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Influence of Varroa Mite (*Varroa destructor*) Infestation Levels and Management Practices on Insecticide Sensitivity in the Honey Bee (*Apis mellifera*)

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ABSTRACT

Varroa mites may cause devastating colony losses throughout the year and especially over winter. In addition to killing honey bees by feeding directly on bodily fluids, these parasites transmit many viral diseases, increase the susceptibility of the honey bee to pathogens, as well as disrupt biochemical and developmental processes. A variety of chemical, mechanical, and cultural practices may be implemented to mitigate mite infestation. While miticide applications are typically the most consistent and efficacious Varroa mite management method, increased sensitivity of honey bees to insecticides via miticide synergism and the eventual evolution of miticide resistance in Varroa mites are reasonable concerns.

We used discriminating potency bioassays to test if mite infestation levels affected sensitivity to three commonly used insecticides. With no other factors considered, higher Varroa mite infestation levels significantly increased sensitivity to naled and imidacloprid, but not to phenothrin. Varroa mite infestation levels measured from newly emerged adults was significantly higher than from sampling from samples of bees on unknown age within the colony.

To test the effects of mite infestation levels and mite management practices on insecticide sensitivity, colonies of Italian honey bee were treated with amitraz (Apivar®) according to the labeled instructions, IPM techniques such as screened bottom boards, drone brood trapping, and powdered sugar grooming stimulation, as well as an untreated control group. Sensitivity to

phenothrin, amitraz, and clothianidin was assessed on a monthly basis from May through October. Measurements of colony health such as adult bee population, brood quantity, brood quality, queen presence, mite infestation levels, pollen collection rates and honey bee weight were also recorded.

Surprisingly, mite infestation levels did not significantly affect the sensitivity to the pesticides we tested. Phenothrin sensitivity was significantly increased by day of the year, pollen collection rate, but significantly decreased by bee weight and queen retention. There was a significant negative relationship between amitraz sensitivity and bee weight. Clothianidin sensitivity was significantly affected by treatment type (amitraz, IPM>Control), and declined with day of the year, brood quality, pollen collection rate, and bee weight. Varroa mite infestation significantly decreased brood quality and honey bee weight. Varroa mite infestation levels were significantly determined by treatment (amitraz < IPM, Control) and day of the year.

These results show that insecticide sensitivity is dynamic throughout the year with fall bees being less sensitive than spring bees likely due to physiological differences between those cohorts. The observation that larger bees and higher pollen collection rates reduce insecticide sensitivity underscores the importance of nutrition on colony health. In-hive amitraz treatment according to the labeled use pattern did not synergize sensitivity to the pesticides tested and should alleviate concern over potential synergistic effects of amitraz observed in laboratory studies. Since IPM practices were largely ineffective at reducing

Varroa mite infestation levels, reliance on chemical methods of Varroa mite management is likely to be prominent in the near future. However, these products must be used judiciously so the long term effectiveness of these compounds could be maximized.

These data demonstrate the complex and dynamics variables that contribute to honey bee colony health. It underscores the importance of controlling for as many of these variables as possible in order to accurately determine the effects of each of these factors as they act alone or in concert with others.

INTRODUCTION

The Varroa mite, *Varroa destructor*, is one of the most important forces responsible for colony declines and increased overwintering colony losses in the honey bee, *Apis mellifera*. Varroa mites feed on the hemolymph of bees at all life stages. This feeding activity can result in premature mortality in all developmental stages of honey bees. Queen loss and starvation are the only factors that are more critical to colony loss than Varroa mite infestation [1,2]. The impacts of Varroa mite infestation can be immediate and profound. In some regions of the US, up to 80% of managed colonies were lost due to Varroa mite infestation in the 1995-96 field season [3]. Varroa mite levels as low as 10 mites per 100 bees can reduce overwintering survival [4].

While the direct damage to honey bees by Varroa mite infestation on its own is evident, Varroa mite infestation indirectly increases the susceptibility to other parasites and diseases. For example, low to moderate Varroa mite infestations can reduce the expression of antimicrobial peptides, dampen immunity function, facilitate virus amplification, and may affect the expression of genes related to behavior [5-7]. High mite infestation can lower pupal and adult weight [8,9], which can lead to lower reproductive output by queens and drones as well as reduced colony maintenance and foraging capabilities by workers.

Varroa mite infestation affects physiological processes that are relevant to insecticide sensitivity. Varroa mite infestation can reduce body size [8,9] and

body size is a universal factor that dictates sensitivity to insecticides. Varroa mites may affect insecticide sensitivity through lowering the titer of vitellogenin in the hemolymph of infested bees [10]. Vitellogenin is a carrier protein that can act to sequester xenobiotics and limit oxidative stress [11], and high vitellogenin levels may account for the different acaricide sensitivities between workers and queens [12]. Varroa infestation mutes expression of genes involved in metabolic detoxification and oxidative stress [13]. Due to its effects on body size, vitellogenin titers, and metabolic gene expression, it is reasonable to conclude that Varroa mite infestation may increase insecticide sensitivity.

The goal of this study was to evaluate if bees infested with high levels of Varroa mites are more sensitive to insecticides than bees with lower levels of mites. In addition, we evaluated colony health indicators in honey bees managed with one of three Varroa mite management strategies.

MATERIALS AND METHODS

Single-Potency Bioassays

Varroa mite infestation was measured in September 2014 from 16 colonies of Italian honey bees (*Apis mellifera ligustica,* Wooten's Golden Bees, Palo Cedro, CA) that were started at nucleus colonies in April 2014 at the USDA-ARS Bee Breeding, Genetics, and Physiology Laboratory in Baton Rouge, LA, under normal field rearing conditions. No mite treatments, antibiotics, or supplemental feedings were administered beyond the scope of this research. Frames with wax-coated foundation and boxes were added based on the needs of the colony. Approximately 300 bees from brood frames were scooped with a 500 mL plastic cup, transferred to a zip-top bag, then stored on ice in the field before freezing overnight at -20°C.

Brood frames with emerging adults were collected from each colony and held at 33±1°C with >70±5% humidity in a dark incubator. One-day-old bees were brushed from the frames and sorted into groups of 20 into disposable wax paper cups and held at environmental conditions listed above with three cotton balls soaked in 50% (w/v) sucrose solution until bees are three days of age. Topical bioassays with phenothrin and naled as well as a feeding bioassay with imidacloprid were performed with diagnostic doses or concentrations that result in 50% (66 ng/bee), 33% (33 ng/bee) and 25% (4 ng/mL) mortality for phenothrin, naled, and imidacloprid, respectively [14]. Topical bioassays with phenothrin and naled were performed by applying a 1 uL drop of insecticide to the

thoracic notum of a bee anesthetized on CO_2 for less than 1 minute with a mechanical Hamilton syringe. The 20 anesthetized bees were weighed after treatment. Bees were held at the environmental conditions listed above. A feeding bioassay with imidacloprid was conducted by placing a perforated microcentrifuge tube with filled with 1 ml of imidacloprid in 50% sucrose solution through the tulle covering the waxed cup and removing the cotton ball soaked with sucrose solution. Control feeding assays were conducted with 50% sucrose solution with 0.001% acetone. Mortality in all bioassays was recorded at 24 hours after insecticide application. Individuals that are ataxic or unable to right themselves were scored as dead.

Bees used in bioassays were frozen at -20°C overnight. Varroa mites left over in the bioassay cups were collected and counted. Varroa mites were dislodged from frozen bees collected from brood frames in the colony and from bioassays by shaking in warm soapy water at 120 rpm on an orbital table shaker for 1hr. Samples were shaken until no additional Varroa mites were dislodged. The Varroa mite infestation level was calculated by dividing the total number of mites collected from bioassay cups and bee washes by the number of bees in the sample (# mites/100 bees).

Seasonal Management Experiments

Thirty-six, deep frame, nucleus colonies of Italian bees were established on 4-May-2015 as described above. The colonies were divided into three equal

157 treatment groups (Control, Amitraz, and IPM) so that Varroa mite infestation
158 levels were equal among groups. Varroa mite levels were not managed in the
159 control group. The amitraz group received treatments in the form of Apivar®
160 strips according to the label instructions. One strip was placed in the brood
161 comb of the colonies from 13-May-2015 through 7-July-2015. A second Apivar®
162 treatment was initiated 1-Sept-2015. One strip was applied for every 5 frames in
163 the brood chambers. Mite levels in the IPM group were managed using non-
164 chemical control methods of screened bottom boards, drone brood Varroa
165 mite trapping, and coating bees with powdered sugar to dislodge Varroa mites.
166 Screened bottom boards were installed at colony establishment on 4-May-2015
167 and remained in place through the duration of the experiment. Drone comb
168 was installed at colony establishment. Drone comb was removed and frozen
169 when sealed drone brood was present and replaced with empty drone comb
170 as needed over the course of the experiment. Bees were treated with
171 powdered sugar by removing frames and coating bees with an even layer of
172 powdered sugar by shaking powdered sugar through a screened shaker.
173 Powdered sugar treatments were administered 6-May-2015, 8-July-2015, and 2-
174 Sept-2015.

175 Colony health was assessed by measuring the population of adult bees,
176 amount of brood, brood quality, queen survivorship, Varroa mite infestation
177 levels, and pollen collection rates using standard procedures [15,16]. Adult
178 population was estimated by counting the number of full deep frames of adult

bees in each colony. Brood quantity was estimated counting the cumulative surface area of all capped worker brood comb in the colony so that for every 100 in brood quantity represents a full side of a deep frame covered with capped worker brood. Brood quality was measured by taking 4 counts of empty brood cells in a 100 total cell survey area. Queen retention was measured by observing if a queen was present on a monthly basis. Queen loss was confirmed by lack of eggs, presence of queen cells, or a supersedure queen. Varroa mite infestation levels were measured by sampling approximately 300 bees from brood bearing comb with a 500 ml plastic cup, placed into a zip top plastic bag on ice in the field, and then frozen at -20°C overnight. Varroa mites were dislodged from bees and infestation rate was calculated as described above. Pollen collection was measured by installing pollen traps at the entrance of each colony. Pollen traps were closed each time frames are removed for bioassays and then opened when frames were replaced. The amount of pollen collected was standardized in grams of pollen collected per day. Due to high mite infestation levels and a large population of bees in the Control and IPM treatments that were symptomatic for deformed wing virus (DWV) and chronic bee paralysis virus (CBPV), our final sample collections occurred in October.

Pesticides

We evaluated the sensitivity of honey bees to the insecticides phenothrin and clothianidin, the miticide amitraz, and the fungicide chlorothalonil.

Phenothrin is widely used in mosquito control programs, while clothianidin is used as a seed treatment in many industrially grown crops. Amitraz is used as an in-hive chemical treatment to control mites. Chlorothalonil is a widely used agricultural fungicide that is commonly found at high levels in honey bee colonies [17]. All materials were >98% purity and were purchased from ChemService (West Chester, PA). Stock solutions of each compound were dissolved in acetone.

Bioassays

Bioassays were performed as previously described [14]. Brood frames were collected from each colony the first Monday of each month (May through October) and held at 33±1°C with >70±1% humidity in a dark incubator. One-day-old bees were brushed from the frames and sorted into groups of 20 into disposable wax paper cups and held at environmental conditions listed above with three cotton balls soaked in 50% (w/v) sucrose solution until bees are three-days of age.

Stock solutions were diluted to include more than four concentrations that provided more than 0% and less than 100% mortality. Dilutions for phenothrin, amitraz, and chlorothalonil stock solutions were done in acetone, while clothianidin was diluted in 50% (w/v) sucrose solution. Topical bioassays with phenothrin, amitraz, and chlorothalonil and feeding bioassays with clothianidin were performed as described above. At least three reps of 20 bees per cup

were used at each dose for each pesticide was used to determine LD_{50} values with a minimum of 200 bees per pesticide per treatment.

Statistical Analyses

All statistics besides probit analysis were performed with JMP 12 (SAS, Cary NC). Correlation of Varroa mite infestation levels with insecticide sensitivity in discriminating potency bioassays was compared with linear regression. Comparison of Varroa mite infestation levels from bees collected from the colony or from bioassays was compared with Wilcoxon Ranked Sum Test. The LD_{50} value for each insecticide for each colony was calculated using probit analysis with Abbot's correction for control mortality [18] and standardized by body weight using Minitab (State College, PA). Toxicity was considered significantly different if the 95% CI of the LD_{50} values did not overlap between colonies or test dates. Differences in the number of frames of adults, amount of brood, brood quality, Varroa mite infestation, honey bee weight, and pollen collection rate were compared using One-Way ANOVA with Fisher's exact test. Queen retention over time among treatment groups was analyzed by Kaplan-Meier survival estimates. The interactions of colony health measurements and LD_{50} values (not standardized by weight) were assessed with a General Linear Model.

RESULTS

Single-Potency Bioassays

Varroa mite infestation measured from newly emerged adults used in bioassays was significantly higher than infestation rates measured from honey bees collected from the colony (Wilcoxon Rank Sum Test, W=-116.0, Z=-2.9, p=0.001). On average, Varroa mite infestation measured from newly emerged bees was 2.2-fold higher than from bees collected from the colony (Figure 1). Sensitivity to naled (df=12, F=6.60, p=0.026, R^2=0.37, Figure 2A) and imidacloprid (df=8, F=7.50, p=0.029, R^2=0.52, Figure 2B) significantly increased with higher Varroa mite infestation levels from newly emerged bees in bioassay cups. Phenothin sensitivity was not significantly correlated with Varroa mite infestation levels from newly emerged bees in bioassay cups (df=15, F=0.03, p=0.853, R^2=0.002, Figure 2C). Control mortality significantly increased with higher Varroa mite infestation levels from newly emerged bees in bioassay cups (df=15, F=5.27, p=0.04, R^2=0.58, Figure 2D). When using Varroa mite infestation levels calculated from honey bees collected from the colony, sensitivity to phenothrin (df=15, F=0.35, p=0.56, R^2=0.02) and naled (df=12, F=0.03, p=0.86, R^2=0.002) were not significantly correlated. Imidacloprid sensitivity (df=8, F=6.18, p=0.04, R^2=0.47) and control mortality (df=15, F=5.72, p=0.03, R^2=0.29) were significantly correlated with Varroa mite infestation levels calculated from bees collected from the colony.

