2017

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Published in *Journal of Invertebrate Pathology* 149 (2017), pp 119–126.
doi 10.1016/j.jip.2017.08.005

Submitted 26 May 2017; revised 28 July 2017; accepted 5 August 2017; online 7 August 2017.

Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection

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Abstract

The health and survival of managed honey bee (*Apis mellifera*) colonies are affected by multiple factors, one of the most important being the interaction between viral pathogens and infestations of the ectoparasitic mite *Varroa destructor*. Currently, the only effective strategy available for mitigating the impact of viral infections is the chemical control of mite populations. Unfortunately, the use of in-hive acaricides comes at a price, as they can produce sublethal effects that are difficult to quantify, but may ultimately be as damaging as the mites they are used to treat. The goal of this study was to investigate the physiological and immunological effects of the formamidine acaricide amitraz and its primary metabolite in honey bees. Using flock house virus as a model for viral infection, this study found that exposure to a formamidine acaricide may have a negative impact on the ability of honey bees to tolerate viral infection. Furthermore, this work has demonstrated that amitraz and its metabolite significantly alter honey bee cardiac function, most likely through interaction with octopamine receptors. The results suggest a potential drawback to the in-hive use of amitraz and raise intriguing questions about the relationship between insect cardiac function and disease tolerance.

Keywords: Honey bee, Heart rate, Virus, Acaricide, Amitraz, DPMF, Octopamine, Phentolamine

1. Introduction

The honey bee (*Apis mellifera*) is valued for providing economically and agriculturally important pollination services, as well as for providing honey and other natural products. Unacceptably high annual losses in the number of managed bee colonies in the United States (Seitz et al., 2016) have increased public awareness of pollinator health issues and focused research efforts on understanding why these losses occur. Although there exist a wide variety of factors that negatively affect pollinator health (Goulson et al., 2015), one of the most significant threats to the survival of managed bee colonies is the risk of acute viral infections (Evans and Schwarz, 2011; Manley et al., 2015). The growing impact of viral infections is associated with the increased prevalence of the ectoparasitic mite *Varroa destructor*, which facilitates the spread of viral pathogens and weakens the immune responsiveness of bees, causing previously covert viral infections to become devastating outbreaks (Genersch and Aubert, 2010; Le Conte et al., 2010; Nazzi et al., 2012). At this time, the only effective strategy that exists for minimizing the spread and impact of viral infections is the management of mite infestations, which relies heavily upon the use of apicultural acaricides such as the organophosphate coumaphos (Checkmite®), the pyrethroids *tau*-fluvalinate (Apistan®) and flumethrin (Bayvarol®), and the formamidine amitraz (Apivar®) (Rosenkranz et al., 2010).

One of the most comprehensive surveys to date of agrochemicals associated with managed bee colonies in the United States found that acaricides used to control *Varroa*, or their associated metabolites, are among the most ubiquitous contaminants of the hive environment (Mullin et al., 2010). Although the acaricides coumaphos and *tau*-fluvalinate have decreased in effectiveness over the years, due to metabolic and target-site resistance in *Varroa* populations (Pettis, 2004), they were the most common hive contaminants detected in the survey (Mullin et al., 2010), likely as a result of their continued use by beekeepers and their lipophilic nature, which allows them to persist in beeswax (Bogdanov, 2006). While amitraz does not persist in the hive environment (Martel et al., 2007), its metabolite *N*-(2,4-dimethylphenyl)-*N*′-methylformamidine (DPMF) does accumulate and was among the ten most commonly detected pesticides in wax, pollen, and the bees themselves (Mullin et al., 2010). This finding is somewhat

surprising, as amitraz was withdrawn from commercial use in 1994 and not registered for apicultural use at the time of the survey (Johnson et al., 2010), which suggests that it continued to be employed as a control measure in many areas. Since amitraz was reregistered for apicultural use by the Environmental Protection Agency in 2013, it is likely that its presence in the hive environment has increased.

Amitraz is a formamidine acaricide that was originally marketed in the United States under the trade name Miticur®, until it was withdrawn from commercial apicultural use. Amitraz, however, remained available as a veterinary acaricide under the trade name Taktic®, which was not labeled for apicultural use, until being reregistered under the name Apivar®. Formamidines act as octopaminergic agonists in arthropods (Evans and Gee, 1980), suggesting that they are likely to influence honey bee behavior, learning, and memory formation, in addition to affecting physiological processes related to various tissues and sensory organs (Roeder, 2005). The biogenic monoamine octopamine is understood to act as a neurotransmitter/neuromodulator in insects and other invertebrates, homologous to the noradrenergic system of vertebrates (Roeder, 1999). High levels of octopamine in the brain of honey bee workers can influence the division of labor within the colony (Schulz and Robinson, 2001) and affect foraging behavior (Barron et al., 2007). Stimulation of octopamine receptors improves kin recognition in honey bees (Robinson et al., 1999), and octopamine receptors appear to play a role in modulating honey bee hygienic behavior (Spivak et al., 2003). Octopamine, a known cardioaccelerant in insects, alters heart rate in isolated honey bee hearts (Papaefthimiou and Theophilidis, 2011), and the acaricide amitraz appears to have similar effects in this model (Papaefthimiou et al., 2013). Acute exposure to amitraz has been shown to cause cell death in the midgut of honey bee larvae (Gregorc and Bowen, 2000), but does not appear to affect learning, short-term memory, or hemolymph octopamine levels in honey bee workers (Rix and Cutler, 2017), nor has it been found to affect the survival or sperm viability of honey bee drones (Johnson et al., 2013). Though some acaricides have been found to reduce honey bee immunocompetence (Boncristiani et al., 2012; Locke et al., 2012), amitraz was not observed to alter the expression profiles of a wide range of metabolic genes involved in detoxification, immunity, and development, nor did it appear to increase pathogen levels in treated honey bee colonies (Boncristiani et al., 2012).

