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Rogan Tokach, Frank Rinkevich, Dan Aurell, Nathan Egnew, Kloe Cargo & Geoffrey R. Williams

Evaluation of late-season *Varroa destructor* treatments and their impact on amitraz resistant mite populations

Rogan Tokach[1,2]*, Frank Rinkevich[3], Dan Aurell[1], Nathan Egnew[3], Kloe Cargo[3], and Geoffrey R. Williams[1]*

[1]Bee Center, Department of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA

[2]School of Natural Resource Sciences, North Dakota State University, Fargo, ND, USA

[3]USDA-ARS Honey Bee Breeding, Genetics and Physiology Laboratory, Baton Rouge, LA, USA

*Corresponding authors Rogan Tokach (rogan.tokach@ndsu.edu) and Geoffrey R. Williams (williams@auburn.edu)

Abstract

Varroa destructor mites are a leading cause of honey bee (*Apis mellifera*) colony losses worldwide. The acaracide amitraz has been among the most preferred *Varroa* treatments for more than a decade because of its high effectiveness and convenience of application. As a result, over reliance on amitraz has led to *Varroa* resistance to amitraz. Here, we evaluated U.S. registered *Varroa* treatments with differing active ingredients on *Varroa* infestation rates and their impact on amitraz resistant *Varroa* to identify alternative treatment options for commercial beekeepers just prior to winter, a critical time of year to have low mite infestation. Treatment groups included an untreated Control, Apivar (active ingredient amitraz), FormicPro (a.i. formic acid), HopGuard 3 (a.i. hops β-acids) and Api-Bioxal vapor (a.i. oxalic acid, "OA" Vapor). Treatments were implemented in late September; colonies were assessed in late November and early February, 63 and 133 days later. We found that the Apivar, FormicPro and HopGuard treatment groups maintained *Varroa* infestation rates, whereas *Varroa* infestation rates significantly increased in the untreated Control and OA Vapor treatment groups. Genetic analysis showed that the frequency of the amitraz resistance genotype significantly increased from Day 0 to Day 63 after the application of Apivar, but this change was reversed by Day 133 after the overwintering period. Comparatively, the amitraz resistance genotype frequency was not impacted by any other treatment group. This indicates there is no cross-resistance of amitraz to other active ingredients tested, and that amitraz resistance may not have a large fitness cost.

Introduction

Varroa destructor (hereafter referred to as *Varroa*) is a parasitic mite of the western honey bee (*Apis mellifera*) and is widely considered to be the leading cause of colony losses in the United States and other areas of the northern hemisphere[1–4]. These mites feed on developing and adult honey bees while transmitting viruses that reduce honey bee colony productivity and survival[5–8]. Failure to manage *Varroa* infestations by beekeepers often results in colony losses in less than one year[9,10]. While multiple management strategies exist for *Varroa* control, large-scale commercial beekeepers primarily rely on chemical treatments to control mites[11,12]. This reliance on chemical treatments has resulted in *Varroa* resistance to some synthetic acaricides, which reduces the effectiveness of these treatments[13–20].

Amitraz has been one of the most widely used acaricides against *Varroa* as it is capable of significantly reducing mite infestations after a single treatment[15,21–24]; however, continued treatments of amitraz has resulted in development of amitraz resistant *Varroa* mites[15,18,25]. Amitraz resistance can be due to increased detoxification via overexpression of the cytochrome P450 isoform CYP3002B2[26] or different mutations in the β2 octopamine receptor (*Octβ2R*) around the world such as N87S found in

France, T115N found in South Korea, Y215H found in the US and Canada, F290L found in Spain, and Y337F found in Turkey [15,18–20,25]. In the U.S., amitraz resistance has been linked with a tyrosine at position 215 to histidine (Y215H) mutation in *Octβ2R*[18,25]. *Varroa* that have the amitraz resistant phenotype overwhelmingly possess the homozygous Y215H mutation[25], and there is justifiable concern that further application of amitraz based products will preferentially select for this resistance genotype, resulting in increases in the amitraz resistant allele frequency[15]. Currently, there is little known about amitraz resistant *Varroa* population dynamics, if there is any potential fitness cost, or how treatment with different active ingredients impacts the resistance allele frequency in *Varroa* populations. These knowledge gaps limit the understanding of how long amitraz resistant alleles may remain in the population if alternative management strategies are employed.

