30.2 (63)	6.3 (79)	9.1 (77)	14.2 (219)
Apidae Total	8.3 (12)	0 (27)	9.4 (32)	5.6 (71)
Apis mellifera	9.1 (11)	0 (16)	11.1 (18)	6.7 (45)
Ceratina dentipes		0 (1)		0 (1)
Ceratina smaragdula	0 (1)	0 (10)	7.1 (14)	4.0 (25)
Colletidae Total	0 (20)	0 (31)	5.3 (19)	1.4 (70)
**Hylaeus nivicola*	0 (17)	0 (22)	6.7 (15)	1.8 (54)
**Hylaeus volatilis*	0 (3)	0 (9)	0 (4)	0 (16)
Halictidae Total	0 (3)	0 (7)	0 (3)	0 (13)
Lasioglossum imbrex	0 (2)	0 (1)		0 (3)
Lasioglossum microlepoides	0 (1)	0 (6)	0 (3)	0 (10)
Ichneumonidae Total		0 (2)	0 (4)	0 (6)
Calliephialtes grapholithae			0 (1)	0 (1)
Diadegma blackburni		0 (2)	0 (3)	0 (5)

Table 7. Continued.

Taxon	**Alginate % marked (n)**	**Poly. % marked (n)**	**TVP % marked (n)**	**All WSG % marked (n)**
Sphecidae Total	65.4 (26)	40.0 (10)	11.1 (18)	42.6 (54)
Bembecinus sp.	70.8 (24)	50.0 (8)	11.8 (17)	46.9 (49)
**Ectemnius nesiotes*	0 (2)	0 (2)	0 (1)	0 (5)
Vespidae Total	50.0 (2)	50.0 (2)	100 (1)	60.0 (5)
**Nesodynerus molokaiensis*	0 (1)	100 (1)		50.0 (2)
**Pachodynerus nasidens*		0 (1)		0 (1)
Polistes aurifer	100 (1)		100 (1)	100 (2)
Lepidoptera Total	1.8 (54)	0 (57)	6.1 (66)	2.8 (177)
Cosmopterigidae Total	3.8 (26)	0 (31)	9.1 (44)	5.0 (101)
**Hyposmocoma* sp.	3.8 (26)	0 (31)	9.1 (44)	5.0 (101)
Lycaenidae Total	0 (2)	0 (2)	0 (3)	0 (7)
Brephidium exilis			0 (1)	0 (1)
Lampides boeticus	0 (1)	0 (2)	0 (2)	0 (5)
**Udara blackburni*	0 (1)			0 (1)
micro-Lepidoptera Total	0 (26)	0 (24)	0 (19)	0 (69)
micro-lep sp.1	0 (3)	0 (4)	0 (3)	0 (10)
micro-lep sp.2	0 (1)	0 (5)	0 (3)	0 (9)
micro-lep other spp.	0 (22)	0 (15)	0 (13)	0 (50)
Grand Total	16.8 (131)	3.9 (154)	8.3 (156)	9.3 (441)

CONCLUSIONS TO DATE

I. Aspects related to efficacy

Drying rates and period of attractiveness

The WSG granules dried more quickly than anticipated under all three scenarios tested. Prior studies reported mean T50 times (time until 50% water loss) of roughly 2 to 15 hours or longer for polyacrylamide crystals or alginate beads, depending on relative humidity and substrate conditions (Buczkowski et al. 2014a, Rust et al. 2015, Tay et al. 2017). In comparison, the WSG tested here exhibited median T50 times of under 2 hours in almost all cases. The prior studies may have underestimated rates of evaporation under field conditions because they either used small clumps of granules, which would typically break apart into more rapidly drying individual granules upon impact if they are broadcast, or were conducted in dessication chambers lacking wind and solar exposure. The results in this study suggest that if T50 times are a reliable indicator of period of attractiveness, WSG should have surprisingly short periods of activity under field conditions. This should be especially true for alginate beads and TVP, which exhibited much shorter median T50 times than polyacrylamide, and also had a much narrower range of T50 times in a typical batch of granules. The poorer water retention performance of alginate beads was related to their smaller size, whereas TVP lost water more rapidly per unit size than polyacrylamide.