At this time, no studies have been published that characterize the physiological or immunological effects of the amitraz metabolite DPMF in honey bees. Furthermore, little is known about the effect of formamidines, or any other class of pesticides, on the ability of bees to resist or tolerate viral infections. A number of challenges are associated with the study of viral infection in bees, including the high prevalence of covert, and often concurrent, viral infections in managed colonies (Chen et al., 2004; de Miranda et al., 2010; Runckel et al., 2011), as well as a lack of availability of infectious clones of bee-specific viruses. These factors pose a challenge for researchers focused on the outcome of infection with a single virus. While some research has been conducted using semi-purified virus preparations (Chen and Siede, 2007), complete removal of contaminating viruses is often impossible, making the accurate characterization of infection dynamics difficult. This represents a significant knowledge gap, given the impact that viruses have on colony health and survival (Cox-Foster et al., 2007; Johnson et al., 2009; McMenamin and Genersch, 2015), the effect of pesticide usage on pollinator health (Mullin et al., 2010), and concerns related to managed bee colony losses (Neumann and Carreck, 2010; Ratnieks and Carreck, 2010). The research described here will begin to address this gap by investigating the effect of amitraz and DPMF on the cardiac function of an agriculturally and economically important pollinator and model social insect. This work will then utilize a recently-described model virus system (O'Neal et al., 2017a) to assess the impact of amitraz and DPMF on the outcome of a viral infection in the honey bee.

2. Materials and methods

2.1. Subjects

European honey bees (*Apis mellifera*) from colonies located at the Virginia Tech Price's Fork Research Facility (Blacksburg, VA) apiary were used for all experiments. Colonies received no pesticide treatments or other exposure to in-hive chemical controls, but otherwise were maintained according to standard beekeeping practices for commercial hives. All bees that were housed in the lab overnight or longer were maintained in incubators at 32 °C with a relative humidity of 50–80%. For all dissection and heart rate assays, worker bees were collected

from brood frames during typical foraging times to ensure collection of predominately nurse bees. Any workers collected from the apiary that were housed in the lab incubators overnight were provided *ad libitum* access to honey and a 50% solution (w/v) of sucrose in water. For all survival experiments, frames of emerging worker brood were removed from the hive and housed in a lab incubator in order to obtain age-matched cohorts of bees. Newly emerged bees were collected from these frames over the course of 24 h and housed in cages in groups of approximately 25 bees per cage with *ad libitum* access to a 50% solution (w/v) of sucrose in water. Cages were maintained in the incubator for the duration of the experiment and were provided with ¼ portions of a queen mandibular pheromone-impregnated strip (Mann Lake Ltd.) to reduce stress by simulating the presence of an egg-laying queen.

2.2. Dissection and heart rate assay

Visualization and pharmacological manipulation of the honey bee heart, as well as measurements of heart rate, were conducted as previously described (O'Neal and Anderson, 2016; O'Neal et al., 2017b). Individual bees were dissected to separate the dorsal abdominal wall and expose the dorsal vessel, which was bathed in an isotonic solution (¼ strength Ringer's solution; Sigma-Aldrich) and given time to allow the heartbeat to stabilize. Baseline heart rate was measured for 1 min prior to treatment, then measured again 2 min later. All test compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted in ¼ strength Ringer's solution to prepare stock solutions. Test compounds were prepared by serial dilution, ensuring a consistent vehicle of 1% DMSO in ¼ strength Ringer's solution. Changes in heart rate were reported as percent change relative to the baseline heart rate, measured in beats per minute (BPM).

2.3. Concentration response experiment

The cardiomodulatory effects of the formamidine acaricide amitraz and its primary metabolite DPMF on bee heart rate were evaluated by testing a range of concentrations for each compound, along with the insect neurotransmitter/neuromodulator octopamine and the octopamine receptor antagonist phentolamine. All test compounds were

obtained from Sigma-Aldrich at the highest purity available and prepared and delivered in 1% DMSO in ¼ strength Ringer's solution, which served as the vehicle control. Test compounds were evaluated across a range of concentrations spanning the high nanomolar to the low millimolar in order to establish a profile for each compound. The sample size for each treatment group consisted of 10 individual bee dissections.

2.4. Phentolamine pretreatment experiment

The ability of amitraz and its metabolite DPMF to modulate honey bee heart rate via interaction with octopamine receptors was examined to determine if phentolamine, a specific octopamine receptor antagonist in insects, including honey bees (Degen et al., 2000), could block their effects. Based on the results of the previous experiment, 100 nM phentolamine was selected to test against 100 µM octopamine, amitraz, and DPMF. Phentolamine was tested at the highest concentration that did not produce a significant effect on heart rate. The concentrations of octopamine, amitraz, and DPMF were selected due to their significant effect on heart rate. The dissection and pharmacological manipulation assay remained unchanged, except that following dissection and visualization, the heart was bathed in either vehicle or vehicle containing 100 nM phentolamine. The protocol then continued as previously described, with the application of 100 µM octopamine, amitraz, DPMF, or vehicle control following 1 min of baseline heart rate assessment. The sample size for each treatment group consisted of 12 individual bee dissections.