Seasonal Mite Management

Colonies receiving IPM treatment for mites had a lower number of frames of bees in May compared to the amitraz treatment ($F=5.14$, $p=0.035$) as well as in June ($F=4.68$, $p=0.017$) compared to the control and amitraz treatments (Figure 3). There were no differences in the number of frames of bees among the three treatment groups from July through September. In October, the IPM treatment group had significantly fewer frames of bees compared to amitraz treated colonies ($F=6.05$, $p=0.029$). Besides the number of frames of bees, the growth dynamics in number of frames of bees varied among treatments. Colonies in the control group grew in a manner best fit with an exponential rise to maximum ($R^2=0.98$, $F=131.13$, $p=0.001$). A linear growth curve best fit the increase in frames of bees in the amitraz treatment ($R^2=0.97$, $F=139.19$, $p=0.0003$). The linear growth in the number of frames of bees in the IPM colonies was significantly altered with the population decline in the October samples ($R^2=0.62$, $F=6.64$, $p=0.06$).

Brood quantity was mostly similar among treatments (Figure 4). The only significant difference was that the IPM group in June had less brood than the control and amitraz treatment groups ($F=3.25$, $p=0.05$). There were no differences in the brood quantity within the control ($F=0.29$, $p=0.91$) and amitraz treatments ($F=0.57$, $p=0.72$) across the duration of the experiment. Brood quantity in the IPM colonies increased significantly in September compared to May and June, but returned to low levels in October ($F=2.77$, $p=0.05$).

There were few significant differences in brood quality score (#uncapped brood cells/100 cells) among or within all treatments mostly owing to high variation among all colonies (Figure 5). Brood quality scores were significantly higher for the IPM colonies compared to the control colonies in June ($F=4.85$, $p=0.04$). The brood quality score in the IPM colonies was significantly higher than the amitraz colonies in October ($F=4.78$, $p=0.02$). The only significant difference within treatments through the duration of the experiment was that brood quality in the control group was higher in July than in May and June ($F=3.05$, $p=0.016$).

Varroa mite infestation rates were variable among treatment groups, but the amitraz treated group was consistently the lowest (Figure 6). Despite starting with equal Varroa mite infestation levels, Varroa mite infestation was significantly lower in the amitraz treated colonies compared to control and IPM colonies in June ($F=6.61$, $p=0.004$) and July ($F=9.65$, $p=0.001$). Amitraz treated colonies had lower Varroa mite infestation levels than the control group, but not the IPM group in August ($F=3.43$, $p=0.05$) and September ($F=3.89$, $p=0.05$). The amitraz treated colonies had lower Varroa mite infestation levels than both the control and IPM groups in October ($F=4.6$, $p=0.02$). Varroa mite infestation levels increased throughout the duration in all treatment groups. The control ($F=7.2$, $p<<0.001$) and amitraz treated colonies ($F=11.31$, $p<<0.001$) had mite levels in September and October that were higher than all of the preceding months. Varroa infestation was higher in September than October in the amitraz treated group. The IPM group Varroa infestation level in October was significantly higher

than the preceding months and the September infestation level was significantly higher than in May ($F=6.62$, $p<<0.001$). The pattern and rate of increase in Varroa mite infestation levels were different among treatments. The pattern of mite growth in the control group was exponential and significant ($R^2=0.63$, $p<0.0001$). A linear relationship was seen in the amitraz treated group, but the relationship was poor and insignificant ($R^2=0.27$, $p=0.28$). Varroa infestation in the IPM group increased in an exponential manner and was highly significant ($R^2=0.95$, $p=0.0009$). The rate of increase in Varroa mite infestation in amitraz treated colonies was significantly lower than in control ($DF=8$, $t=2.39$, $p=0.044$) and IPM colonies ($DF=8$, $t=2.33$, $p=0.047$).

Much like other measurements of colony health, pollen collection was variable between treatments and time (Figure 7). Pollen collection in June was significantly lower in the IPM group compared to the control ($F=2.85$, $p=0.05$). The amitraz treated colonies collected significantly more pollen in October compared to the control and IPM colonies ($F=3.36$, $p=0.05$). Pollen collection varied within the control ($F=3.01$, $P=0.027$), amitraz ($F=9.34$. $p<0.001$), and IPM colonies ($F=2.7$, $p=0.047$). Within all treatments, there were no differences in pollen collection from June through August. The control group collected significantly less pollen in September compared to June and October, while the October pollen collection was also significantly higher than in July. Pollen collection in the amitraz group was significantly lower in September compared to August and October, but October pollen collection was significantly higher

than all other months. October pollen collection in the IPM group was significantly higher than all other months besides August. There were no differences in pollen collection from June through September in the IPM group.

Honey bee weight varied among treatments and through time (Figure 8). Honey bee weight in the IPM colonies was significantly lower than the control colonies in May ($F=6.19$, $p=0.024$), but to both control and amitraz colonies in June ($F=7.91$, $p=0.001$). In September ($F=5.46$, $p=0.026$) and October ($F=4.57$, $p=0.05$), honey bee weight in control colonies was significantly lower compared to amitraz treated colonies. Honey bee weight increased peaked in July and August in all treatments. Bee weight in the control colonies was highest in July and August, while bee weight in May, June, and October were not significantly different as was bee weight in June, September and October.

Queen loss was constant and linear throughout the experiment in all treatment groups (Figure 9). However, the rate of queen loss was significantly higher in the control group compared to the amitraz treatment and the IPM treatment (Kaplan-Meier, Wilcoxon $\chi^2=6.60$, $df=2$, $p=0.037$) There was no difference in the rate of queen loss among the amitraz and IPM treatments. Based on the linear equation of the line of queen survivorship over time, control, amitraz, and IPM colonies would go extinct by 263 days (95%CI=239-288 days), 691 days (95%CI=652-732 days), and 422 days (95%CI=383-461 days), respectively. There was no difference in the rate of queen supersedure among treatments ($df=6$, $\chi^2=6.01$, $p=0.42$).

Bioassays varied among and within treatment groups over time. Chlorothalonil was unable to kill bees in any treatment group in any month when applied at the dose of 100 ug per bee, which was near the solubility limit of chlorothalonil. Phenothrin sensitivity was equal among all treatments in May and July (Table 1). Bees in the IPM treatment group had significantly higher phenothrin sensitivity in June compared to the control and amitraz treatments. In August, both the control and IPM treatments had higher phenothrin sensitivity than the amitraz treatment. However, the amitraz treated bees were more sensitive to phenothrin in September and October than the control. Within the control group, phenothrin sensitivity was not significantly different from the initial sensitivity evaluated in May in any month. The highest phenothrin sensitivity in the control group in August was significantly different than all other months besides May, while the lowest sensitivity was seen in July and September. Phenothrin sensitivity in the amitraz treated group was significantly higher in October compared to any other month. Furthermore, phenothrin sensitivity in the amitraz treated group was significantly higher in June compared to May and August. Phenothrin sensitivity was highest in the IPM group in June and it was significantly different compared to July, August, and September. August's LD_{50} value was significantly different compared to June, July, and September.

Sensitivity to amitraz varied with no consistent pattern among and between treatment groups (Table 2). In May, amitraz sensitivity was higher in the control and amitraz groups compared to the IPM group. Amitraz sensitivity was

highest in the amitraz treated group in June compared to the control and IPM groups. The IPM group was more sensitive to amitraz than the amitraz treated group in August. In September, amitraz sensitivity was highest in the amitraz treated and IPM colonies. The control group was more sensitive to amitraz than the amitraz treated group in October and the LD_{50} for amitraz was unable to be calculated from the IPM group in October.

Clothianidin sensitivity was significantly higher in the control and amitraz group than the IPM group at in May (Table 3). The IPM group was more than 11-fold more sensitive to clothianidin than the control group in June. The IPM group was more sensitive to clothianidin than the amitraz group in August. In September, the amitraz group was more sensitive to clothianidin than the control group. The amitraz and IPM groups were more sensitive to clothianidin than the control group in October. Within the control group, clothianidin sensitivity was highest in May, June, and July, intermediate in August and October, and lowest in September. Clothianidin sensitivity in the amitraz group was highest in May and June which was significantly different from July, which was significantly different from October, which was significantly different from August and September. The IPM group had the highest sensitivity to clothianidin in June while the lowest sensitivity was in May and September.

Interactions of Measurements of Colony Health

The number of frames of bees was significantly increased by day (F=6.97,

p=0.009, m=0.021), brood quantity (F=39.73, p<0.001, m=0.0094), and pollen collection (F=3.71, p=0.05, m=0.013) and the model had an R^2 of 0.546.

The model for brood quantity with an R^2 of 0.661 showed that day (F=6.35, p=0.013, m=-1.032), and frames of bees (F=39.73, p<0.001, m=24.94) significantly increased brood quantity, while brood quality (F=84.78, p<0.001, m=-4.24) significantly decreased brood quantity.

Brood quantity (F=84.78, p<0.001, m=-0.094) significantly decreased brood quality, while Varroa mite infestation level (F=8.92, p=0.003, m=0.529) significantly increased brood quality, and the model yielded an R^2 of 0.604.

Varroa mite infestation was significantly influenced by treatment (F=5.51, p=0.005, m_{Treat2}=5.63, m_{Treat3}=2.35), and significantly increased with day (F=6.52, p=0.012, m=0.0747), and significantly decreased with brood quality (F=8.92, p=0.003, m=0.122) and weight (F=6.96, p=0.009, m=-0.285). The mite model yielded an R^2 of 0.408.

Pollen collection rate was significantly increased with day (F=16.82, p<0.001, m=0.391) and queen retention (F=13.36, p<0.001, m=104.8) with a model R^2 of 0.343.

The model for honey bee weight (R^2=0.143) varied by treatment (F=3.82, p=0.024, m_{Treat2}=0.44, m_{Treat3}=-4) and significantly decreased with mite infestation level (F=6.96, p=0.009, m=-0.179).

Queen loss was well described by the model (R^2=0.711) with treatment varied (F=30.36, p<0.001, m_{Treat2}=-0.041, m_{Treat3}=-0.147), day significantly

decreased queen survival ($F=136.71$, $p<0.001$, $m=-0.0024$), and pollen collection ($F=13.36$, $p<0.001$, $m=0.0009$) significantly increased queen survival.

Phenothrin sensitivity was significantly increased by day ($F=5.01$, $p=0.032$, $m=0.00008$), significantly decreased by pollen collection ($F=10.49$, $p=0.003$, $m=-0.00011$), bee weight ($F=9.71$, $p=0.004$, $m=0.0003$), and queen retention ($F=7.56$, $p=0.01$, $m=0.0324$). The phenothrin sensitivity model yielded an $R^2=0.512$.

The only factor that was significant for amitraz sensitivity was bee weight ($F=15.64$, $p<0.001$), but it was a negative relationship ($m=-0.013$). The model produced an $R^2=0.444$.

The model showed that treatment varied clothianidin sensitivity ($F=4.68$, $p=0.016$, $m_{Treat2}=0.00003$, $m_{Treat3}=-0.000017$), and significant decreases with day ($F=22.9$, $p<0.001$, $m=0.000001$), brood quality ($F=6.19$, $p=0.018$, $m=-0.000002$), pollen collection ($F=7.0$, $p=0.12$, $m=-0.000001$), and bee weight ($F=9.75$, $p=0.004$, $m=0.000002$). This model was highly descriptive with an R^2 of 0.837.

DISCUSSION

Honey bee colony health are complex and dynamic manifestations of an increasingly nuanced summation of biotic and abiotic factors [19]. Understanding the interactions of these factors that promote colony health is of utmost importance to the $20B in the commercial pollination industry in the US [20].

The results of the single-potency bioassays demonstrate the influence of Varroa mite infestation levels on insecticide sensitivity and the need to control for factors that can affect bioassay results and interpretation. The >2-fold difference in Varroa mite infestation levels between honey bees collected from the colony or from newly emerged adult bees is expected as newly emerged adults are more likely to harbor Varroa mites that parasitize the larval and pupal stages. Upon emergence, Varroa mites may disassociate from the infested adult and attach to other adults in the colony, thereby diluting the Varroa mite infestation levels measured from bees of various age and behavioral state within colony as in commonly used methods to collect bees to measure Varroa mite infestation. Measuring Varroa mite infestation levels from newly emerged adults may be a more accurate method when using those bees in bioassays according to our method as the significant relationship of naled sensitivity with Varroa mite infestation level would have been overlooked using calculations from colony-collected bees.

While mite infestation affects bioassay mortality, the fact that Varroa

infestation levels were also correlated with control mortality has toxicological and practical implications. Control mortality needs to be accounted for in bioassays to accurately assess insecticide induced mortality [18]. Measurements of insecticide potency in bioassays with high levels of control mortality are statistically challenging and toxicologically questionable [21]. From a practical standpoint, for every Varroa mite/100 bees, there is a corresponding 1.5% increase in mortality of 4-day old bees. This is important to note as Varroa mite infestation levels are highest in the fall and honey bee reproduction is declining. This finding may help explain why Varroa mite infestation has their most profound effects on colony health late in the season and overwinter.

Varroa mite infestation effects on the sensitivity to naled and imidacloprid, but not phenothrin are curious. The slopes of the increased mortality for naled and imidacloprid with higher Varroa mite infestation levels are identical, suggesting a uniform mechanism of increased insecticide sensitivity to these two classes of insecticides with distinctly different modes of action, target sites, and detoxification pathways [22,23]. The reduction in weight [8,9], lower vitellogenin levels [10], and decrease expression of detoxification enzymes [13] with Varroa mite infestation may explain this shared increase in sensitivity to naled and imidacloprid. However, the fact that Varroa mite infestation did not affect phenothrin sensitivity demonstrates this possible mechanism of increased sensitivity is not universal to all insecticides.