While other chemical *Varroa* treatments with different active ingredients exist, many of them have limitations that have prevented widespread adoption[12]. *Varroa* behavior and biology can be a limiting factor for treatments as mites have two phases, the dispersal and reproductive phase[27,28]. During the reproductive phase, foundress mites reproduce and feed on pupating honey bees while protected by the wax brood cell capping, thereby limiting effectiveness of contact treatments when capped brood is present[12,29–34]. Rapid acting treatments that rely on contact to kill *Varroa* mites, such as oxalic acid and HopGuard (active ingredient of hops β-acids), are most effective when *Varroa* is present on adult honey bees in the dispersal phase and there is a low amount or no brood is present allowing the acaricide to contact and kill mites[28,35–40]. Meanwhile, formic acid is the only acaricide that has shown the ability in some studies to penetrate cell cappings, killing mites within brood cells during their reproductive phase[27,41,42]. However, FormicPro (active ingredient of formic acid) has a narrow temperature range. The product has about a 20 degree temperature window from 10ºC to 29.4ºC (50°F to 85°F) limiting its opportunities for use in areas that see fluctuations in temperatures outside that threshold. Furthermore, adult honey bees, appear to be at higher risk when conditions are >29.4°C (85°F), especially within 2-3 days of first application, thus limiting its potential use in regions with elevated temperatures most of the year[43–45]. Additionally, cost of treatment applications is an important factor for beekeepers. Treatments like FormicPro and HopGuard are more expensive than other OA based treatments, thus, they are less economical for beekeepers especially at a commercial scale[11].

This experiment was designed to test the effectiveness of various *Varroa* treatments with differing active ingredients in the southeastern region of the U.S. Due to climatic conditions, this region often has high temperatures and moderate amounts of brood later into the fall and early winter. This characteristic limits *Varroa* treatment selections and reduces effectiveness of treatments for colonies going into winter, a critical time for

Varroa treatments[10,23]. Therefore, we tested the effectiveness of four different registered acaricides (Apivar, FormicPro, HopGuard, and Api-Bioxal) on their ability to reduce *Varroa* infestation rates. Additionally, *Varroa* mites were collected and genotyped to determine how treatment with various active ingredients impacts amitraz resistance genotype frequency.

Results

Varroa infestation rate

There was a significant *Day* and *Treatment* interaction for *Varroa* infestation rate ($\chi^2_8 = 27.47$, $P < 0.001$). On Day 0, we did not observe any significant differences between treatment groups (F ratio$_{4,\infty} = 0.02$, $P = 0.99$) (Figure 1), where *Varroa* infestation rates were estimated at 2.47%, 2.43%, 2.53%, 2.51%, and 2.32% for Control, Apivar, FormicPro , HopGuard, and OA Vapor treatment groups, respectively. On Day 63, there were significant differences between treatment groups (F ratio$_{4,\infty} = 5.85$, $P < 0.001$). The Control group had an estimated *Varroa* infestation rate of 9.20%, which was significantly higher than the 2.95% infestation estimated for the Apivar group (z-ratio$_\infty = 3.29$, $P = 0.009$), 2.58% for the FormicPro group (z-ratio$_\infty = 3.33$, P = 0.008), and 2.74% for the HopGuard group (z-ratio$_\infty = 3.30$, $P = 0.008$). The OA Vapor group had a *Varroa* infestation rate on Day 63 of 7.80%, which was also significantly higher than the Apivar, FormicPro , and HopGuard groups (z-ratio$_\infty = -2.78$, $P = 0.044$; z-ratio$_\infty = -2.87$, $P = 0.033$; z-ratio$_\infty = -2.82$, $P = 0.038$, respectively). No other significant differences in *Varroa* infestation rate existed between treatment groups on Day 63. As on Day 0, we did not find any significant differences among treatment groups on Day 133 (F ratio$_{4,\infty} = 0.75$, P = 0.56) – *Varroa* infestation rates were estimated at 2.53%, 1.63 %, 1.28 %, 1.32 %, and 1.38 % for Control, Apivar, FormicPro , HopGuard, and OA Vapor treatment groups, respectively (Figure 1). Model estimates can be seen in Table S1.

We did not observe any significant changes over time of *Varroa* infestation rate across experimental days for the Apivar (F ratio$_{2,\infty} = 2.56$, $P = 0.077$), FormicPro (F ratio$_{2,\infty} = 2.42$, $P = 0.089$), or HopGuard (F ratio$_{2,\infty} = 2.78$, $P = 0.062$) treatment groups. For the Control and OA Vapor treatment groups we found significant differences for *Varroa* infestation rate across experimental days (F ratio$_{2,\infty} = 16.88$, $P < 0.001$; F ratio$_{2,\infty} = 19.45$, $P < 0.001$). The Control treatment groups had significantly higher *Varroa* infestation rates on Day 63 compared to Day 0 or Day 133 (z-ratio$_\infty = -4.88$, $P < 0.001$; z-ratio$_\infty = -5.39$, $P < 0.001$). Similarly, the OA Vapor treatment group had significantly higher *Varroa* infestation rates on Day 63 compared to Day 0 or Day 133 (z-ratio$_\infty = 3.89$, $P < 0.001$; z-ratio$_\infty = 5.33$, $P < 0.001$).