2.5. Acaricide exposure

Bees were exposed to acaricides either through oral administration or exposure to a portion of an Apivar® Miticide Strip (Mann Lake Ltd.) in the cage. For oral administration, stock solutions of amitraz and DPMF (Sigma-Aldrich) were initially prepared in DMSO. Test groups received 50% sucrose solution (w/v) supplemented with either amitraz or DPMF (100 µM final concentration) in DMSO (1% final concentration), while vehicle control groups received sucrose solution supplemented only with 1% DMSO. In order to avoid complications related to the poor solubility of amitraz, supplemented solutions were

prepared fresh daily. The concentration of amitraz and DPMF (100 μM) was selected based on preliminary testing, which revealed this to be the highest concentration of either compound that did not significantly affect bee survival over a 10 d period. Preliminary testing also demonstrated no significant effects of sucrose solution supplemented with 1% DMSO on bee survival over a 10 d period. In order to simulate the type of exposure that bees would typically have to an in-hive acaricide treatment, test cages were equipped with a 0.5 cm × 4 cm portion of an amitraz-impregnated acaricide strip (3.33% active ingredient). Preliminary testing demonstrated that the presence of the acaricide strip did not have any effect on bee survival over a 10 d period.

2.6. Viral infection

Viral infections were performed using flock house virus (FHV), generously provided by Dr. Anette Schneemann (The Scripps Research Institute, La Jolla, California), that was purified as previously described (Marshall and Schneemann, 2001). FHV has been shown to pathogenically infect honey bees and has been used as a model for the study of viral infections in bees (O'Neal et al., 2017a). Viral stocks were prepared in 10 mM Tris-HCl, pH 7.5. Infections were performed by injection (Nanoject II apparatus; Drummond Scientific) of 50.6 nl of a 2×10^7 plaque-forming units (pfu)/μl viral suspension into the thorax of each bee, resulting in the delivery of 1×10^6 pfu of FHV/bee. This virus titer was selected based on previous work (O'Neal et al., 2017a), which suggested that it would produce a moderate infection that would permit the observation of changes in survival over time in different treatment groups. Injection of the same volume of 10 mM Tris-HCl, pH 7.5, was used as a vehicle control. Preliminary testing compared the survival of bees following vehicle control injections and sham injections, in which the bee thorax was punctured by the needle without delivery of fluid, and found that neither group experienced significant changes in survival relative to uninjected bees over a 10 d period.

2.7. Oral dosing survival experiment

The effects of amitraz and its metabolite DPMF on the survival of virus-challenged honey bees was examined by orally exposing individuals to these compounds and then infecting them with FHV. For all

survival experiments, six replicates of 25 bees each were used for each treatment group. Bees were injected with either vehicle or virus following 24 h of exposure to amitraz-supplemented, DPMF-supplemented, or DMSO-supplemented sucrose solution as the only source of food and water. Bees were provided access to the same sucrose solution, which was prepared fresh each day, for the duration of the test. Survival was observed daily following injection for 10 d.

2.8. Acaricide strip survival experiment

The effects of contact exposure to amitraz on the survival of virus-challenged honey bees was examined by exposing bees to an amitraz-impregnated plastic strip, a commonly-used treatment in mite-infested hives, and then infecting them with FHV. As in the previous survival experiments, six replicates of 25 bees each were used for each treatment group. Bees were injected with either vehicle or virus following 24 h of exposure to the amitraz strip and remained exposed to the strip throughout the duration of the test. Survival was observed daily following injection for 5 d.

2.9. Statistical analysis

All heart rate assay analyses and calculations were conducted using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). All heart rate assay results are expressed as the mean ± standard deviation (SD). Each heart rate assay treatment group was subjected to a D'Agostino-Pearson test for normality ($P < 0.05$) and not all groups were found to come from a normally distributed population; therefore, a nonparametric, two-tailed Mann-Whitney test ($P < 0.05$) was used for all comparisons between heart rate assay treatment groups. A one-way analysis of variance (ANOVA) was used to test for differences ($P < 0.05$) in the mean baseline heart rate of groups in the concentration response experiment. All survival experiment results are reported as Kaplan-Meier survival curves, calculated using GraphPad Prism 7, with significant differences between the survival curves determined by the log-rank (Mantel-Cox) test. For the acaricide strip survival experiment, the treatment mortality (Untreated/Virus and Amitraz/Virus) was corrected for control mortality (Untreated/Vehicle and Amitraz/Vehicle) using

Abbott's formula (Abbott, 1925). A mixed model repeated-measures analysis of the corrected mortality data was performed as previously described (Singh et al., 2016) using JMP Version 13 (SAS Institute Inc., Cary, NC) to determine differences between treatments and their interaction with time. In the model, treatment, day, and treatment by day were the fixed effects and replicate was the random effect.