We found that Varroa mite infestation levels increased sensitivity in

discriminating potency bioassays but not in the seasonal mite management. This discrepancy may have arisen because the two measures of sensitivity we used in these two experiments are conceptually divergent. In the discriminating potency bioassays, a single treatment yielded a range of mortalities that was regressed against Varroa mite infestation levels treated as a continuous, untransformed variable, while in the seasonal mite management experiments, multiple concentrations yielded mortality that was converted to probits and regressed against the logarithm of the insecticide concentration. Therefore, there is a fundamental difference in the kinds of data generated between these experiments. There are more chances for type II error when using discriminating potency bioassays as variation increases at insecticide concentrations that are above or below the LD_{50}/LC_{50} as the those values have the least amount of variation by definition [21]. Furthermore, populations may have similar LD_{50}/LC_{50} values, but concentrations above and below LD_{50}/LC_{50} values may produce dramatically different mortalities due to the slope of the line. For example, in the seasonal mite management experiment, the control group had the exact same LD_{50} for phenothrin in both May and June, but the slopes were very different (Table 1). Therefore, if we used the LD_{20} to compare these groups rather than the LD_{50}, the LD_{20} for the control group in May (0.21 (0.11-0.28 95% CI) ng phenothrin/mg bee) and June (0.34 (0.32-0.36 95% CI) ng phenothrin/mg bee) would be significant different by approximately 60%. While using concentrations besides the LD_{50}/LC_{50} values to compare populations may be statistically

unsatisfying, it may be useful to accurately determine the maximum sublethal concentration when comparing sublethal effects between populations as opposed to simply using a dose or concentration that is arbitrarily lower than the LD_{50}/LC_{50} values.

The practical implications of the increased insecticide sensitivity with Varroa mite infestations as measured in our discriminating potency bioassays are likely to be minimal. The use of organophosphates in general are being greatly reduced [24] and naled specifically is usually applied by aircraft to control mosquito populations only in the event of a regional outbreak of mosquito-borne disease (Randy Vaeth, personal communication). Exposure to neonicotinoids (i.e. imidacloprid, clothianidin) typically occurs by encounters with dust emitted from planters depositing neonicotinoid treated seed in late April [25] or when corn and soy shed pollen in July [26], which is separated in time from peak Varroa infestation levels in October. Furthermore, neonicotinoids in honey bee colonies are typically found very infrequently and at very low concentrations [17,27,28], far below the concentration we used in our single-potency bioassays. The fact that insecticide sensitivity was not correlated with Varroa mite infestation levels in our seasonal mite management experiments validates the assertion of minimal practical impacts.

While our seasonal mite management experiments were terminated in October, we originally planned to continue our experiments through December. However, the control and IPM colonies in October displayed a high frequency

of overt symptoms of deformed wing virus (DWV) and chronic bee paralysis virus (CBPV), presumably due to high mite infestation rates in those treatment groups. Although viral titers were not measured, the high prevalence of infection leading to poor adult emergence would have confounded our bioassay results, especially in the case of amitraz bioassays in the IPM group in October. We did not use any colonies that had a high prevalence of virus-mediated symptoms. Future experiments to assess insecticide sensitivity in bees of known virus infection rates will determine possible interactions of these factors.

The difference in the colony growth dynamics was somewhat unexpected. The control and IPM groups followed normal colony growth dynamics with a stable rise and slight drop off late in the year [29]. The linear increase in the number of frames of bees in the amitraz group suggested that the amitraz treatment released those colonies from the seasonal factors that slow or inhibit colony growth. This is probably due to reduced mite pressure or possibly an octopaminergic pathway. Octopamine levels in honey bee brains decrease after peaking early in the summer [30], which is correlated with colony size over that time. However, a causal relationship between octopamine levels and colony population levels remains unresolved. It is possible that amitraz may inhibit the signal to slow colony growth over time because although mite infestation levels were lower in the amitraz treatment group, they are not a significant factor in determining the number of frames of bees. While the IPM treatment had less frames of bees than the control and amitraz treatment

groups in May and June, this was the result of ensuring the colonies were distributed among groups with equal mite infestation rate as this was the major hypothesis being tested.

Much like frames of bees, the age of the colony, frames of bees, and brood quality scores were significant determinants of brood quantity. Despite the potential collinearity of these variables, none of them could be excluded from the model with the as the Variable Inflation Factors (VIF) never exceeded 3.5 for any factor in any model.

Spotty brood patterns are a tell-tale symptom of high levels of mite infestation [31]. In our experiments, mite infestation level was a significant factor in determining brood quality, demonstrating that mite infestation leads to patchier brood patterns despite not affecting overall brood quantity.

The differences in Varroa mite infestation level between treatments showed that amitraz has a significant impact on mite populations compared to control and IPM treatments. The control and IPM colonies reached Varroa mite infestation levels by September of 21.2 and 9.5 Varroa mites/100 bees, respectively, that are strong indications that those colony will die out [32,33]. The screened bottom boards and powdered sugar treatments administered in the IPM treated group were not adequate to suppress mite levels that were different than control measures. IPM treatment did slow the rate of Varroa mite infestation in August and September to the point where it was not statistically different from the amitraz treatment. However, the IPM treatment did not stop

566 the dramatic increase in Varroa mite infestation so that in October the IPM
567 treatment produced the highest Varroa mite infestation levels seen in the
568 experiment. These findings are largely in line with previous reports that IPM
569 measures provide limited effectiveness at controlling Varroa mite populations at
570 the colony level through the season [34,35]. Drone brood trapping was likely to
571 not very effective in our treatment scheme because very little drone brood
572 (<200 capped drone cells/comb) was present at any time it was removed from
573 the colony. Other studies of drone brood trapping in reducing Varroa mite
574 infestation levels were effective when large number of drone brood were
575 removed (>7000 capped drone cells [36], >3000 cells [37]). The lack of
576 consistently efficacious and easily administered IPM techniques results in an
577 increased emphasis on chemical control in colonies headed by non-hygienic
578 queens. It is likely that Varroa mite suppression by amitraz treatment would have
579 been enhanced if treatment would have been continuous throughout the year.
580 The gap between amitraz treatments (to mimic a honey harvest) allowed for the
581 mite population to rebound dramatically. For practical purposes concerning
582 managing mites with chemical means, the beekeeper may have to balance
583 taking a honey crop with colony survival. The current Apivar® label limits
584 application to 2 treatments annually with a 56 day maximum treatment interval.
585 Amendment of the Apivar® label to allow uninterrupted, year round, treatment
586 would very likely improve product effectiveness in the short term. However, a
587 constant treatment regime would also increase selection pressure for amitraz

588 resistance in Varroa mites. The loss of effective amitraz treatments to control
589 Varroa mites is a disconcerting prospect due to the low rate of product
590 development to specifically and effectively control Varroa mites.

591 Queen loss was significantly higher in the control group compared to the
592 amitraz and IPM group. It is likely this difference is due to the differences in
593 queen establishment between the treatment groups with all the queens
594 surviving initially in the control group in May and June. Excluding those time
595 points, the amitraz treatment experiences significantly less queen loss than both
596 the control and IPM treatment. While it is highly probable that reduced mite
597 pressure shortens the life span of the queen, this result seems to suggest that
598 octopamine may play a role on queen longevity, which is largely dominated by
599 biochemical pathways involving insulin-like peptides, juvenile hormone, and
600 vitellogenin [38]. The significance of treatment type is curious because
601 treatment was a significant factor in mite levels and weight, but both of those
602 factors were not significant factors to explain queen loss. Queen loss was
603 significantly negatively correlated with pollen collection rate. Pollen collection
604 rate is typically driven by brood quantity [39] and brood pheromone [40]. The
605 presence of a queen or queen pheromone such as 9-oxodecenoic acid
606 stimulate nectar foraging, but not pollen foraging [41]. The influence of the
607 queen on pollen collection is transitive via brood deposited by the queen [42].
608 However, there was no relationship between brood quantity and queen
609 survivorship in our model, which was highly descriptive. The fact that queen loss

610 was significantly affected by treatment, day, and pollen collection rate
611 underscore the manner in which external factors influence queen longevity.
612 Worker bee weight was significantly influenced by treatment and mite
613 infestation level, with those two factors having already been established as
614 significantly interacting. The influence of mite infestation on bee weight has
615 been previously demonstrated. For example, drone brood with 1-3 mites per
616 pupa significantly reduced pupal weight by the red eye stage as well as smaller
617 adult drones compared to uninfested pupae. In cases of extreme Varroa mite
618 infestation of 20 mites per pupae, newly emerged adult drones were 50% lighter
619 than adults from uninfested pupae [8]. In addition to weight, Varroa infestation
620 levels also reduced protein and carbohydrate concentrations [9] as well as
621 vitellogenin titers [10] in newly emerged workers.
622 The fungicide chlorothalonil is commonly found at high concentrations in
623 wax and pollen [17]. This frequent and copious detection of chlorothalonil may
624 be concerning because at concentrations of 34 mg/L (i.e. 34,000 ppb),
625 chlorothalonil results in >50% mortality to 6-day old honey bee larvae in chronic
626 toxicity bioassays and synergizes toxicity of fluvalinate and coumaphos at low
627 concentrations [43]. Chlorothalonil was unable to kill adult bees under our
628 experimental conditions. Despite chlorothalonil treatment of 100 ug/bee, this
629 dose was below the reported LD_{50} of 181 ug/bee for chlorothalonil from the EPA-
630 OPP Pesticide Ecotoxicity Database
631 (http://www.ipmcenters.org/Ecotox/Details.cfm?RecordID=29837). It is important

to note that this LD_{50} is presented without slope or confidence intervals, so its quality as a reference value is reasonably questionable. Although high concentrations of chlorothalonil were associated with entombed pollen (~1300 ppb), there were no effects of entombed pollen with high concentration of chlorothalonil on adult bee survivorship or larval growth [44]. It is important to note that chlorothalonil concentrations the study by vanEnglesdorp et al. were 10-fold lower than the study of Zhu et al. showing high larval mortality. The concentration found in entombed pollen also is near the mean concentration of chlorothalonil found in pollen within colonies (1593 ppm [17]). Therefore, it seems chlorothalonil poses very little hazard to adult bees, but may be detrimental to larvae at unusually high concentrations.

As classical toxicological principals would predict, weight was a significant factor in determining insecticide sensitivity in all cases. However, mite infestation level was not. This incongruence is surprising considering the highly significant interaction of weight and mite infestation levels in our model. A further confounding issue is that mite infestation causes the downregulation of cytochrome P450 monooxygenases [13,45] that are involved in detoxification of insecticides such as pyrethroids and neonicotinoids [46,47]. In the case of amitraz, sensitivity increased with an increase in body weight, which is counterintuitive. While this is an unusual observation, there was a negative correlation between body size and abamectin and β-cypermethrin in the oriental fruit fly, *Bactrocera dorsalis* [48].

There was a significant influence of date in the sensitivity of honey bees to phenothrin and clothianidin, but not amitraz. The positive relationship seen in both cases show that fall bees are less sensitive to insecticides than spring bees. A similar pattern of seasonal sensitivity was seen with sensitivity to diazinon that correlated with cytochrome P450 activity [49]. Since both phenothrin and clothianidin are capable of being detoxified by P450s [14,50,51], it is likely the seasonal variation in P450 activity may underlie the seasonal sensitivity to these insecticides. Amitraz sensitivity does not follow the same seasonal variation presumably due to P450 detoxification because it appears that P450s do not detoxify amitraz in honey bees as the P450 inhibitors piperonyl butoxide (PBO) or prochloraz do not synergize amitraz sensitivity in honey bees [52].

The significant effect of pollen collection on phenothrin and clothianidin sensitivity showed a negative correlation between these factors. This observation is at odds with previous work showing that pollen feeding reduces pesticide sensitivity [53]. However, our experiment was different in that we used 3-day old bees raised on 50% sugar water for 3 days and pollen amount was measured from pollen traps, while the study of Wahl and Ulm used 8-day old bees raised on defined pollen regimes.

While clothianidin sensitivity was higher in the amitraz group compared to the control group in September and October, clothianidin sensitivity was also higher in the IPM group compared to the control in those same two months. Taken together, this shows that amitraz treatment did not synergize clothianidin

676 sensitivity.

677 Queen survivorship was another significant factor in phenothrin sensitivity.
678 Colonies headed by queens were less sensitive to phenothrin than queenless
679 colonies. While the interaction of the multitude of queen pheromones have on
680 worker behavior and development have been well documented [54,55], little
681 data exists on the influence of queen pheromone on insecticide sensitivity and
682 detoxification. While the expression of cytochrome P450s CYP4AA1, CYP4G11,
683 and CYP18A1 are not increased by queen presence [56], those enzymes are
684 involved in caste-specific fatty acid hydroxylation or chemoreception and not
685 detoxification. The possible influence of queens on insecticide sensitivity is
686 particularly concerning as queen longevity is typically less than 1 year in many
687 commercial operations and queen losses are the most often cited factor in
688 colony losses [2,57].

689 This study highlights how many real world practices can affect insecticide
690 sensitivity. These results underscore the difficulty in comparing results of honey
691 toxicology from study to study due to the difficulty in controlling all these
692 variables. As our, and many other studies show, Varroa mites are the major
693 factor affecting colony health and losses. The fact that amitraz strips were a
694 significantly more effective method of controlling Varroa mite than the IPM
695 measures we implemented ensures amitraz will be used more intensely in the
696 short term. Overuse of this product will undoubtedly select for amitraz resistant
697 Varroa mites, thus ensuring the loss of highly effective mite management tool.

Use of Varroa-resistant bees (i.e. Varroa-Sensitive Hygienic (VSH) bees) [58,59] and development of novel, more consistently effective, non-chemical Varroa mite control will likely be long term, sustainable colony management practices.