Despite this poor predicted performance, all three WSG types yielded good results in field efficacy trials (see below). This discrepancy may indicate that 1) ants continue to feed substantially on WSG even after 50% water loss, 2) that a sufficient portion of broadcast granules fall in shaded or other sheltered locations that slow evaporation rates, 3) that uptake of the bait prior to the T50 time is sufficient to achieve good control, or 4) a combination of these is true. Regardless, any provisions that slow rates of evaporation, such as treatment under humid conditions or in the late afternoon for nocturnally active ants, could be expected to increase efficacy.

Bait preference among WSG

There was not much evidence of strong preference for any of the WSG types for any of the ant species tested. The rate of decline in attractiveness of the WSG was faster for AA than YCA, perhaps because of faster drying under drier conditions at HALE. The increasing recruitment over time for LFA was unexpected, but may suggest that WSG will have a longer period of activity with LFA in humid regions like Puna. Overall, the trials suggest that all three WSG types should work well as carriers of the sucrose bait from the perspective of palatability.

Pesticide repellency

The pesticide repellency trials suggest that YCA is quite sensitive to thiamethoxam, while indoxacarb and dinotefuran are not repellant to YCA until concentrations are relatively high. This is consistent with poor results using thiamethoxam and good results using dinotefuran against YCA on Johnston Atoll (Peck et al. 2016). In contrast, AA appear to be quite sensitive to dinotefuran, but exhibited much lower repellency to indoxacarb and thiamethoxam. Good results with thiamethoxam have previously been demonstrated for AA in California and South Africa (Buczkowski et al. 2014b, Rust et al. 2015, Boser et al. 2017). Based on the repellency results, efficacy tests for YCA in Hawaii focused on formulations with indoxacarb and dinotefuran, whereas efficacy tests for AA focused on formulations with indoxacarb and thiamethoxam. Repellency tests with LFA suggest that indoxacarb, and possibly dinotefuran, would be good candidate AI's with which to conduct efficacy tests because of relatively low repellency, whereas LFA exhibited strong repellency towards thiamethoxam.

Efficacy of WSG for controlling ants

The AA efficacy test suggests that both thiamethoxam at 0.0005% concentration and indoxacarb at 0.05% concentration are highly effective at reducing ant densities: numbers dropped sharply (>98%) after a single application in all six plots testing these formulations. Although reductions in plots treated with indoxacarb at the lower concentration of 0.005% were not significantly different from the other two formulations, the number of replications with each formulation, and thus statistical power to detect differences, was low. The average reduction of 87% across the three 0.005% indoxacarb plots may represent substantial differences in control relative to the other two formulations. Although mean reduction increased to 94% after the second application, the lower concentration indoxacarb formulation may still be a less effective option.

For YCA, there were more substantial differences in efficacy among the AI formulations. Both concentrations of dinotefuran tested (0.05% and 0.005%) yielded good results, with >90% reductions in ant numbers with a single application. Multiple applications would be necessary to achieve eradication, and this was not tested here. Dinotefuran has been applied previously in polyacrylamide granules at 0.05% concentration at Johnston Atoll, where eradication is the goal, with highly promising results to date (Peck et al. 2016, 2017). The present results suggest that a concentration of 0.005% may be equally effective for YCA, and perhaps that even lower concentrations may be worth testing. In contrast, indoxacarb formulations performed more poorly. While the higher concentration indoxacarb formulation (0.05%) was not significantly different from the dinotefuran formulations, the mean percent reduction in ant numbers (75.8%) after a single application was substantially lower, and would likely be significantly different from the dinotefuran formulations with a larger sample size. A second application of the 0.05% indoxacarb formulation yielded generally similar results. A clear knock-down effect occurs immediately after treatment, but ant numbers rebound relatively quickly. Relative to the dinotefuran formuations, indoxacarb was apparently not able to kill enough ants to prevent renewed active foraging from surviving nests and/or rapid recolonization by nests outside the plots. The weaker results with indoxacarb could be related to lower solubility of this compound relative to dinotefuran, which may result in less complete availability of the AI in the liquid bait. Furthermore, the poorer indoxacarb results with YCA relative to AA may be attributed in part to extremely high densities of YCA at the study site, or perhaps to the larger size of this ant. Additional efficacy testing with indoxacarb against AA should clarify whether this compound provides consistently good results for this species.