3. Results

3.1. Concentration response experiment

Phentolamine treatment decreased honey bee heart rate, whereas octopamine, amitraz, and DPMF treatment increased heart rate (**Fig. 1**). Phentolamine produced a concentration-dependent effect on heart rate, as the application of increasing concentrations resulted in greater decreases in heart rate with no evidence of an increase at any concentration. A significant decrease in heart rate relative to the vehicle control was observed at concentrations of 300 nM and above (Mann-Whitney test; $P < 0.01$) with maximal effect (complete cessation of heart beat) observed at 1 mM. Mean baseline heart rate (±SD) across phentolamine treatment groups was 105.0 ± 2.5 BPM. Octopamine significantly increased heart rate relative to the vehicle control at concentrations of 3 μM and above (Mann-Whitney test; $P < 0.01$). Mean baseline heart rate (±SD) across octopamine treatment groups was 101.7 ± 9.6 BPM. Amitraz significantly increased heart rate relative to the vehicle control at concentrations of 30 μM and above (Mann-Whitney test; $P < 0.01$). Mean baseline heart rate (±SD) across amitraz treatment groups was 102.8 ±4.7 BPM. DPMF significantly increased heart rate relative to the vehicle control at concentrations of 1 μM and above (Mann-Whitney test; $P < 0.01$). Mean baseline heart rate (±SD) across DPMF treatment groups was 105.4 ± 3.1 BPM. Maximal effects could not be determined for octopamine, amitraz, and DPMF due to solubility issues at concentrations greater than 1 mM. No statistically significant differences were detected between the mean baseline heart rates of each experimental group.

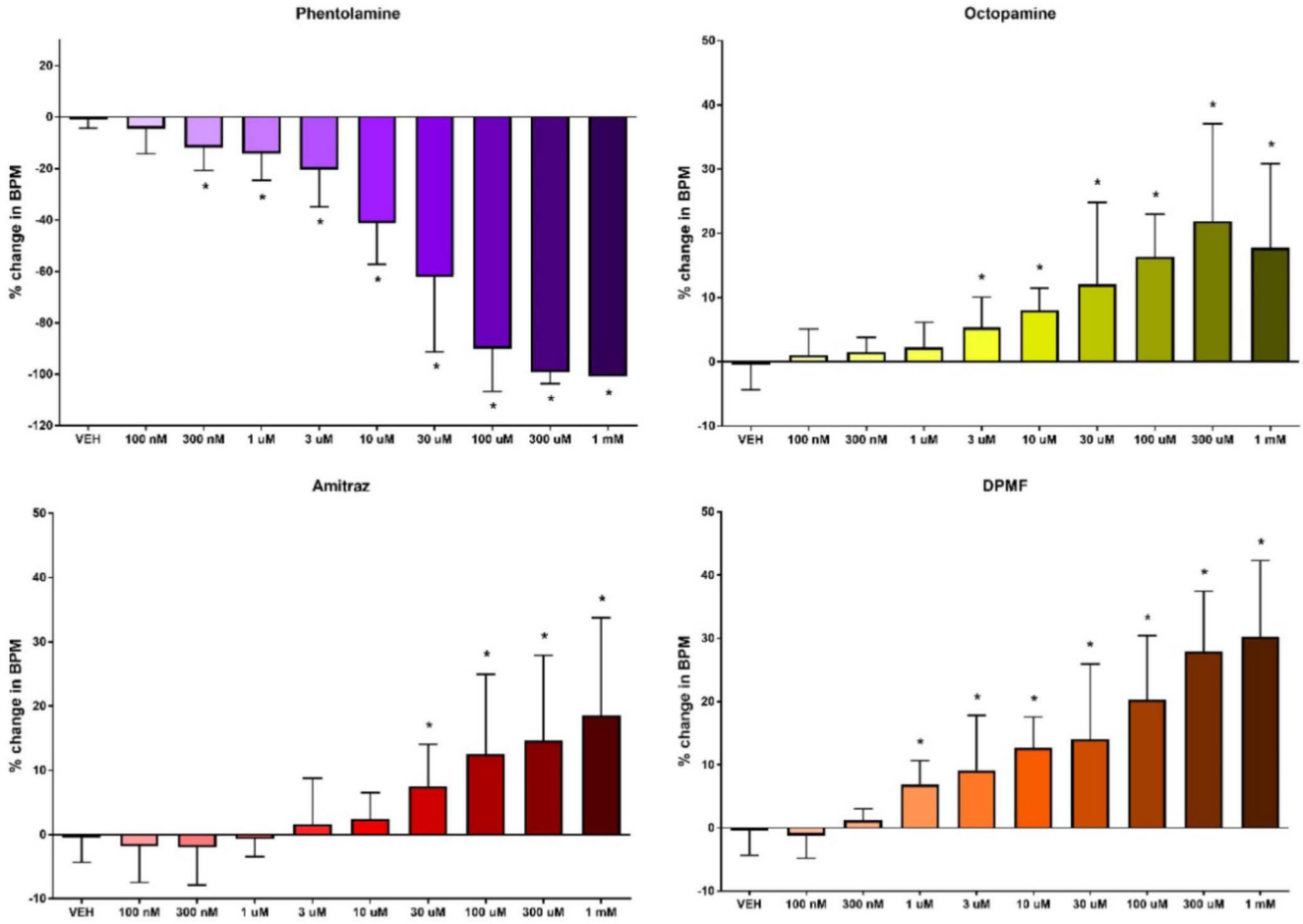

Fig. 1. Percent change in heart rate (beats per minute, BPM) resulting from the application of increasing concentrations of amitraz, DPMF, octopamine, and phentolamine. Bars represent mean change in heartbeat frequency ± standard deviation relative to baseline heart rate (n = 10). The mean treatment values were compared to a vehicle control using a nonparametric Mann-Whitney test where $P < 0.05$ was considered significant, as represented by *. Data were analyzed using GraphPad Prism 7 software.