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Figure Legend.

Figure 1. Difference in mite infestation levels calculated from unaged bees of unknown behavioral status collected off of frames within the colony and from newly emerged adults collected the lab. Infestation levels were significantly more than 2-fold higher in newly emerged adults in bioassay cups than from the colony. Data are shown as the average ± SEM and letters indicate significant differences.

Figure 2. Correlation of Varroa mite infestation levels with sensitivity to naled (A), imidacloprid (B), phenothrin (C), as well as control mortality in 4-day old bees (D). Higher Varroa mite infestation levels were significantly correlated with control mortality and sensitivity to naled and imidacloprid, but not phenothrin.

Figure 3. Frames of adult honey bees over time among the Control, Amitraz, and IPM groups. Data are shown as the average ± SEM. Bars with different letters within the same sampling date indicate significant differences between treatment groups at that sampling date. Bars with different numbers indicate significant differences within treatment groups over sampling dates.

Figure 4. Brood quantity over time among the Control, Amitraz, and IPM groups. Data are shown as the average ± SEM. Significant differences between and within treatment groups is as described in Figure 1.

Figure 5. Brood quality over time among the Control, Amitraz, and IPM groups. Data are shown as the average ± SEM. Significant differences between and within treatment groups is as described in Figure 3.

Figure 6. Varroa mite infestation levels over time among the Control, Amitraz, and IPM groups. Data are shown as the average ± SEM. Significant differences between and within treatment groups is as described in Figure 1.

Figure 7. Pollen collection rates over time among the Control, Amitraz, and IPM groups. Data are shown as the average ± SEM. Significant differences between and within treatment groups is as described in Figure 1.

Figure 8. Adult honey bee weight over time among the Control, Amitraz, and IPM groups. Data are shown as the average ± SEM. Significant differences between and within treatment groups is as described in Figure 1.

Figure 9. Queen survivorship over time among the Control, Amitraz, and IPM groups. Queen survivorship was significantly lower in the Control group compared to the Amitraz and IPM groups.

Table 1. Honey bee phenothrin bioassay summary. The LD_{50} values are in shown in units of ng phenothrin/mg bee. Letters and numbers beside LD_{50} values indicate significant differences in rows and columns, respectively.

	Control			Amitraz			IPM		
Month	**n**	**LD_{50} (95% CI)**	**Slope (SE)**	**n**	**LD_{50} (95% CI)**	**Slope (SE)**	**n**	**LD_{50} (95% CI)**	**Slope (SE)**
May	308	0.45 (0.35-0.52)^{a123}	2.6 (0.4)	239	0.48 (0.45-0.52)^{a1}	6.4 (0.7)	229	0.47 (0.32-0.57)^{a123}	3.1 (0.6)
June	489	0.45 (0.42-0.46)^{a13}	7.2 (0.5)	576	0.42 (0.40-0.44)^{a2}	7.6 (0.5)	531	0.32 (0.29-0.34)^{b3}	5.8 (0.6)
July	448	0.41 (0.38-0.43)^{a1}	5.3 (0.5)	374	0.43 (0.40-0.48)^{a12}	3.7 (0.5)	329	0.45 (0.41-0.49)^{a1}	5.0 (0.6)
August	383	0.33 (0.29-0.35)^{b2}	4.7 (0.6)	303	0.50 (0.46-0.54)^{a1}	4.8 (0.6)	543	0.35 (0.34-0.38)^{b2}	4.7 (0.4)
September	297	0.48 (0.45-0.51)^{a3}	7.3 (0.7)	252	0.42 (0.39-0.45)^{b12}	6.3 (0.7)	284	0.47 (0.44-0.50)^{ab1}	6.7 (0.7)
October	362	0.41 (0.37-0.44)^{a1}	4.3 (0.6)	464	0.34 (0.31-0.36)^{b3}	4.8 (0.5)	73	0.43 (0.31-0.52)^{ab123}	4.7 (1.4)

Table 2. Honey bee amitraz bioassay summary. The LD_{50} values are in shown in units of ng amitraz/mg bee. Letters and numbers in beside LD_{50} values indicate significant differences in rows and columns, respectively. The LD_{50} value for the IPM treatment group in October was not reported as the data were not well represented by a line.

	Control			Amitraz			IPM		
Month	**n**	**LD_{50} (95% CI)**	**Slope (SE)**	**n**	**LD_{50} (95% CI)**	**Slope (SE)**	**n**	**LD_{50} (95% CI)**	**Slope (SE)**
May	373	20.0 (18.4-21.8)^{b3}	4.9 (0.4)	339	17.6 (15.8-19.6)^{b5}	3.7 (0.3)	320	27.1 (24.7-29.7)^{a4}	4.2 (0.4)
June	380	59.4 (51.5-72.9)^{a1}	3.4 (0.4)	416	33.7 (31.1-36.6)^{b23}	4.1 (0.4)	368	46.8 (42.0-53.5)^{a12}	3.5 (0.4)
July	429	20.1 (18.5-21.9)^{a3}	4.2 (0.3)	238	19.5 (17.6-21.8)^{a5}	5.0 (0.5)	218	18.4 (16.3-20.9)^{a3}	4.4 (0.5)
August	324	23.8 (19.9-28.5)^{ab3}	2.2 (0.3)	328	25.3 (22.7-28.1)^{a4}	3.5 (0.3)	568	18.5 (16.7-20.5)^{b3}	2.6 (0.2)
September	327	60.6 (55.7-65.8)^{a1}	4.3 (0.5)	180	41.0 (35.8-46.1)^{b123}	4.0 (0.6)	255	36.4 (31.1-41.2)^{b2}	4.0 (0.5)
October	321	30.6 (28.5-32.3)^{b2}	9.1 (1.3)	350	42.4 (38.9-46.3)^{a12}	4.4 (0.5)	NA	NA	NA

Table 3. Honey bee clothianidin bioassay summary. The LC_{50} values are in shown in units of ng clothianidin/mL/mg bee. Letters and numbers in beside LD_{50} values indicate significant differences in rows and columns, respectively.

	Control			Amitraz			IPM		
Month	**n**	**LC_{50} (95% CI)**	**Slope (SE)**	**n**	**LC_{50} (95% CI)**	**Slope (SE)**	**n**	**LC_{50} (95% CI)**	**Slope (SE)**
May	308	1.12 (0.78-1.43)^{b3}	2.4 (0.3)	269	0.48 (0.09-0.93)^{b4}	1.5 (0.3)	293	2.78 (2.37-3.16)^{a1}	4.0 (0.5)
June	280	0.81 (0.51-1.10)^{a3}	1.9 (0.3)	460	0.35 (0.09-0.64)^{ab4}	1.2 (0.2)	479	0.07 (0.001-0.25)^{b5}	1.0 (0.2)
July	357	1.20 (1.07-1.37)^{a3}	3.3 (0.3)	253	1.14 (1.04-1.28)^{a3}	5.6 (0.7)	276	1.39 (1.22-1.65)^{a34}	3.7 (0.4)
August	325	2.19 (1.95-2.49)^{ab2}	3.7 (0.4)	287	2.47 (2.24-2.71)^{a1}	5.8 (1.0)	416	1.97 (1.81-2.15)^{b23}	4.5 (0.4)
September	370	3.25 (2.94-3.55)^{a1}	4.3 (0.4)	356	2.29 (1.95-2.62)^{b1}	2.8 (0.3)	340	2.59 (2.23-2.97)^{ab1}	2.6 (0.3)
October	296	2.62 (2.33-2.93)^{a2}	4.0 (0.4)	447	1.75 (1.60-1.90)^{b2}	5.3 (0.4)	54	1.60 (0.89-2.04)^{b234}	7.3 (2.2)

LITERATURE CITED

1. vanEngelsdorp, D.; Hayes, J.; Underwood, R.M.; Pettis, J. A survey of honey bee colony losses in the us, fall 2007 to spring 2008. *PLoS One* **2008** *3*, e4071.

2. Steinhauer, N.A.; Rennich, K.; Wilson, M.E.; Caron, D.M.; Lengerich, E.J.; Pettis, J.; Rose, R.; Skinner, J.A.; Tarpy, D.R.; Wilkes, J.T.*, et al.* A national survey of managed honey bees 2012-2013 annual colony losses in the USA: Results from the bee informed partnership. *J. Apic. Res.* **2014**, *53*, 1-18.

3. Finley, J.; Camazine, S.; Frazier, M. The epidemic of honey bee colony losses during the 1995-1996 season. *Am. Bee J.* **1996**, *136*, 805-808.

4. Siede, R.; Konig, M.; Buchler, R.; Failing, K.; Thiel, H.J. A real-time pcr based survey on acute bee paralysis virus in german bee colonies. *Apidologie* **2008**, *39*, 650-661.

5. Gregory, P.G.; Evans, J.D.; Rinderer, T.; de Guzman, L. Conditional immune-gene supression of honeybees parasitized by *varroa* mites. *J. Ins. Sci.* **2005**, *5*, 7-11.

6. Yang, X.; Cox-Foster, D.L. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *PNAS* **2005**, *102*, 7470-7475.

7. Navajas, M.; Migeon, A.; Alaux, C.; Martin-Magniette, M.L.; Robinson, G.E.; Evans, J.D.; Cros-Arteil, S.; Crauser, D.; Le Conte, Y. Differential gene expression of the honey bee *apis mellifera* associated with *varroa destructor* infection. *BMC Genomics* **2008**, *9*, 301-311.

8. Duay, P.; De Jong, D.; Engels, W. Weight loss in drone pupae (*apis mellifera*) multiply infested by *varroa destructor* mites. *Apidologie* **2003**, *34*, 61-65.

9. Bowen-Walker, P.L.; Gunn, A. The effect of the ectoparasitic mite, *varroa destructor*, on adult worker honey bee (*apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels. *Entomologia Experimentalis et Applicata* **2001**, *101*, 207-217.

10. Amdam, G.V.; Hartfelder, K.; Norberg, K.; Hagen, A.; Omholt, S.W.

Altered physiology in worker honey bees (hymenoptera: Apidae) infested with the mite *varroa destructor* (acari: Varroaidae): A factor in colony loss during overwintering? *J. Econ. Entomol.* **2004**, *97*, 741-747.

11. Seehuus, S.C.; Norberg, K.; Gimsa, U.; Krekling, T.; Amdam, G.V. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *PNAS* **2005**, *103*, 962-967.

12. Dahlgren, L.; Johnson, R.M.; Siegfried, B.D.; Ellis, M.D. Comparative toxicity of acaricides to honey bee (hymenoptera: Apidae) workers and queens. *J. Econ. Entomol.* **2012**, *105*, 1895-1902.

13. Alaux, C.; Dantec, C.; Parrinello, H.; Le Conte, Y. Nutrigenomics in honey bees: Digital gene expression analysis of pollen's nutritive effects on healthy and varroa-parasitized bees. *BMC Genomics* **2011**, *12*, 496-508.

14. Rinkevich, F.D.; Margotta, J.W.; Pittman, J.M.; Danka, R.G.; Tarver, M.R.; Ottea, J.A.; Healy, K.B. Genetics, synergists, and age affect insecticide sensitivity in the honey bee, *apis mellifera*. *PLoS One* **2015**, *10*, e0139841.

15. Imdorf, A.; Buehlmann, G.; Gerig, L.; Kilchenmann, V.; Willie, H. A test of the method of estimation of brood areas and number of worker bees in free flying colonies. *Apidologie* **1987**, *18*, 137-146.

16. Delaplane, K.S.; van der Steen, J.J.M.; Guzman-Novoa, E. Standard methods for estimating strength parameters of *apis mellifera* colonies. *J. Apic. Res.* **2013**, *52*, 1-12.

17. Mullin, C.A.; Frazier, M.; Frazier, J.L.; Ashcraft, S.; Simonds, R.; vanEngelsdorp, D.; Pettis, J.S. High levels of miticides and agrochemicals in north american apiaries: Implications for honey bee health. *PLoS ONE* **2010**, *5*, e9754.

18. Abbott, W.S. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **1925**, *18*, 265-267.

19. vanEngelsdorp, D.; Meixner, M.D. A historical review of managed honey bee populations in europe and the united states and the factors that may affect them. *J. Invert. Pathol.* **2010**, *103*, S80-S95.

20. Gallai, N.; Salles, J.M.; Settele, J.; Vaissiere, B.E. Economic valuation of the vulnerability of world agriculture confronted with pollinator

decline. *Ecol. Econ.* **2009**, *68*, 810-821.

21. Robertson, J.L.; Preistler, H.K. *Pesticide bioassays with arthopods*. CRC Press: Boca Raton, FL, 1992; p 127.

22. Casida, J.E.; Durkin, K.A. Neuroactive insecticides:Targets,selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* **2013**, *58*, 99-117.

23. Jeschke, P.; Nauen, R. Neonicotinoid insecticides. In *Comprehensive molecular insect science*, Gilbert, L.I.; Iatrou, K.; Gill, S.S., Eds. Elsevier: New York, 2005; Vol. 5, pp 53-105.

24. Jeschke, P.; Nauen, R.; Schindler, M.; Elbert, A. Overview of the status and global strategy for neonicotinoids. *Agri. Food Chem.* **2011**, *59*, 2897-2908.

25. Krupke, C.H.; Hunt, G.J.; Eitzer, B.D.; Andino, G.; Given, K. Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS one* **2012**, *7*, e29268.

26. Long, E.Y.; Krupke, C.H. Non-cultivated plants present a season-long route of pesticide exposure for honey bees. *Nature Comm.* **2016**, *7*.

27. Chauzat, M.-P.; Faucon, J.-P.; Martel, A.-C.; Lachaize, J.; Cougoule, N.; Aubert, M. A survey of pesticide residues in pollen loads collected by honey bees in france. *J. Econ. Entomol.* **2006**, *99*, 253-262.