Colony strength metrics

There was no significant *Day* by *Treatment* interaction on frames of adult honey bees ($\chi^2_8 = 2.74$, $P = 0.95$) or frames of capped honey bee brood ($\chi^2_8 = 3.89$, $P = 0.87$). For both frames of adult honey bees and frames

of capped honey bee brood, there was a significant effect of *Day* (χ^2_2 = 130.47, $P < 0.001$; χ^2_2 = 774.49, $P < 0.001$), but no significant effect of *Treatment* throughout the study (χ^2_4 = 0.65, $P = 0.96$; χ^2_4 = 2.47, $P = 0.65$) (Figures 2 and 3). There was a significant *Day* by *Treatment* interaction on colony weight (χ^2_8 = 36.74, $P < 0.001$) (Figure 4). There was no significant difference between treatment groups for colony weight on Day 0 (F ratio$_{4,179}$ = 0.69, $P = 0.60$), where average colony weights ranged from 39.6-42.2 kg. There was a significant difference between treatment groups on Day 63 (F ratio$_{4,179}$ = 4.46, $P = 0.002$). On Day 63, colonies in the FormicPro treatment group had an estimated weight of 37.1 kg, which was significantly less than the Control (t-ratio$_{179}$ = 2.85., $P = 0.039$), Apivar (t-ratio$_{179}$ = 3.49., $P = 0.006$), and OA Vapor (t-ratio$_{179}$ = -2.89., $P = 0.035$) treatment groups, which had model estimates of 42.1, 43.2, and 42.2 kg respectively. The FormicPro treatment group did not significantly differ on Day 63 from the HopGuard treatment group (t-ratio$_{179}$ = -0.91., $P = 0.89$), which had a model estimate of 38.7 kg, and there were no other significant differences between treatment groups for colony weight on Day 63. On Day 133, there were no significant differences between treatment groups (F ratio$_{4,179}$ = 1.29, $P = 0.27$), and mean colony weights ranged from 36.0-39.2 kg. Model estimates for weight can be seen in Table S1.

Final colony status

The final colony status on Day 133, and reason for removal from the trial, varied among treatment groups; the Apivar treatment group was the only one in which all 15 colonies survived throughout the experiment (Figure 5). The Control treatment group had six colonies removed on Day 63 because *Varroa* mite counts exceeded the pre-determined limit of 45 *Varroa* mites in the alcohol wash. The FormicPro treatment group had five colonies removed from the experiment due to queen failure, all prior to Day 63 in the six weeks after initial FormicPro application. This treatment group also had one colony removed due to weak colony strength between Days 63 and 133. The HopGuard treatment group had a total of four colonies removed from the study. This included one colony removed due to a high *Varroa* count in the alcohol wash, one for queen failure, one for weak colony size, and one colony due to a beekeeping error where a queen was collected in an alcohol wash. The OA Vapor treatment group had four colonies removed from the trial on Day 63 for high *Varroa* mites. One colony was also removed due to queen failure prior to Day 63. All colonies removed due to high *Varroa* counts in the alcohol wash were moved to a secondary location and were inspected on Day 133; however, none of these colonies survived to this point. Final colony numbers on Day 133 remaining in the experiment were 15 for the Apivar treatment group, 9 in the untreated Control, 9 in the FormicPro treatment group, 11 in the HopGuard treatment group, and 10 in the OA Vapor treatment group.

Amitraz resistance genotype

Pretreatment field resistance levels from the Day 0 amitraz resistance field tests showed an average of 32.8% amitraz resistance in the Apivar treatment group, illustrating that colonies in this treatment group had amitraz resistant *Varroa* mites before treatments.

There were no statistical differences in frequency of genotype classes (i.e., susceptible (SS), heterozygous (SR), or resistant (RR)) across the three sampling periods for the Control (F ratio$_{2,\infty}$ = 1.62, P = 0.20; F ratio$_{2,\infty}$ = 1.37, P = 0.25; F ratio$_{2,\infty}$ = 0.54, P = 0.58), FormicPro (F ratio$_{2,\infty}$ = 0.089, P = 0.92; F ratio$_{2,\infty}$ = 0.24, P = 0.78; F ratio$_{2,\infty}$ = 0.38, P = 0.68), HopGuard (F ratio$_{2,\infty}$ = 2.30, P = 0.10; F ratio$_{2,\infty}$ = 0.27, P = 0.76; F ratio$_{2,\infty}$ = 1.60, P = 0.20), or OA Vapor (F ratio$_{2,\infty}$ = 0.67, P = 0.51; F ratio$_{2,\infty}$ = 0.12, P = 0.89; F ratio$_{2,\infty}$ = 0.44, P = 0.65) treatment groups (Figure 6). Model estimates can be seen in Table S2.