3.2. Phentolamine pretreatment experiment

Pretreatment with phentolamine uniformly blocked the cardioaccelerator effects of octopamine, amitraz, and DPMF (**Fig. 2**). Pretreatment with 100 nM phentolamine followed by treatment with vehicle resulted in no significant change in heart rate relative to the vehicle

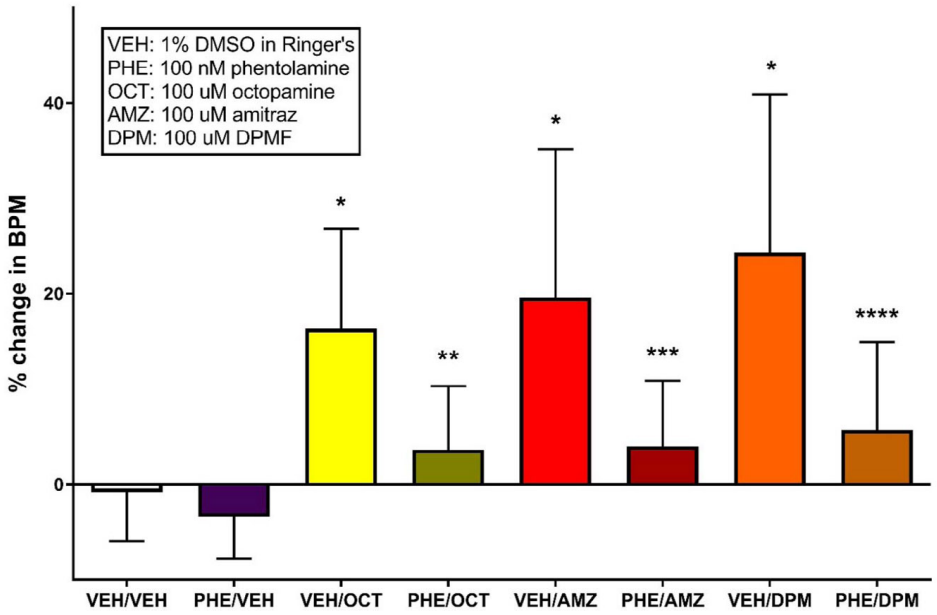

Fig. 2. Percent change in heart rate (beats per minute, BPM) resulting from the application of phentolamine followed by octopamine, amitraz, or DPMF. Bars represent mean change in heartbeat frequency ± standard deviation relative to baseline heart rate (n =12). The mean values were compared using a nonparametric Mann-Whitney test where $P < 0.05$ was considered significant. * indicates a significant difference from VEH/VEH group. ** indicates no significant difference from VEH/VEH, but a significant difference from VEH/OCT. *** indicates no significant difference from VEH/VEH, but a significant difference from VEH/AMZ. **** indicates no significant difference from VEH/VEH, but a significant difference from VEH/DPM. Data were analyzed using GraphPad Prism 7 software.

control. Pretreatment with vehicle followed by 100 µM octopamine, 100 µM amitraz, or 100 µM DPMF resulted in a significant increase in heart rate (Mann-Whitney test; $P < 0.001$), with effects comparable to what was observed in the previous experiment (Fig. 1). Pretreatment with 100 nM phentolamine followed by treatment with 100 µM octopamine, 100 µM amitraz, or 100 µM DPMF resulted in a complete loss of effect, with the observed heart rate not significantly different from vehicle treatment, but significantly reduced when compared to the corresponding drug-treated replicates without phentolamine (Mann-Whitney test; $P< 0.01$). Mean baseline heart rate (±SD) across treatment groups measured 98.0 ± 2.7 BPM.

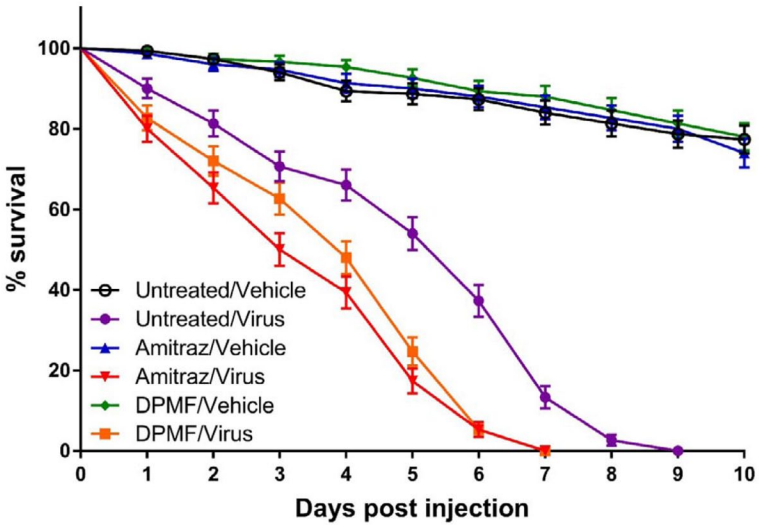

Fig. 3. Effect of orally-dosed amitraz and its metabolite DPMF on honey bee survival following a virus challenge. Data presented as Kaplan-Meier survival curves with points representing mean values ± standard error for 150 bees (6 replicate groups of 25 adult bees each per treatment). Amitraz/Virus and DPMF/Virus groups experienced significantly higher mortality than Control/Virus group (Kaplan-Meier log-rank test; $P < 0.0001$). Data were analyzed using GraphPad Prism 7 software.