28. Pohorecka, K.; Skubida, P.; Miszczak, A.; Semkiw, P.; Sikorski, P.; Zagibajło, K.; Teper, D.; Kołtowski, Z.; Skubida, M.; Zdańska, D. Residues of neonicotinoid insecticides in bee collected plant materials from oilseed rape crops and their effect on bee colonies. *Journal of Apicultural Science* **2012**, *56*, 115-134.

29. Mattila, H.R.; Seeley, T.D. Genetic diversity in honey bee colonies enhances productivity and fitness. *Science* **2007**, *317*, 362.

30. Harris, J.W.; Woodring, J. Effects of stress, age, season, and source colony on levels of octopamine, dopamine, and serotonin in the hone bee (*apis mellifera* l.) brain. *J. Ins. Physiol.* **1992**, *38*, 29-35.

31. Snyder, R. Parasitic mite syndrome. https://beeinformed.org/2013/10/15/parasitic-mite-syndrome-pms/

32. Guzman-Novoa, E.; Eccles, L.; Calvete, Y.; McGowan, J.; Kelly, P.G.;

Correa-Benitez, A. *Varroa destructor* is the main culprit for the death and reduced populations of overwintered honey bee (*apis mellifera*) colonies in ontario, canada. *Apidologie* **2010**, *71*.

33. Boeking, O.; Genersch, E. Varroosis- the ongoing crisis in bee keeping. *J. Verbrauch. Lebensm.* **2008**, *3*, 221-228.

34. Berry, J.A.; Afik, O.; Nolan, M.P.; Delaplane, K.S. Revisiting powdered sugar for varroa control on honey bees (*apis mellifera* l.). *J. Apic. Res.* **2012**, *51*, 367-368.

35. Calderone, N.W. In *A three-year study of screened bottom boards in the northeast*, Proceedings of the American Bee Research Conference, 2007; pp 436-437.

36. Calderone, N.W. Evaluation of drone brood removal for management of *varroa destructor* (acari: Varroidae) in colonies of *apis mellifera* (hymenoptera: Apidae) in the northeastern united states. *J. Econ. Entomol.* **2005**, *98*, 645-650.

37. Charriere, J.D.; Imdorf, A.; Bachofen, B.; Tschan, A. The removal of capped drone brood: An effective means of reducing the infestation of varroa in honey bee colonies. *Bee World* **2003**, *84*, 117-124.

38. Corona, M.; Velarde, R.A.; Remolina, S.; Moran-Lauter, A.; Wang, Y.; Hughes, K.A.; Robinson, G.E. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *PNAS* **2007**, *104*, 7128-7133.

39. Al-Tikrity, W.S.; Benton, A.W.; Hlllman, R.C.; Clark, W.W. The relationship between the amount of unsealed brood in honeybee colonies and their pollen collection. *J. Apic. Res.* **1972**, *11*, 9-12.

40. Pankiw, T.; Page, R.E.; Fondrk, M.K. Brood pheromone stimulates pollen foraging in honey bees (*apis mellifera*). *Behav. Ecol. Sociobiol.* **1998**, *44*, 193-198.

41. Jaycox, E.R. Honey bee queen pheromones and worker foraging bahavior. *Annals Entom. Soc. Am.* **1970**, *63*, 222-228.

42. Jaycox, E.R. Honey bee foraging behavior: Response to queens, larvae, and extracts of larvae. *Annals Entom. Soc. Am.* **1970**, *63*, 1689-1694.

43. Zhu, W.; Schmehl, D.R.; Mullin, C.A.; Frazier, J.L. Four common

pesticides, their mixtures, and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. *PLoS One* **2014**, *9*, e77547.

44. VanEngelsdorp, D.; Evans, J.D.; Donovall, L.; Mullin, C.; Frazier, M.; Frazier, J.; Tarpy, D.R.; Hayes, J.; Pettis, J. "Entombed pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. *J. Invert. Pathol.* **2009**, *101*, 147-149.

45. Gregorc, A.; Evans, J.D.; Scharf, M.; Ellis, J.D. Gene expression in honey bee (*apis mellifera*) larvae exposed to pesticides and varroa mites (*varroa destructor*). *J. Ins. Physiol.* **2012**, *58*, 1042-1049.

46. Karunker, I.; Benting, J.; Lueke, B.; Ponge, T.; Nauen, R.; Roditakis, E.; Vontas, J.; Gorman, K.; Denholm, I.; Morin, S. Over-expression of cytochrome p450 *cyp6cm1* is associated with high resistance to imidacloprid in the b and q biotypes of *bemisia tabaci* (hemiptera: Aleyrodidae). *Insect Biochem. Molec. Biol.* **2008**, *38*, 634-644.

47. Scott, J.G. Cytochromes p450 and insecticide resistance. *Insect Biochem. Mol. Biol.* **1999**, *29*, 757-777.

48. Lin, Y.; Jin, Y.; Zeng, L.; Lu, Y. Insecticide toxicity to oriental fruit fly *bactrocera dorsalis* (diptera:Tephrididae) is influenced by environmental factors. *J. Econ. Entomol.* **2013**, *106*, 353-359.

49. Smirle, M.J.; Winston, M.L. Intercolony variation in pesticide detoxification by the honey bee (hymenoptera: Apidae). *J. Econ Entomol.* **1987**, *80*, 5-8.

50. Komagata, O.; Kasai, S.; Tomita, T. Overexpression of cytochrome p450 genes in pyrethroid-resistant *culex quinquefasciatus*. *Insect Biochem. Mol. Biol.* **2010**, *40*, 146-152.

51. Puinean, A.M.; Denholm, I.; Millar, N.S.; Nauen, R.; Williamson, M.S. Characterisation of imidacloprid resistance mechanisms in the brown planthopper, *nilaparvata lugens* stål (hemiptera: Delphacidae). *Pestic. Biochem. Physiol.* **2010**, *97*, 129-132.

52. Johnson, R.M.; Dahlgren, L.; Siegfried, B.D.; Ellis, M.D. Acaricide, fungicide and drug interactions in honey bees (*apis mellifera*). *PLoS One* **2013**, *8*, e54092.

53. Wahl, O.; Ulm, K. Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *apis mellifera*

carnica. *Oecologia* **1983**, *59*, 106-128.

54. Slessor, K.N.; Winston, M.L.; Le Conte, Y. Pheromone communication in the honeybee *J. Chem. Ecol.* **2005**, *31*, 2731-2745.

55. Le Conte, Y.; Hefetz, A. Primer pheromones in social hymenoptera. *Annu. Rev. Entomol.* **2008**, *53*, 523-542.

56. Malka, O.; Karunker, I.; Yeheskel, A.; Morin, S.; Hefetz, A. The gene road to royalty – differential expression of hydroxylating genes in the mandibular glands of the honeybee. *FEBS Letters* **2009**, *276*, 5481-5490.

57. Lee, K.V.; Steinhauer, N.A.; Rennich, K.; Wilson, M.E.; Tarpy, D.R.; Caron, D.M.; Rose, R.; Delaplane, K.S.; Baylis, K.; Lengerich, E.J.*, et al.* A national survey of managed honey bee 2013-2014 annual colony losses in the USA. *Apidologie* **2015**, *46*, 292-305.

58. Harbo, J.R.; Harris, J.W. Responses to varroa by honey bees with different levels of varroa sensitive hygiene. *Journal of apicultural research* **2009**, *48*, 156-161.

59. Danka, R.G.; Harris, J.W.; Dodds, G.E. Selection of vsh-derived "pol-line" honey bees and evaluation of their varroa-resistance characteristics. *Apidologie* **2015**, 1-8.

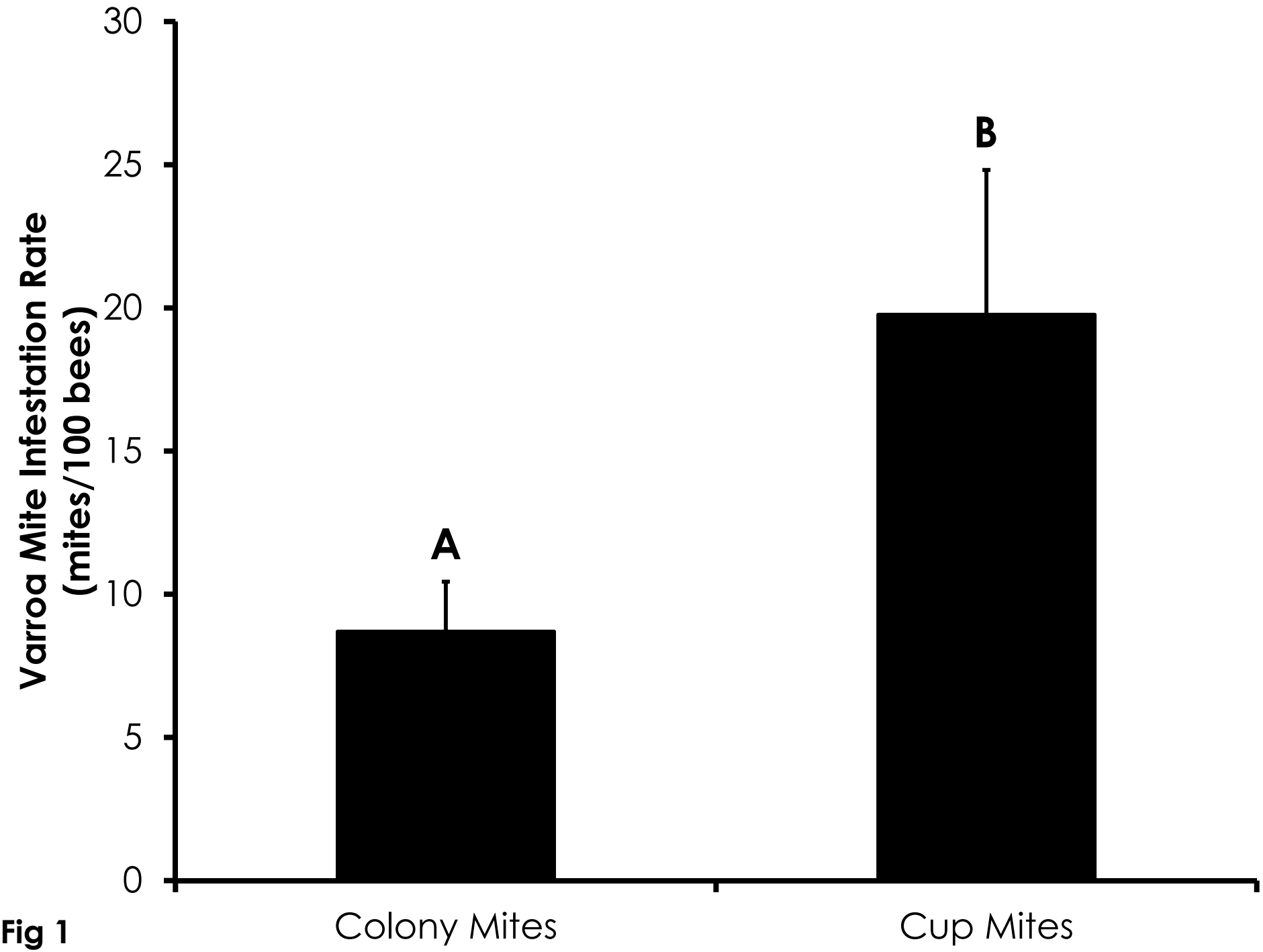
Varroa Mite Infestation Rate
(mites/100 bees)
30
25
20
15
10
5
0
A
B
Colony Mites
Cup Mites

Fig 1

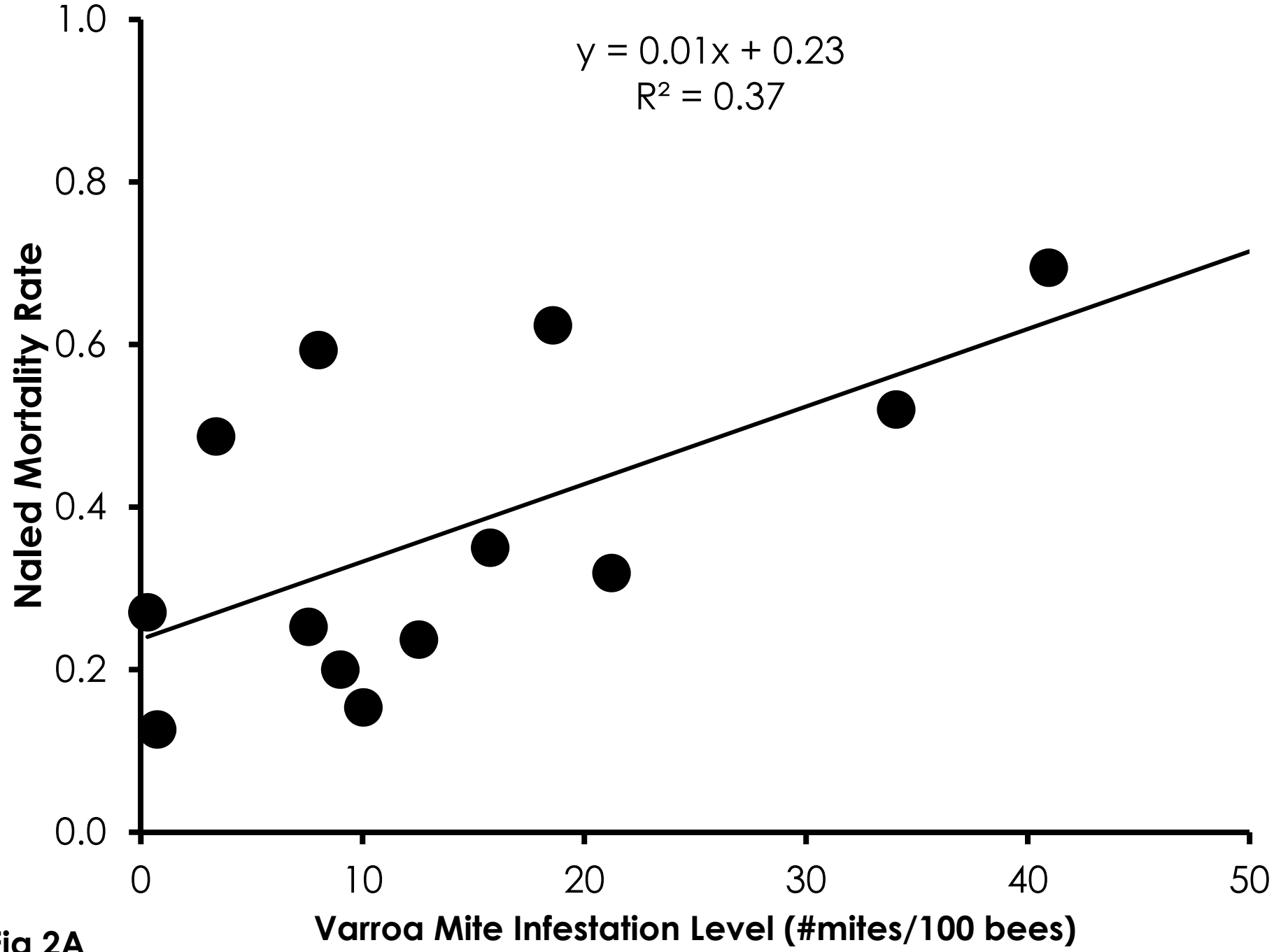
y = 0.01x + 0.23
R² = 0.37
Naled Mortality Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Varroa Mite Infestation Level (#mites/100 bees)