There was a significant difference of genotype class frequencies across sampling days for the Apivar treatment group (F ratio$_{2,\infty}$ = 17.20, P < 0.001; F ratio$_{2,\infty}$ = 18.43, P < 0.001) (Figure 7). The amitraz susceptible genotype (homozygous susceptible (SS)) frequency declined to an estimated 0.22 on Day 63 after treatment compared to 0.52 on Day 0 (z-ratio$_\infty$ = -5.83, P < 0.001) or 0.46 on Day 133 (z-ratio$_\infty$ = 3.83, P < 0.001). There was no difference in the amitraz susceptible genotype frequency between Day 0 and Day 133 (z-ratio$_\infty$ = -0.83, P = 0.68). Meanwhile, the homozygous amitraz resistant genotype (RR) frequency significantly increased from 0.39 on Day 0 (z-ratio$_\infty$ = 6.05, P < 0.001) to an estimated 0.71 on Day 63 after treatment then decreased significantly to 0.47 on Day 133 (z-ratio$_\infty$ = -3.58, P = 0.001). Again, there was no difference in amitraz resistant genotype frequency between Day 0 and Day 133 (z-ratio$_\infty$ = 1.18, P = 0.46). There was no observed difference in heterozygous genotype (SR) frequency for the Apivar treatment group across sampling days (F ratio$_{2,\infty}$ = 0.26, P = 0.77) (Figure 7).

Total numbers of *Varroa* mites sampled per treatment group from each sampling day are shown in Table 1. The Apivar treatment group had a larger sample size of mites on Day 0 due to the additional amitraz resistance tests for those colonies and on Day 133 due to higher colony survival throughout the experiment. Total number of mites per genotype can be seen in Table S2.

Discussion

Control of *Varroa* before winter is a critical timepoint, integral to limiting honey bee colony losses over winter[2,46]. Continued increases in amitraz resistance[15,25] and narrow treatment application windows, especially in the Southeastern U.S., limit the treatment options for beekeepers. This experiment demonstrated that Apivar, FormicPro, and HopGuard could all be potential viable treatments for use in the fall to limit increases *Varroa* infestation rate in the Southeastern US when infestation rates are around 2-3%. In contrast, OA Vapor at 2 g per brood box allowed *Varroa* infestation rates to increase even when three applications were

used. This experiment demonstrated that treatments of Apivar were associated with an increase in the amitraz resistance allele frequency in *Varroa* populations, albeit briefly, while treatments with other active ingredients were not. These results show that further research needs to be conducted to find effective treatments at reducing *Varroa* infestation rates in the fall and understand how different treatments impact amitraz resistance allele frequencies in *Varroa* populations.

No treatment group significantly reduced *Varroa* infestation rate at posttreatment sampling times, as infestation rates either remained consistent near 3% or increased at the midpoint sampling. While the limited amount of capped brood area could have artificially increased *Varroa* infestation rates on adult honey bees on Day 63 since less brood represents more mites on adult honey bees[47], the lack of a decline in infestation rate after 63 days could necessitate an additional treatment as colonies reduce brood production and begin preparing for winter[10,48]. Additionally, the absence of difference between treatment groups in *Varroa* rates on Day 133 is likely due to survivorship bias, as multiple colonies were removed from the original yard and later failed due to high mite infestation rates on Day 63 in both the OA Vapor (4 colonies) and the Control (6 colonies) treatment groups; this limited the sample solely to surviving colonies. For this reason, we believe that the Day 63 results are a better guide to understanding the true effects of treatment on *Varroa* infestation. All colonies in the Apivar treatment group survived despite the moderate levels of amitraz resistance, and the *Varroa* infestation levels were maintained <3% adult bee infestation, thus reinforcing the importance of keeping *Varroa* infestations below this level to provide a higher probability of colony survival. Comparatively, both the Control and OA Vapor groups had colonies with high *Varroa* infestation rates that were removed from the study and ultimately died further illustrating that low levels of *Varroa* are critical for colony survival.

These results provide further evidence that the Y215H mutation in the *Octβ2R* is associated with amitraz resistance, and that Apivar application selects for individuals with the amitraz resistant genotype thereby increasing resistance levels in populations. We observed a slight numerical increase in *Varroa* infestation rate (though not significant) along with a significant increase in the resistant genotype frequency. While the *Varroa* infestation rate was significantly lower after treatment with Apivar compared to the Control, the *Varroa* infestation rate in the Apivar treatment group slightly increased from Day 0 to Day 63 (Day 0 Pretreatment = 2.43% vs Day 63 Posttreatment = 2.95%). It is important to recognize that maintaining, instead of decreasing, *Varroa* infestation rates after treatment can be considered a treatment failure since amitraz susceptible *Varroa* populations should significantly decline after Apivar application[15,23]. Furthermore, this research shows that while *Varroa* infestations may maintain in quantitative terms, the populations can change in a qualitative

sense in that the frequency of resistance genotypes significantly increases, thus increasing the probability of a subsequent treatment failure.

