Journal of Invertebrate Pathology 149 (2017) 119–126

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

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

Scott T. O'Neal[a,*], Carlyle C. Brewster[a], Jeffrey R. Bloomquist[b], Troy D. Anderson[c]

[a] Department of Entomology, Virginia Tech, Blacksburg, VA, USA
[b] Department of Entomology and Nematology, Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA
[c] Department of Entomology, University of Nebraska, Lincoln, NE, USA

ARTICLE INFO

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

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.

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

* Corresponding author.
E-mail address: onealst@vt.edu (S.T. O'Neal).

http://dx.doi.org/10.1016/j.jip.2017.08.005
Received 26 May 2017; Received in revised form 28 July 2017; Accepted 5 August 2017
Available online 07 August 2017
0022-2011/

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