3.3. Oral dosing survival experiment

Treatment with amitraz and the amitraz metabolite DPMF decreased the survival of honey bees following infection with 1×10^6 pfu of FHV/bee, relative to the infected control group (**Fig. 3**). Bees in the uninfected control groups all experienced approximately 10% mortality by 5 d post-injection and 25% mortality by the end of the study at 10 d post-injection. Infected controls experienced 46% mortality by 5 d postinjection and reached 100% mortality at 9 d post-injection, whereas infected bees receiving amitraz treatment experienced 83% mortality by 5 d post-injection and reached 100% mortality at 7 d post-injection. Similarly, infected bees receiving DPMF treatment experienced 75% mortality by 5 d post-injection and reached 100% mortality at 7 d postinjection. Log-rank tests of the Kaplan–Meier survival curves indicated a significant difference in survival between infected bees that were treated with amitraz and infected bees that were treated with vehicle ($\chi^2 = 54.32$; df = 1; $P < 0.0001$). Similarly, a significant difference in survival was also detected between infected bees

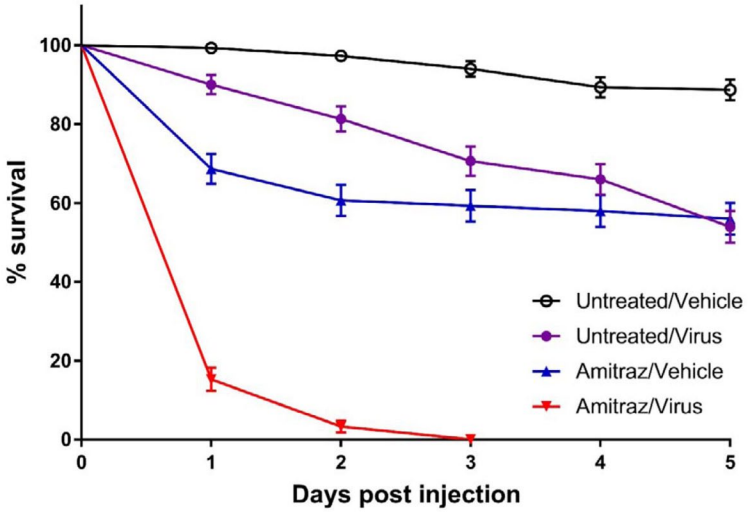

Fig. 4. Effect of exposure to Apivar® (amitraz) strips on honey bee survival following a virus challenge. Data presented as Kaplan-Meier survival curves with points representing mean values ± standard error for 150 bees (6 replicate groups of 25 adult bees each per treatment). The Amitraz/Virus group experienced significantly higher mortality than all other groups, while Amitraz/Vehicle and Control/Virus did not differ from one another, but both experienced greater mortality than Control/Vehicle (Kaplan-Meier log-rank test; P <0.0001). Data were analyzed using GraphPad Prism 7 software.

that were treated with DPMF and infected bees that were treated with vehicle (χ^2 = 41.45; df = 1; P < 0.0001). There was no significant difference in survival detected between the uninfected control groups treated with vehicle, amitraz, or DPMF, nor was there any difference between the infected groups treated with amitraz and DPMF.

3.4. Acaricide strip survival experiment

Exposure to amitraz-impregnated plastic strips decreased the survival of bees challenged with virus and vehicle injections alike, relative to the respective control groups (**Fig. 4**). Infected bees exposed to amitraz experienced 85% mortality just 1 d after infection, compared to only 10% for the infected control group. Bees injected with vehicle and exposed to amitraz experienced 32% mortality after just 1 d, compared to only 1% mortality for the uninfected control group. Infected bees exposed to amitraz experienced 100% mortality by 3 d after injection, whereas the curve leveled off for bees injected with

vehicle and exposed to amitraz starting at 2 d after injection and their survival remained consistent at approximately 40% mortality for the remainder of the study. Log-rank tests of the Kaplan–Meier survival curves indicated a significant difference in survival between the Untreated/Vehicle and Untreated/Virus groups ($\chi^2 = 44.43$; df = 1; $P < 0.0001$), between the Untreated/Vehicle and Amitraz/Vehicle groups ($\chi^2 = 43.57$; df = 1; $P < 0.0001$), and between the Untreated/Vehicle and Amitraz/Virus groups ($\chi^2 = 329.20$; df = 1; $P < 0.0001$). Significant differences were also detected between the Untreated/Virus and Amitraz/Virus groups ($\chi^2 = 240.70$; df= 1; $P < 0.0001$) and between the Amitraz/Vehicle and Amitraz/Virus groups ($\chi^2 = 147.90$; df = 1; $P < 0.0001$), but no difference was detected between the Untreated/Virus and Amitraz/Vehicle groups. When the treatment mortality was corrected for the control mortality, amitraz was found to have a significant effect on the survival of virus-infected bees ($F = 1997.39$; df = 1, 13; $P < 0.0001$).