Observing that Apivar treatment affected resistance allele frequency supports previous findings that the Y215H mutation in the *Octβ2R* is a cause of target site resistance consistent with other classic examples, such as the numerous *kdr*-type mutations in the sodium channel that provides resistance to pyrethroids[49,50] or truncated nicotinic acetylcholine receptors that provide spinosad resistance[51]. Consistent with previous results[25], these findings reinforce the utility of genotyping assays for the Y215H mutation as a valuable tool for resistance monitoring. However, other mutations in *Octβ2R* exist in European and Asian *Varroa* populations[18–20], so frequent genomic reevaluations should assess if other resistance alleles become important in the population, as has happened with further *kdr* resistance mutations occurring in house flies[52] and Colorado potato beetles[53]. Furthermore, additional causes of amitraz resistance should also be explored[26] to assist in broadening the understanding of resistance development within *Varroa* populations. While this study demonstrated an association of the Y215H mutation with amitraz resistance, further investigation needs to be done to determine what other mutations or supplementary changes may influence amitraz resistance in *Varroa*[54,55].

The decline in the amitraz resistant genotypes and the resistant allele frequency during the overwintering period from Day 63 to Day 133 in the Apivar treatment group can be taken as an indication that the Y215H mutation may impose a fitness cost on *Varroa* because the resistance allele frequency significantly decreased from Day 63 to Day 133 along with a subsequent increase in susceptible allele frequency between those observations. However, if this was a real fitness cost, this trend of reduced resistant genotypes and resistant allele frequencies over time should have been observed in all colonies regardless of treatment group. Contrary to expectations, this was only observed in the Apivar treatment group. This opens the question as to why overwintering amitraz resistant allele frequency on Day 133 declined in the Apivar treatment group to pretreatment levels after initially increasing by Day 63 following an Apivar application. One potential explanation is that small population sizes can increase the impact of genetic drift and result in the loss of resistance alleles from populations[56]. There is the possibility that *Varroa* infestation rates were low enough that the resistance genotype was not maintained and the overall gene pool of *Varroa* was homogenized from Day 63 to Day 133 due to random drift of genes or movement of *Varroa* mites on adult honey bees from neighboring colonies[9,57,58]. While genetic drift is a possibility to explain observed results, the equalization of allele frequencies occurred over a short period, likely in less than five *Varroa* generations[47], limiting the time for random genetic drift to occur, especially in a primarily inbred system like *Varroa*. The impact or potential fitness cost of the Y215H mutation remains an interesting component of amitraz resistance, and

further research should be done to explore the changing allele frequency dynamics between resistant and susceptible *Varroa* populations over time.

The lack of change in the frequency of the resistant genotype in other treatment groups suggests that the Y215H mutation does not provide cross resistance to the other tested treatments, nor does it make individuals with this mutation more susceptible to other treatments. Therefore, this implies that *Varroa* populations displaying amitraz resistance via the Y215H mutation in *Octβ2R* can be managed by application of other acaricides. Data from Day 63 of this study showed that FormicPro and HopGuard were similarly effective as Apivar in managing amitraz resistant *Varroa* infestations. Additionally, at higher levels of amitraz resistance, it is possible that the effect of these alternative treatments would be superior to an amitraz based treatment.

In this experiment, none of the treatments significantly reduced *Varroa* infestation. To increase the likelihood of overwintering colony success, *Varroa* infestation rates should be reduced prior to overwintering to prevent mites from feeding on adult bees while in the dispersal phase, an important component in honey bee winter survival[59,60]. Without the initial 32.8% amitraz resistance observed, we would have expected to see the amitraz based treatment of Apivar to significantly reduce *Varroa* infestation rates to near zero levels making it more effective than the FormicPro and HopGuard treatment groups[15,22,24,61,62]. Consequently, this experiment demonstrated that regardless of treatment used, additional follow-up treatments may be necessary to reduce *Varroa* infestations in the Southeastern U.S., particularly if beekeeping operations have some level of amitraz resistance present. These results also show the importance of posttreatment monitoring to ensure that the treatment was effective in reducing or keeping *Varroa* infestation rates manageable. Oxalic acid is a common treatment used late in the season to control *Varroa* in the dispersal phase[30,36,63,64]. This experiment illustrates the importance of avoiding treating with OA when colonies have brood present since the OA Vapor treatment group had significantly higher *Varroa* infestation rates compared to other treatment groups and did not differ from the Control on Day 63[22,33,34]. This is reinforced by the observation that at Day 63, during the week of 20 November 2023, the colonies in this study still maintained some amount of brood, thus extending the reproductive period for *Varroa* later in the year. While both the HopGuard and FormicPro treatment groups were able to prevent increases in *Varroa* infestation rates, both treatment groups had multiple colonies removed before the end of the study. The HopGuard treatment group had some colony loss, but there was no single major cause of loss; we saw better survival than a previous overwintering study utilizing HopGuard[40]. Comparatively, the FormicPro treatment group had five of the 15 colonies losing their queens before Day 63. This illustrates that FormicPro remains a difficult treatment to employ in the Southeast due to its brief application window resulting in potential queen losses as evidenced in this experiment[43,44]. This was despite appropriate temperatures during

the application period as per label instructions, although temperatures were close to label maximum when they reached a high of 29.3ºC four days posttreatment, right at the label maximum of 29.4ºC[45]. While further impact on colony strength has been observed in other studies using formic acid[65,66], we only saw reduced colony weight on Day 63 in the FormicPro treatment group which was not associated with any significant differences in frames of capped honey bee brood or frames of adult honey bees.