Fig 2A

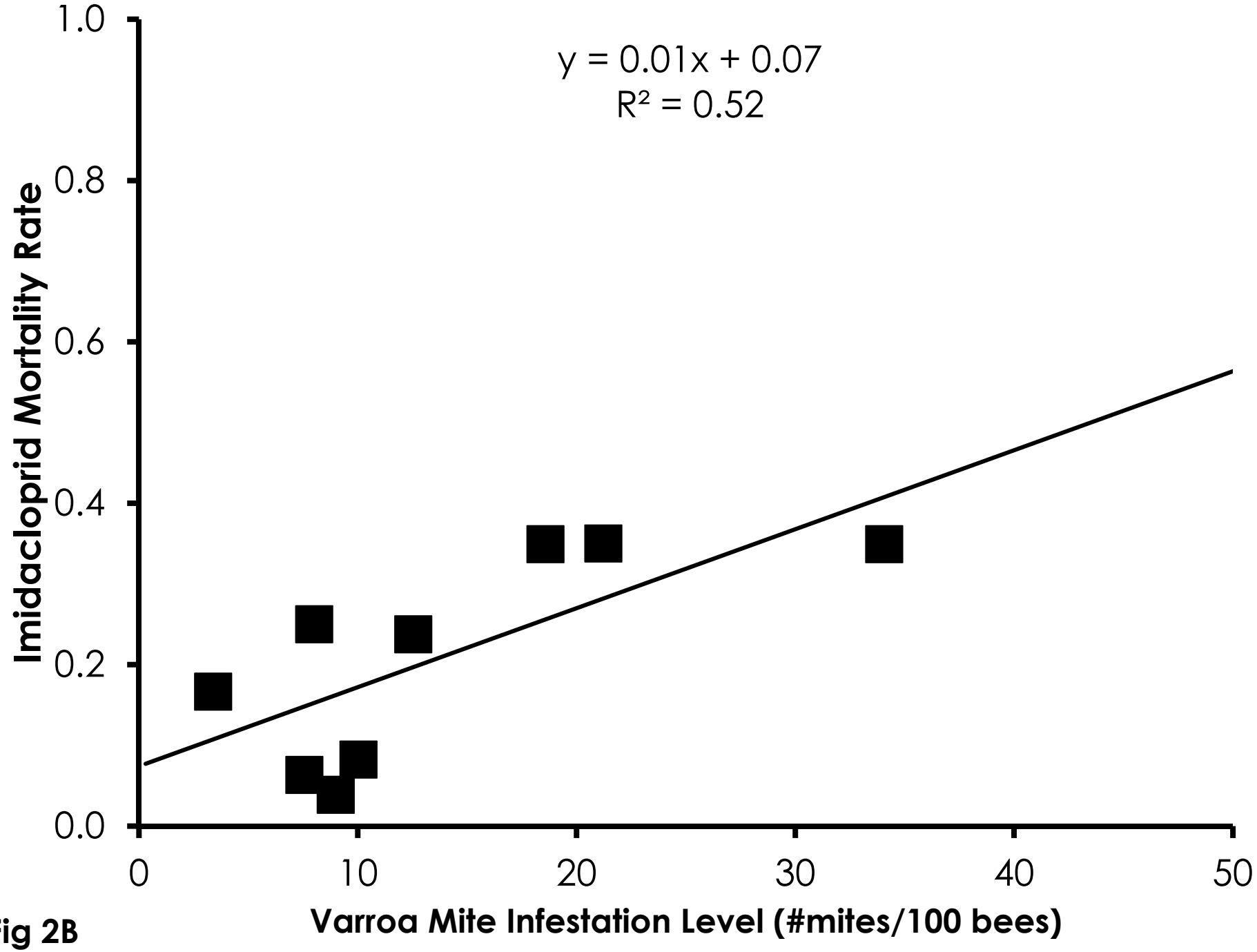
y = 0.01x + 0.07
R² = 0.52
Imidacloprid Mortality Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Varroa Mite Infestation Level (#mites/100 bees)

Fig 2B

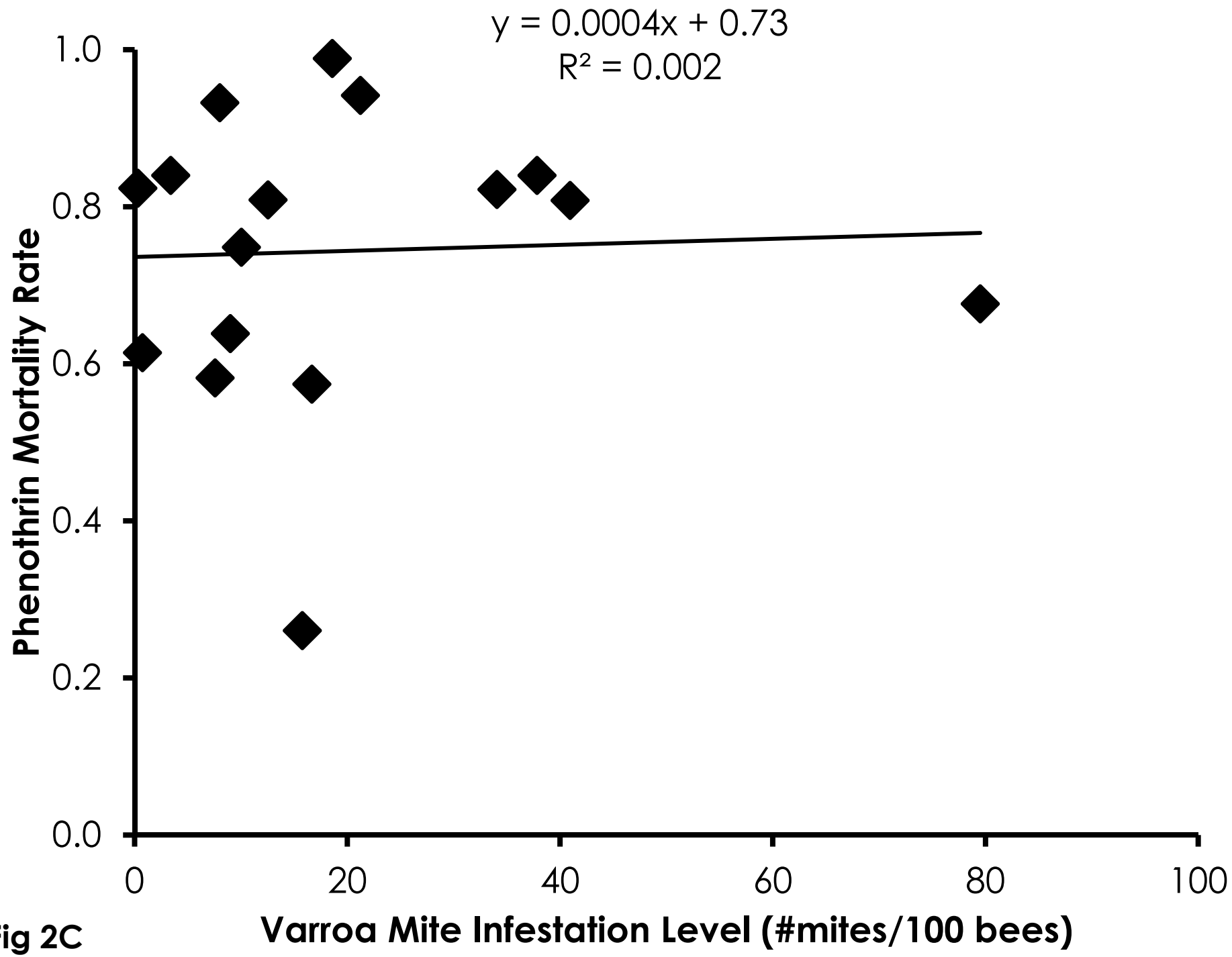
y = 0.0004x + 0.73
R² = 0.002
1.0
0.8
0.6
0.4
0.2
0.0
Phenothrin Mortality Rate
0
20
40
60
80
100
Varroa Mite Infestation Level (#mites/100 bees)

Fig 2C

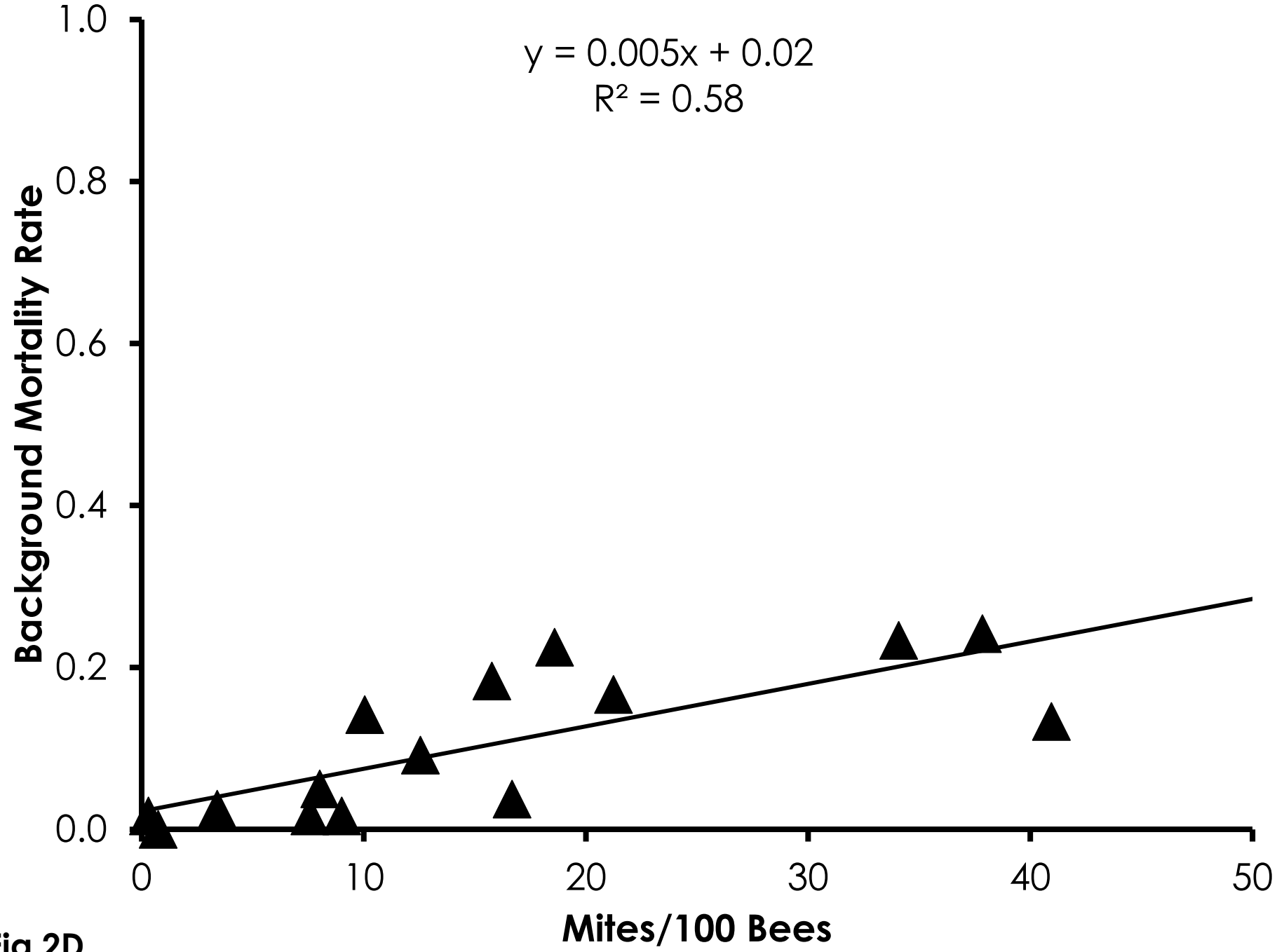
y = 0.005x + 0.02
R² = 0.58
Background Mortality Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Mites/100 Bees