Both efficacy trials found no significant differences in reductions of ants among the three WSG granule types. However, mean percent reductions were inversely related to drying rates of the granule types for both ant species following most applications, with reductions generally following the pattern of highest with polyacrylamide, intermediate with alginate, and lowest with tvp. This may suggest that there are small differences in efficacy among the granule types that could be tied to differences in drying rates and hence longevity of attractiveness. Yet, such differences were fairly minor, particularly for the most effective AI formulations, perhaps owing to the reasons enumerated in the section on drying rates above. Overall, the two efficacy trials indicate that all three types of WSG can successfully deliver sugar water baits laced with pesticides to ants, and yield good results when formulated at the right concentrations with the right AI.

<u>Other considerations</u>

Polyacrylamide was by far the easiest and cheapest WSG type to use. Whereas only 20 g of polyacrylamide crystals are needed to absorb 1 L of sucrose bait, at least 350 g of TVP is needed to absorb the same volume. For a single application at the rate used in this study (55 L bait/ha), this translates to approximately $32/ha for the polyacrylamide crystals used in this study, compared to approximately $183/ha for the TVP used (including shipping). The additional weight and volume of the TVP carrier is another disadvantage. Alginate beads cost approximately $240/ha for materials, but that does not include the considerable labor time needed to produce them. In their current state of development, alginate beads also need to be manufactured fresh for each application, have a short shelf life once manufactured, and present additional logistical challenges compared to the other two granule types.

The main advantage of both the alginate beads and TVP over polyacrylamide is their biodegradable characteristics. Alginate beads disintegrate rapidly in the field, and TVP can be expected to break down fairly quickly as well. The duration of persistence of polyacrylamide granules in the field is unknown, but is clearly longer than the other two granule types, and some of the degradates of polyacrylamide are deemed toxic (Tay et al. 2017). The longer persistence of polyacrylamide granules, however, could increase their efficacy for ant control if they can reabsorb moisture from the environment after their initial application and dessication, and thereby regain some activity (Peck et al. 2016).

II. Non-target risks

Attraction to and consumption of WSG baits by non-target insects

The video observation data suggest that WSG granules that fall to the ground pose relatively low risk to common pollinating insects, as there were few visits by bees, moths and hover flies to baits placed on the ground. However, baits on the ground do attract flies, especially the TVP bait which has a strong odor. WSG that lodge in the vegetation near flowers, in contrast, pose a much higher risk to pollinating insects. Granules located immediately adjacent to flowers attracted many visits by bees, including native and non-native solitary bees and non-native honey bees. Moths and wasps were also relatively common visitors to the granules. When discovered, these insects clearly fed on the granules, in many cases extensively. However, the fact that granules were visited more often on plant species that received higher rates of flower visitation suggests that insects discovered the granules not because they were strongly attractive, but because they were located near a primary source of attraction (flowers). This might suggest that the vast majority of granules that lodge in vegetation at some distance from flowers will not often be discovered by pollinators and other flying insects, which could be tested with further observations.

The latter hypothesis is supported by the results of the non-target broadcast plots. Frequency of consumption of the broadcast baits, as judged by detection of the protein marker placed in the baits, was low among most insect groups including bees and other common pollinators. This likely occurred because most of the broadcast granules were observed to fall to the ground, and relatively little lodged near flowers. Several taxa, however, appeared to find and consume the baits much more readily, in particular the newly detected non-native wasp in the genus *Bembecinus*. Hence, some mortality of non-target insects, including native species, through direct consumption of baits can inevitably be expected. The magnitude of this non-target risk to pollinators may be reduced by broadcasting the baits, when possible, in such a way as to minimize lodging in vegetation frequented by flower-visiting insects.

Attraction to and consumption of WSG baits by birds

Efforts to assess attraction to WSG baits by birds were inconclusive. It is safest to assume that some types of birds will consume at least some of the bait, even if they are not strongly attracted to it. Chickens were observed to eat some of the baits in the bait preference tests, and crows feed on polyacrylamide baits used in Australia (B. Hoffmann, CSIRO Australia, pers. comm.). Risk of WSG baits to birds is therefore best assessed by the toxicity of the active ingredients used in them. Although completely speculative at this point, the stronger odor and organic nature of TVP granules might make them more attractive to birds than the polyacrylamide or alginate granules.

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