This experiment demonstrates that Apivar is effective at maintaining *Varroa* infestation levels despite moderate levels of amitraz resistance (32.8%), but we tentatively expect that in operations with high (40-60%) to very high levels amitraz resistance (>60%), beekeepers are unlikely to see successful reduction or maintenance of *Varroa* infestation rates. Similarly, this experiment again showed that repeated applications of OA Vapor at the previous label rate of 2 g per brood box is an insufficient treatment when capped brood is present[33,34]. Meanwhile, FormicPro may be best utilized in the spring when beekeepers can more easily recover from potential queen loss. Comparatively, in conditions such as these, HopGuard may be appropriate to maintain *Varroa* infestation levels, but not to reduce them. As amitraz resistance continues to rise beekeepers need to optimize alternative *Varroa* treatment methods to reduce reliance on amitraz to ensure it can remain viable in future years. Overall, more research needs to be done to investigate the persistence of resistance genotypes in *Varroa* populations to assist beekeepers in managing colonies with these resistance pressures.

Materials and Methods

Experimental Design

The experiment used a total of 75 *Apis mellifera* honey bee colonies in two apiaries, located 6.5 km apart in Auburn, Alabama, USA. All colonies were established in April 2023 and maintained according to standard beekeeping practices based on the needs of the colonies. At the beginning of the experiment, all colonies were queenright with Italian-type queens and housed in two deep 10-frame Langstroth boxes. Colonies were initially assessed the week of 25 September 2023 for *Varroa* infestation rate on adult honey bees, capped honey bee brood area, frames of adult honey bees, and colony weight. *Varroa* infestation rate was determined by shaking adult honey bees from a frame containing open worker brood into a plastic tub and scooping ~ 120 mL (1/2 cup) of adult honey bees (~ 300 honey bees) into a 946 mL (32 oz) polypropylene container containing 35% isopropyl alcohol[67]. Triple rinse mite washes were performed using methods described in Aurell et al. (2024), and mites were collected in 1.5 mL microcentrifuge tubes and stored in a freezer at –20°C. Adult honey bees were also collected after mite washes and counted to record *Varroa* infestation rate (% adult bees infested). Colony strength assessments of frames of capped honey bee brood and adult honey bees were done using the methods described in Guzman-Novoa et al. (2024). All colonies were

weighed to determine differences in accumulated resources like honey, nectar, pollen, brood, and adult honey bees, by using a tripod pulley system to lift colonies on a spring scale. After initial colony inspections, colonies were assigned to one of five treatment groups based on *Varroa* infestation rate through stratified randomization[69–74]. Colonies were organized by descending order of *Varroa* infestation rate with five colonies per stratum and randomly assigned so each treatment was represented once per stratum. The proposed treatment assignments were reviewed and rerandomized several times to assist in the homogenization of colony strength variables and to evenly distribute treatment groups between the two locations. Treatments were applied the week of 25 September 2023. The daily high temperatures during the first week of treatment ranged from 23.4ºC to 29.3ºC with the highest daily temperature being recorded 4 days after treatment. Colonies were reassessed for the same four metrics using previously described methods the week of 27 November 2023 and the week of 5 February 2024 (63 and 133 days after treatments were applied, respectively). These dates were selected to evaluate *Varroa* infestation rates after the treatment and overwintering periods. In the U.S. Southeast, late November presents a time when brood production is reduced, allowing for the opportunity of an additional treatment, while early February constitutes a point when brood production begins increasing and spring management starts (Winter Capped Brood Monitoring in Honey Bee Colonies 2025). Colonies were also monitored monthly through December to assess queen presence. Colonies were removed from the study and designated as queen failure if they were queenless on two consecutive inspections, as determined by absence of eggs. Colonies were also removed and designated as weak if they had less than one frame of adult honey bees by field assessment. Lastly, colonies were moved to a secondary location 18.6 and 24.4 km away from established apiaries after Day 63 assessments if more than 45 *Varroa* mites were found in the mite wash (~15 *Varroa* mites per 100 honey bees) to prevent reinfestation of previously treated colonies[9,57,58,76]. Data collected up until the point of colony removal were kept in the analysis.