4. Discussion

This work has demonstrated that the formamidine acaricide amitraz and its metabolite DPMF significantly alter honey bee cardiac function, most likely through interaction with octopamine receptors. Treatment with the octopamine receptor antagonist phentolamine decreased honey bee heart rate, whereas treatment with octopamine, amitraz, and DPMF increased heart rate. Furthermore, pretreatment with a low concentration of phentolamine was found to block the effects of octopamine, amitraz and DPMF. This work has also demonstrated that exposure to a formamidine acaricide may have a negative impact on the ability of honey bees to tolerate viral infection. Oral treatment with amitraz and DPMF similarly decreased the survival of virus-challenged bees, as did exposure to amitraz-impregnated plastic strips routinely used in the treatment of mite-infested hives. These findings are significant as they highlight the complex nature of insect immunity and disease tolerance, as well as the inter-relatedness of diverse physiological systems.

The changes in heart rate observed with this approach are comparable in many respects to those observed using other methods, most notably two studies that tested the effects of octopamine,

phentolamine, and amitraz on *ex vivo* honey bee heart preparations using a force-displacement transducer and intracellular recordings of heart muscle fibers using microelectrodes (Papaefthimiou et al., 2013; Papaefthimiou and Theophilidis, 2011). In these studies, octopamine was observed to have a cardioacceleratory effect in bees at concentrations in the picomolar range and above, while phentolamine blocked contractions in the micromolar range (Papaefthimiou and Theophilidis, 2011). Amitraz was also observed to have a cardioacceleratory effect on *ex vivo* bee hearts at concentrations in the nanomolar and micromolar ranges and above, as well as in experiments examining *in vivo* effects via injection or oral administration (Papaefthimiou et al., 2013). These studies, however, reported biphasic effects of both octopamine and amitraz, noting that at lower concentrations, both compounds could have inhibitory effects. Octopamine was reported to act as an antagonist in the femtomolar range (Papaefthimiou and Theophilidis, 2011), suggesting extreme sensitivity of the bee heart, though even the authors acknowledge that this might be unrealistically low. Amitraz was observed to have inhibitory effects on bee cardiac activity at concentrations in the picomolar range, while at higher concentrations, an initial inhibitory response preceded the observed cardioacceleration (Papaefthimiou et al., 2013).

The biphasic effects and the extremely high sensitivity detected in these two studies may be due in part to the significant differences between the recording methods used in these studies and the work reported here. Another significant difference, however, is that both of these studies reported that recordings of *ex vivo* heart preparations did not begin until an hour or more following dissection, during which time significant changes in bee heart rate were reported to take place (Papaefthimiou and Theophilidis, 2011), resulting in the development of a bursting pattern that appears quite different from the steady heart rate observed using the method reported here. Although the dissection and visualization method employed in this work (O'Neal and Anderson, 2016) has been observed to allow heart preparations to continue beating for two hours or more, provided periodic renewal of the isotonic solution bathing the heart, qualitative differences suggesting tissue degradation can be observed, in addition to slowing of heart rate and localized or partial loss of heart function. To avoid this, observations of heart rate typically began within approximately 5 min of dissection, which provided sufficient time for the heart rate

to stabilize. Consequently, these differences in the observed effects of octopamine and amitraz are likely due to the significant differences in methodology employed. As DPMF has not been previously tested in a similar model, no such comparisons can be made.

Exposure to cardiomodulatory compounds such as amitraz can have a significant impact on a variety of physiological processes, as the insect circulatory system is understood to play a role in thermoregulation, ventilation, and the maintenance of homeostasis. Several studies have also found that insect circulatory and immune systems can closely interact to regulate infections (King and Hillyer, 2012; Sigle and Hillyer, 2016), reinforcing the idea that there exists an extensive level of integration between cardiac function and the insect immune response. The primary insect antiviral immune response is the post-transcriptional gene silencing mechanism known as RNA interference (RNAi), which initiates targeted degradation of RNA in response to the presence of double-stranded RNA (dsRNA) (Ding, 2010; Ding and Voinnet, 2007). While bees have been shown to employ an antiviral RNAi response (Chejanovsky et al., 2014; Desai et al., 2012; Maori et al., 2009; Wang et al., 2013), there is also evidence that the presence of nonspecific dsRNA is sufficient to reduce virus production in bees (Flenniken and Andino, 2013). Another difference noted during a comprehensive examination of honey bee immune responses is that bees were found to only express about one third as many genes associated with insect immunity as have been observed in fruit flies and mosquitoes (Evans et al., 2006). One explanation for this discrepancy could be the effectiveness of colony-level, social immune barriers to infection. Another possibility is that bees also rely on tissue-specific, homeostatic mechanisms to simply tolerate infection (Schneider and Ayres, 2008). This idea is supported by findings that demonstrate an essential role for the evolutionarily conserved cardiac ion channel known as the ATP-sensitive inwardly rectifying potassium (K_{ATP}) channel in the resistance to infection by a cardiotropic virus in *Drosophila*, through modulation of RNAi (Eleftherianos et al., 2011). More recent work has provided evidence of K_{ATP} channel regulation of viral infections in bees (O'Neal et al., 2017a) and proposed an important role for this ion channel in connecting the antiviral immune response of bees to changes in cellular metabolism induced by exposure to environmental stressors.