Fig 2D

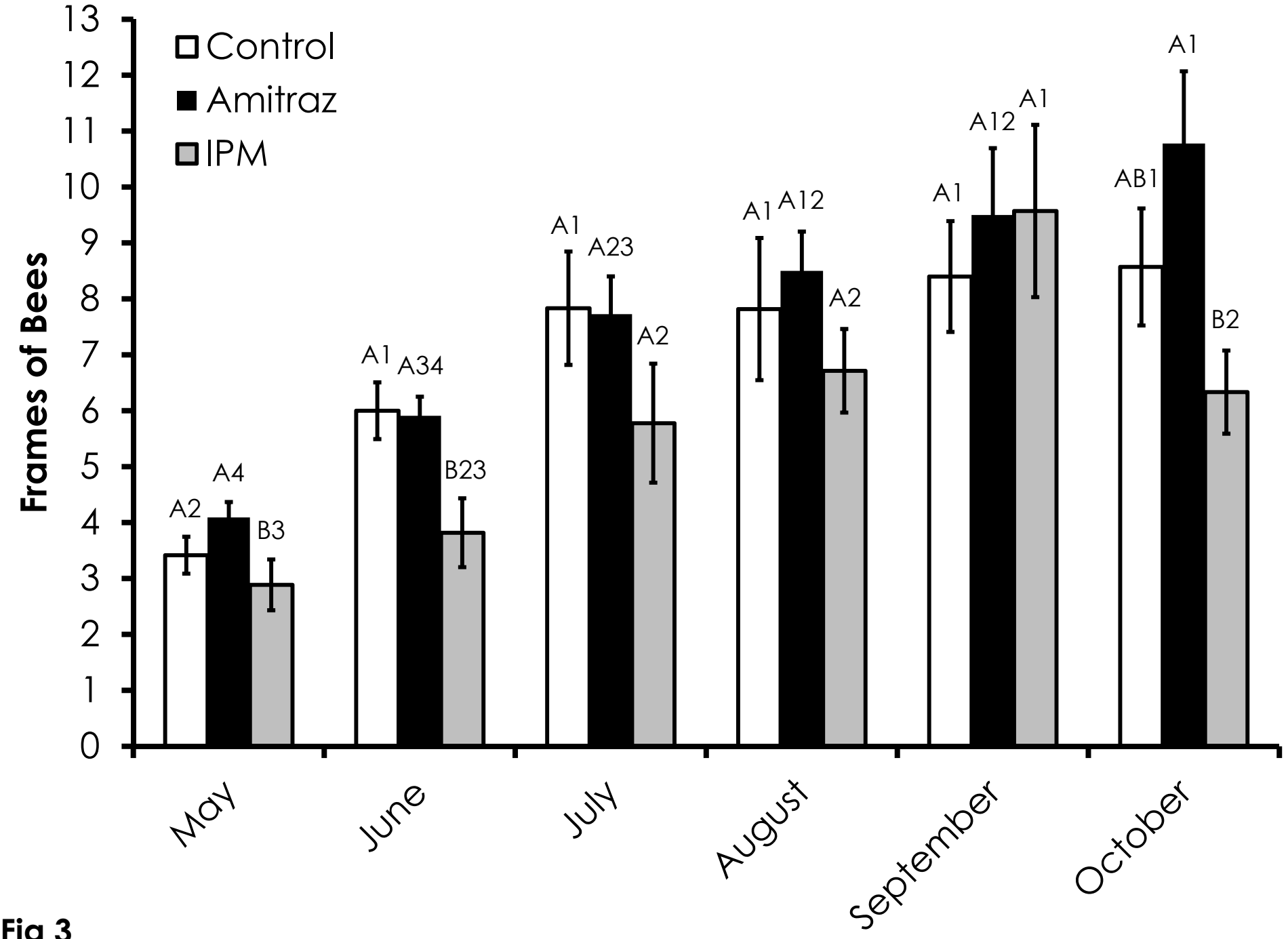
Control
Amitraz
IPM
Frames of Bees
0
1
2
3
4
5
6
7
8
9
10
11
12
13
A2
A4
B3
A1
A34
B23
A1
A23
A2
A1
A12
A2
A1
A12
A1
AB1
A1
B2
May
June
July
August
September
October

Fig 3

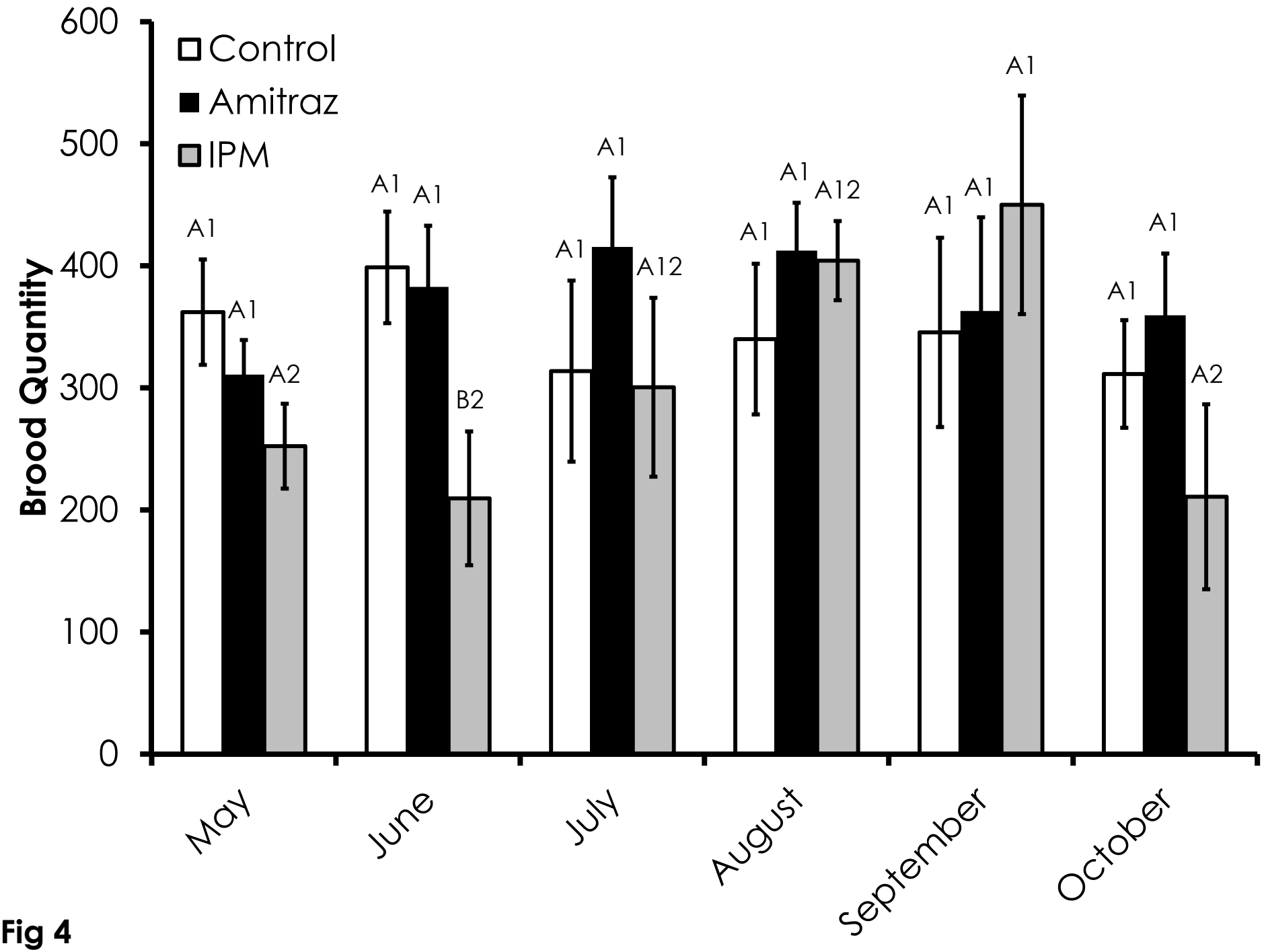
600
500
400
300
200
100
0
Brood Quantity
Control
Amitraz
IPM
A1
A1
A2
A1
A1
B2
A1
A1
A12
A1
A1
A12
A1
A1
A1
A1
A1
A2
May
June
July
August
September
October

Fig 4

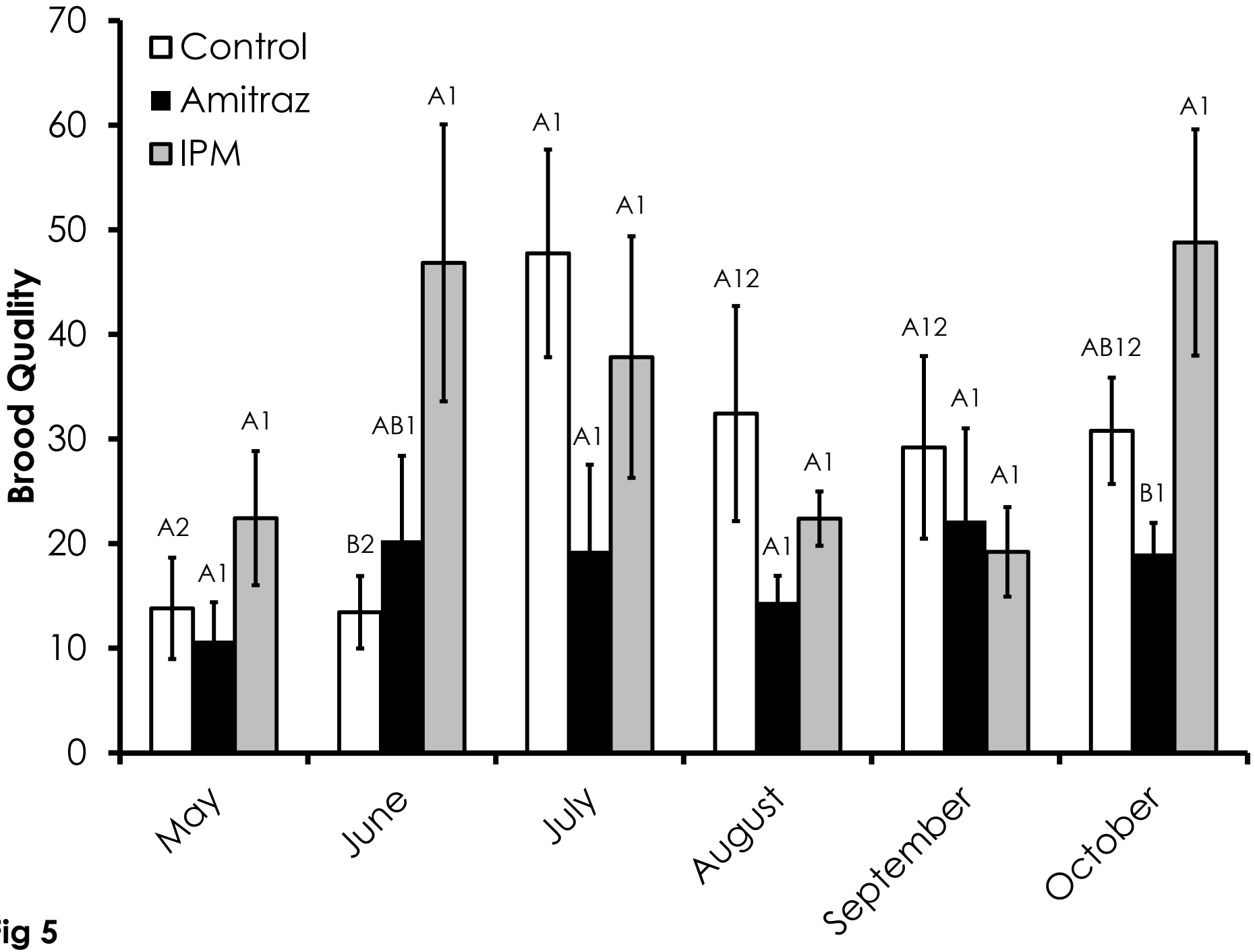
Control
Amitraz
IPM
Brood Quality
70
60
50
40
30
20
10
0
A2
A1
A1
B2
AB1
A1
A1
A1
A1
A12
A1
A1
A12
A1
A1
AB12
B1
A1
May
June
July
August
September
October

Fig 5

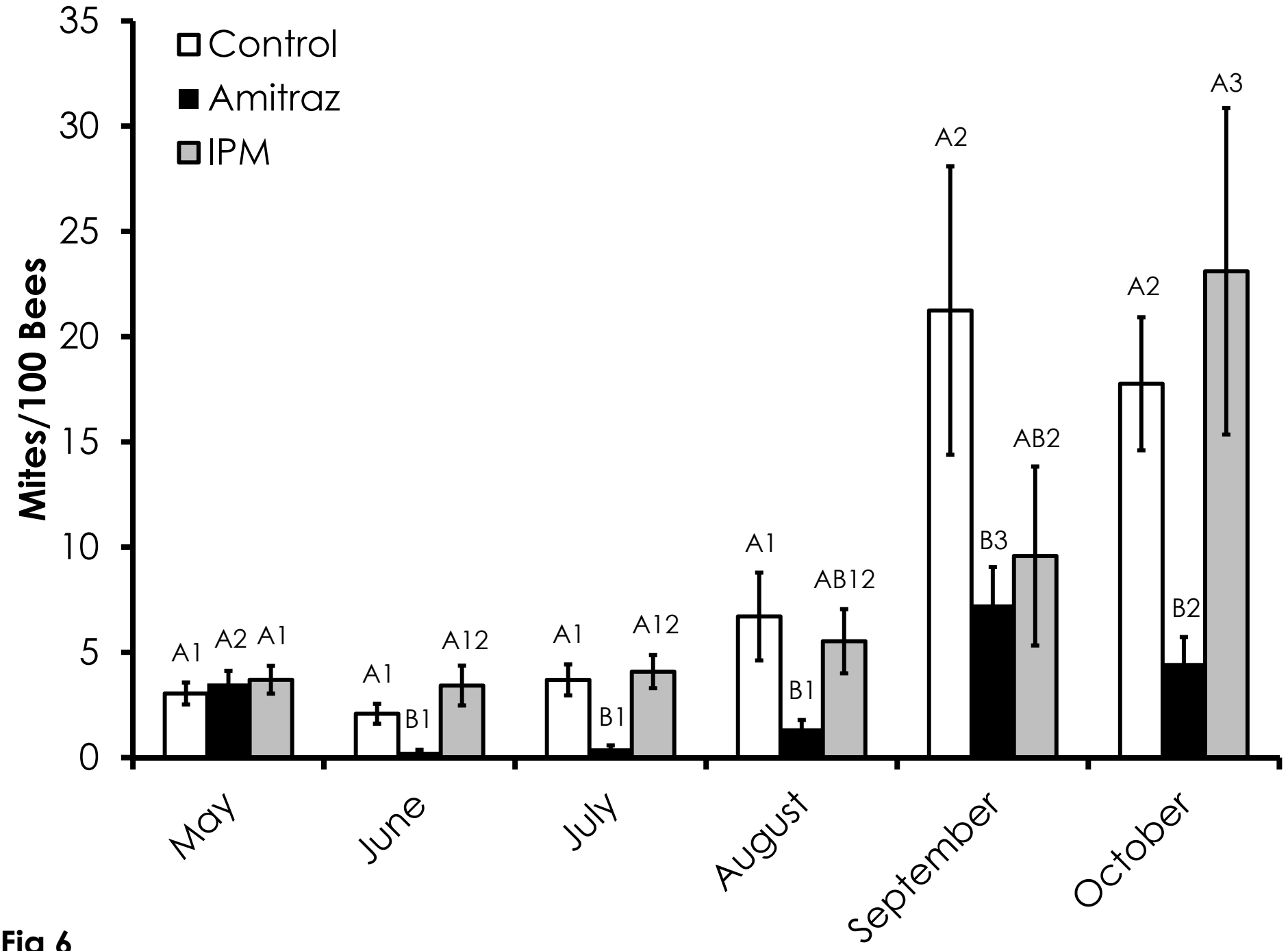
Control
Amitraz
IPM
Mites/100 Bees
35
30
25
20
15
10
5
0
A1
A2
A1
A1
B1
A12
A1
B1
A12
A1
B1
AB12
A2
B3
AB2
A2
B2
A3
May
June
July
August
September
October