Varroa treatments

This experiment included five different treatment groups with 15 colonies per group. The treatments included an untreated Control, Apivar (active ingredient (a.i.) amitraz at 3.3%), FormicPro (a.i. formic acid at 65%), HopGuard 3 (a.i. hops β-acids at 16%), and an Api-Bioxal oxalic acid vapor (OA Vapor) regimen (a.i. oxalic acid at 99%). For the Apivar group, two strips of Apivar per deep hive body for a total of four strips per colony were placed in the brood nest and removed after 42 days. Prior to treatment with Apivar, all colonies in this treatment group (n = 15) had an amitraz resistance test performed using previously described methods[15] to give a baseline understanding of amitraz resistance levels which would help explain the performance of Apivar. In brief, this involved collecting ~120 mL (1/2 cup) of adult honey bees into a 946 mL (32 oz) polypropylene

container using previously described methods. The container had a 4 cm by 4 cm square of Apivar glued to the bottom of the container and after honey bee collection it was fitted with a lid of 3.2 mm (1/8th in.) hardwire cloth. The cup had small binder clips added around the lid and was inverted over a plastic weigh boat coated in petroleum jelly for three hours. After three hours, the polypropylene container was flipped again, 35% isopropyl alcohol was added, and an alcohol wash was completed using previously described methods. *Varroa* mites that had fallen on the weigh boat were collected and considered amitraz susceptible while mites collected in the alcohol wash were collected and considered amitraz resistant. The results of this field-relevant bioassay is a highly reliable indicator of amitraz resistance measured by the LC_{50} from glass vial laboratory bioassays[15]. The cup bioassay is much more convenient than the glass vial bioassay since it can be performed in the field, does not require an incubator (although the test does need to be performed between 20-30C to ensure reliable results)[77], can be completed in 3 hours, and the results much more relatable to beekeepers. The FormicPro group had two FormicPro pads placed in between the deep hive bodies, and the pads were removed 14 days later. The HopGuard group had two strips of HopGuard 3 applied per deep hive body for a total of four strips per colony placed in the brood nest. These strips were removed after 14 days and another round of HopGuard 3 was applied in the same manner which was also subsequently removed after another 14 days. The OA Vapor group received 2 grams of Api-Bioxal per brood box for a total of 4 grams per colony three times with treatments spaced 7 days apart[78]. OA Vapor was administered using a ProVap device (OxaVap). The vaporizer was allowed to reach 230°C which took approximately one minute before it was removed. Prior to application, all cracks in the hive were sealed with tape, and a rag was used to plug the entrance of the hive. The rag was removed ten minutes after the OA Vapor treatment had been applied. All treatments and dosages were applied according to U.S. labels.

Amitraz Resistance Genotyping

All *Varroa* from all assessments and treatment groups that were collected in alcohol washes were shipped to the USDA-ARS Honey Bee Breeding, Genetics, and Physiology Lab, Baton Rouge, LA, where they were stored at -80°C until genotyping. Genotyping was done to assess genotype frequency of the Y215H mutation in the β2 octopamine receptor (*Octβ2R*)[25] for each different treatment group individually per sampling day: 1757 *Varroa* were genotyped before treatment (September, n=742), after treatment (November, n=763), and after overwintering (February, n=252). Genomic DNA was extracted from individual *Varroa* after homogenizing in a Bead Ruptor Elite Mill (OMNI-International) using the Maxwell RSC PureFood GMO and Authentication Kit (Promega) according to the manufacturer's directions. DNA concentration was measured on a NanoDrop (ThermoFisher). Genotyping for the Y215H mutation in the β2

octopamine receptor (*Octβ2R*) was performed using a previously described TaqMan assay[18,25]. Genotyping was performed only for Y215H mutation as it was the only mutation previously identified in the US and Canada[18,79]. Other research has supported regionally exclusive mutations in association with amitraz resistance[18,20,80], and previously tested mites from the US did not have the N87S mutation as found in France[18,79].

Statistics

All statistics were conducted using R version 4.3.3[81]. Packages used included *lme4*, *dplyr*, *tidyverse*, *glmmTMB*, *emmeans*, *DHARMa*, and *ggplot2*[82–87]. *Varroa* infestation rate was analyzed using a generalized linear mixed model with negative binomial distribution, and fixed effects of *Day*, *Treatment*, and a *Treatment* by *Day* interaction. A log offset was used to account for variation in the number of adult honey bees per sample[83], and, because pseudoreplication can be an issue[88], a random effect of *Hive* was used to account for repeated measurements[33,89,90]. Colony weight was analyzed with a linear mixed model with normal (Gaussian) distribution, fixed effects of *Day*, *Treatment*, and a *Treatment* by *Day* interaction, and a random effect of *Hive*. Since both models had significant interactions between *Treatment* and *Day*, models were tested using the *joint_tests* function in the *emmeans* package to determine if there were significant differences between treatment groups within day[87]. Pairwise comparisons were then made using the *emmeans* package, where Tukey-HSD adjusted p-values were used and significance was determined with an alpha of 0.05. Frames of adult honey bees and capped honey bee brood were analyzed using a linear mixed model with normal distribution, fixed effects of *Day* and *Treatment*, and a random effect of *Hive*. An F-test of nested models indicated that the *Treatment* by *Day* interaction did not significantly improve model fit for either the frames of adult honey bees ($\chi^2_8 = 2.74$, P = 0.95) or capped honey bee brood models ($\chi^2_8 = 3.89$, P = 0.87). Consequently, descriptive statistics were used to describe differences among treatment groups within day. Additionally, scaled quantile residuals were used and QQ plots were visualized utilizing the *DHARMa* package to test for model fit and ensure an appropriate model distribution was selected[86].