It is understood that exposure to chronic stress gradually weakens the immune response and reduces the metabolic activity of an organism until it is no longer able to survive (McEwen, 2000). This holds true for bees as well, since physiological stress can have a wide range of detrimental consequences for bee health and survival (Even et al., 2012). Harmful synergistic interactions between simultaneous exposure to pesticides, dietary toxins, and pathogens have been demonstrated in bees, though the specific mechanisms that explain these interactions have yet to be revealed (Alaux et al., 2010; Aufauvre et al., 2012; Kohler et al., 2012; Vidau et al., 2011). Especially relevant to managed colony health is the evidence that apicultural pesticides can negatively impact bee immunity, as the acaricide *tau*-fluvalinate has been shown to have an effect on host susceptibility to viral infection (Locke et al., 2012), while the acaricides thymol, coumaphos, and formic acid were found to alter the expression of genes related to immunity, detoxification, and development (Boncristiani et al., 2012). The work presented here provides the first evidence that the formamidine acaricide amitraz, as well as its primary metabolite DPMF, may have a negative impact on honey bee antiviral resistance or tolerance to viral infection. This work also demonstrates the physiological effect that amitraz and DPMF can have on bee cardiac function, describing a cardioacceleratory role for both.

Although these findings do not provide direct evidence of a relationship between octopamine receptor-mediated modulation of bee cardiac function and weakened immunity or tolerance to viral infection, it is reasonable to hypothesize that such a relationship could exist, given the integration between insect immune and circulatory systems, as well as the important role of cardiac function in maintaining homeostasis. These findings demonstrate that DPMF is at least as cardioactive as the parent compound amitraz and appears to function in the same manner as amitraz, as the activity of each is blocked by the octopamine receptor antagonist phentolamine. This is important to note, as amitraz is quickly metabolized, but DPMF is less easily degraded and is one of the most commonly encountered agrochemical contaminants in the hive environment, as well as the bees themselves (Mullin et al., 2010). This means that managed bees may experience long-term exposure to residual DPMF present in the hive, even after amitraz treatment has been discontinued. This is significant, given

that this study reveals a potentially harmful synergistic relationship between exposure to amitraz, as well as DPMF, and the ability to resist or tolerate a viral infection. Although these findings are significant and dramatic, it can be argued that this experimental approach does not truly reflect the typical exposure of bees during an in-hive treatment with amitraz.

In order to simulate in-hive amitraz treatment, caged bees were exposed to small portions of a commercial miticide strip impregnated with amitraz. While preliminary testing found that this type of exposure had no effect on the survival of caged bees, this treatment did have a significant effect on the survival of bees challenged with virus, as well as bees in the control group that were challenged with a vehicle injection. The effects are quite pronounced after just 1 d post-injection, as there was considerable mortality in both the amitraz-treated groups. Interestingly, mortality levels off at that point in the amitraz-treated group that received a vehicle challenge, whereas the rate of mortality continues in both virus-challenged groups. It is very likely that this observation is related to the age of the bees, which had eclosed between 24 and 48 h prior to injection. One possibility is that the cuticular layers were still in a state of transition following eclosion, making the cuticle more susceptible to penetration by amitraz during this state (Noble-Nesbitt, 1970). Another possibility is that cuticular injury from the injection also facilitated amitraz penetration. In either case, however, there appears to be a synergistic interaction between exposure to amitraz and either the vehicle or the injury from the injection, which could imply that there is a trade-off, or a competition for resources, that limits the ability of the bee to both detoxify the acaricide and respond to the injury. This interaction warrants a more thorough examination.

These findings have some immediate implications for apiculture practices, as there is likely a trade-off to be considered when the decision is being made to treat a mite-infested hive with amitraz. Unfortunately, the data provided here are insufficient for determining when the economic benefits of treating with amitraz outweigh the possible drawbacks of reducing immune responsiveness. More research is needed to determine the practical implications of the interactions between amitraz, DPMF, and honey bee resistance or tolerance to viral infection at the colony level, but the evidence supports a policy

of minimizing chemical interventions within the hive. These findings also raise intriguing questions about the nature of this interaction. Is this relationship the result of octopaminergic interference with cardiac function, thereby resulting in a loss of homeostasis and the ability to tolerate infection? Or, do these compounds act through some unknown mechanism to regulate the innate immune response of the insect? Furthermore, are there age-dependent effects that must also be considered here? The answers to these questions would provide significant insight into honey bee physiology and represent a promising area for future research intended to improve understanding of honey bee antiviral immunity, disease tolerance, and their relationship to factors that negatively impact pollinator health.

Acknowledgments — Jennifer R. Williams for her technical assistance. Dr. Anette Schneemann for providing FHV. This material is based upon work that is supported by the National Institute of Food and Agriculture, United States Department of Agriculture, under award number 2017-67011-26048. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions — Conceptualization: STO, JRB, TDA. Investigation: STO. Formal Analysis: STO, CCB, TDA. Original Draft Preparation: STO. Review and Editing: STO, CCB, JRB, TDA.

Author competing interests — The authors declare no competing financial interests.

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