Fig 6

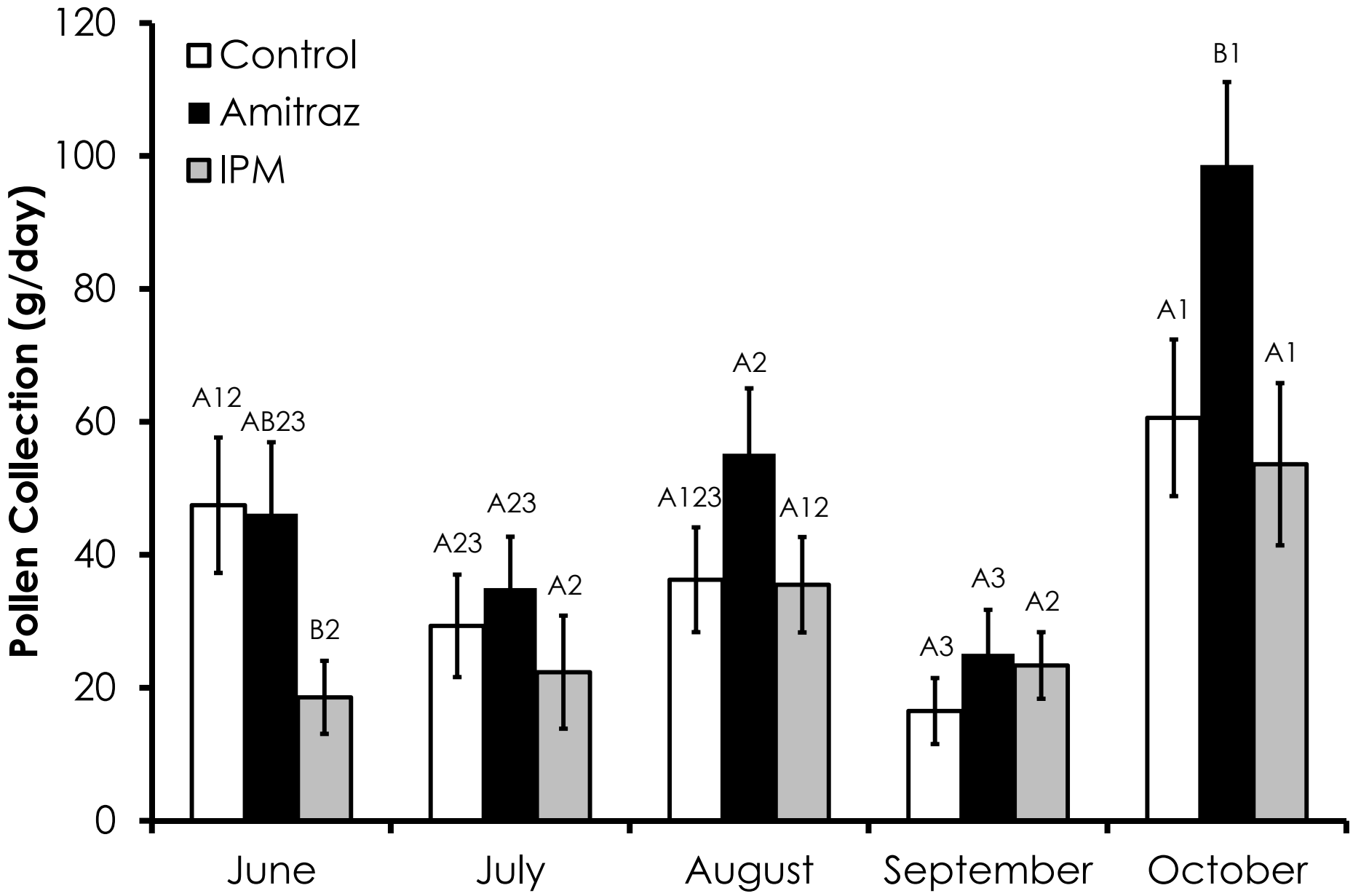
120
100
80
60
40
20
0
Pollen Collection (g/day)
Control
Amitraz
IPM
A12
AB23
B2
A23
A23
A2
A123
A2
A12
A3
A3
A2
A1
B1
A1
June
July
August
September
October

Fig 7

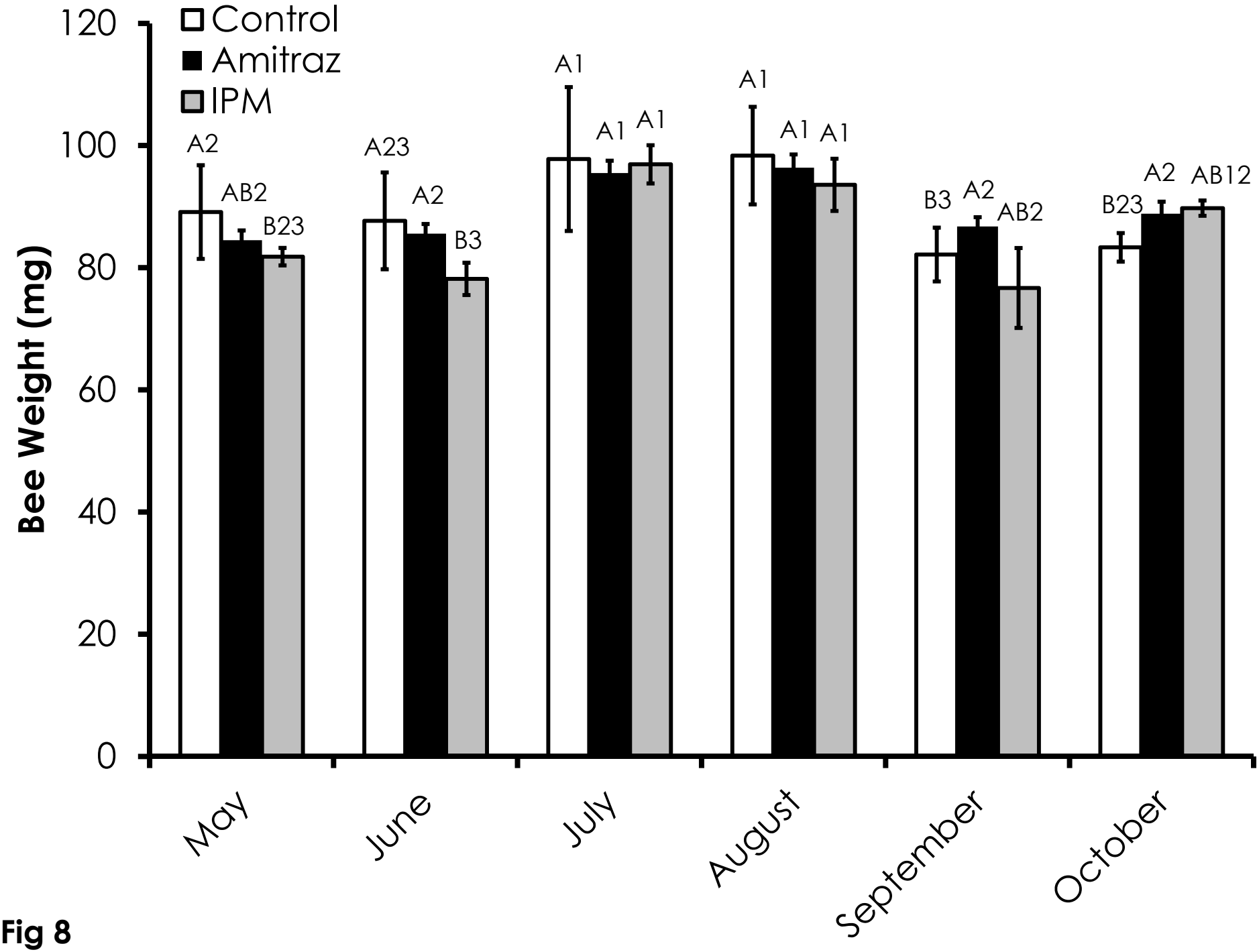
Control
Amitraz
IPM
Bee Weight (mg)
120
100
80
60
40
20
0
A2
AB2
B23
A23
A2
B3
A1
A1
A1
A1
A1
A1
B3
A2
AB2
B23
A2
AB12
May
June
July
August
September
October

Fig 8

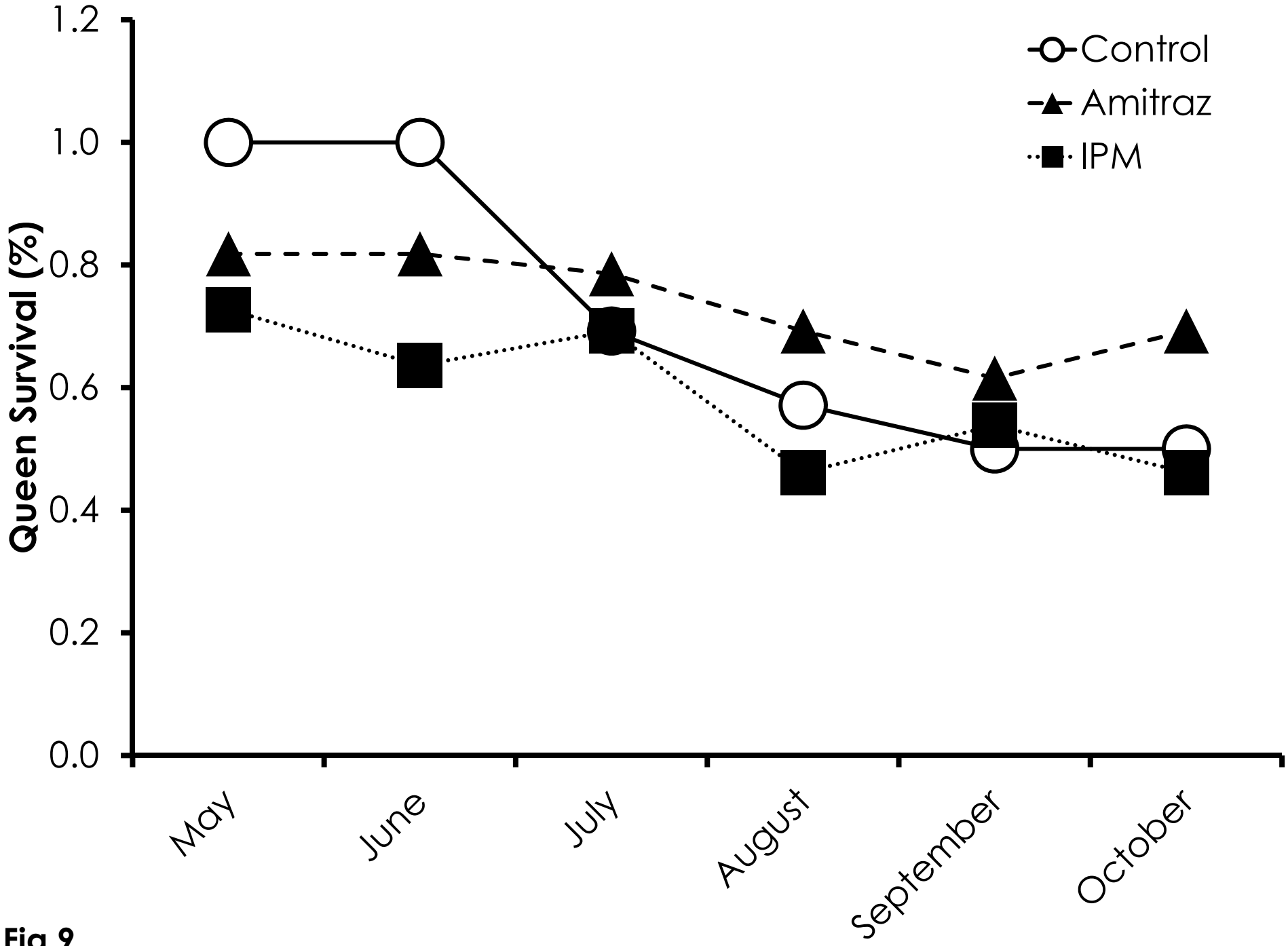
Queen Survival (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Amitraz
IPM
May
June
July
August
September
October

Fig 9