Genotype data were summarized by treatment group and genotype. Generalized linear mixed models with binomial distribution with a fixed effect of *Treatment* by *Day* interaction and a random effect of *Hive* were used to analyze differences among resistance genotype for homozygous resistant, heterozygous, and homozygous susceptible genotypes over time[83,88]. Since the FormicPro treatment group did not have any *Varroa* mites with a heterozygous genotype on Day 133, the heterozygous genotype for the FormicPro treatment group was given an estimate of zero[74]. Pairwise comparisons were made using the *emmeans* package for each genotype, where Tukey-HSD adjusted p-values were used and significance was determined with an alpha of 0.05. All pairwise comparisons were done

as specific model estimates were of interest for all allele frequencies in this study. The *DHARMa* package was again used to visualize QQ plots utilizing quantile residuals to test for model fit and confirm the use of the appropriate model distribution[86].

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Author Contributions

RT: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. FR: Conceptualization, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – Reviewing & Editing. DA: Conceptualization, Investigation, Methodology, Writing – review & editing. NE: Investigation, Methodology. KC: Investigation, Methodology. GW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – reviewing & editing.

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Data Availability

Datasets generated from this experiment will be made available upon reasonable request to the corresponding author.

Competing Interests

The authors have no competing interests to declare. We also do not endorse any beekeeping practice, product, or active ingredient included in this study.

Figure Legends

Figure 1. *Varroa destructor* infestation rate on adult honey bees (*Apis mellifera*) among all treatment groups on each experimental day. Model estimated means are shown with 95% confidence limits. Letters indicate significant differences between treatments for each individual sampling period. ($P < 0.05$) (OA = oxalic acid).

Figure 2. Boxplots showing frames of adult honey bees (*Apis mellifera*) among all treatment groups on each experimental day. No significant differences were observed between treatments for any individual sampling period. (All $P > 0.05$) (OA = oxalic acid).

Figure 3. Boxplots showing frames of capped honey bee (*Apis mellifera*) brood among all treatment groups on each experimental day. No significant differences were observed between treatments for any individual sampling period. (All $P > 0.05$) (OA = oxalic acid).

Figure 4. Mean honey bee (*Apis mellifera*) hive weight among treatment all groups on each experimental day. Model estimated means are shown with 95% confidence limits. Letters indicate significant differences between treatments for each individual sampling period. ($P < 0.05$) (OA = oxalic acid).

Figure 5. Final honey bee (*Apis mellifera*) colony status on Day 133 across all different treatment groups. All treatment groups started with 15 colonies. Colonies removed due to high *Varroa destructor* infestation had > 45 *Varroa* mites in the wash representing approximately >15% *Varroa* infestation rate of adult bees. Colonies removed due to being weak had <1 frame of adult honey bees during an inspection. (OA = oxalic acid).

Figure 6. Amitraz resistance genotype frequency in *Varroa destructor* mites from honey bee (*Apis mellifera*) colonies in the Control, FormicPro, HopGuard, and OA Vapor treatment groups. Relative amitraz genotype frequency for the *Octß2R* Y215H resistance genotype for different treatment groups across experimental days. (SS = homozygous susceptible, SR = heterozygous, RR = homozygous resistant). Model estimates are shown. No significant differences were observed among resistance genotype classes for any treatment across the three sampling periods. (All $P > 0.05$) (OA = oxalic acid).

Figure 7. Amitraz resistance genotype frequency in *Varroa destructor* mites from honey bee (*Apis mellifera*) colonies in the Apivar treatment group. Relative amitraz resistance genotype frequency for the *Octß2R* Y215H resistance genotype for the Apivar treatment group across experimental days. (SS = homozygous susceptible, SR = heterozygous, RR

= homozygous resistant). Model estimated means are shown with 95% confidence limits. Letters indicate significant differences between resistance genotype class across each sampling period. ($P < 0.05$).

Tables

Table 1. Total number of *Varroa destructor* mites genotyped per treatment group on each individual sampling day. A total of 1757 individual *Varroa* were genotyped.

Treatment	Day 0	Day 67	Day 133	Treatment Total
Control	119	231	52	**402**
Apivar	290	154	88	**532**
FormicPro	116	90	33	**239**
HopGuard	111	100	38	**249**
OA Vapor	106	188	41	**335**
Day Total	**742**	**763**	**252**	**1757**