

**The role of ATP-sensitive inwardly rectifying potassium channels in
the honey bee (*Apis mellifera* L.)**

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ABSTRACT (Academic)

Honey bees are economically important pollinators of a wide variety of crops that have attracted the attention of both researchers and the public alike due to unusual declines in the numbers of managed colonies in some parts of the world. Viral infections are thought to be a significant factor contributing to these declines, along with exposure to agricultural and apicultural pesticides, but viruses have proven a challenging pathogen to study in a bee model and interactions between viruses and the bee antiviral immune response remain poorly understood. Recent studies have demonstrated an important role for inwardly-rectifying ATP-sensitive potassium (K_{ATP}) channels in the cardiac regulation of the fruit fly antiviral immune response, but no information is available on their role in the heart-specific regulation of bee immunity. The results of this work demonstrate that K_{ATP} channel modulators have an observable effect on honey bee heart rate that supports their expected physiological role in bee cardiac function. Here, it is also reported that the entomopathogenic flock house virus (FHV) infects adult bees, causing rapid onset of mortality and accumulation of viral RNA. Furthermore, infection-mediated mortality can be altered by pre-exposure to K_{ATP} channel modulators. Finally, this work shows that exposure to environmental stressors such as commonly used in-hive acaricides can impact bee cardiac physiology and tolerance to viral infection. These results suggest that K_{ATP} channels provide a significant link between cellular metabolism and the antiviral immune response in bees and highlight the significant impact of environmental stressors on pollinator health.

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ABSTRACT (Public)

Honey bees are economically important pollinators of a wide variety of crops that have attracted the attention of both researchers and the public alike due to unusual declines in the numbers of managed colonies in some parts of the world. Viruses are thought to be an important contributor to these declines, along with exposure to pesticides used in farming and beekeeping, but viruses have proven challenging to study in honey bees, and the bee immune response remains poorly understood. Recent work using fruit flies has shown an important role for a type of ion channel known as the inwardly-rectifying ATP-sensitive potassium (K_{ATP}) channel in the insect heart and regulation of virus infection, but this ion channel has not been studied in honey bees. The results of this work demonstrate that drugs targeting K_{ATP} channels affect honey bee heart rate and support their expected role in the function of the honey bee heart. Here, it is also shown that a model insect virus can be used to study viral infections in bees, which helps to overcome several major challenges to the study of how honey bees respond to such infections. This model system provides evidence that K_{ATP} channel drugs can be used to improve the survival of bees that have been infected with a virus. Finally, this work demonstrates that exposure to pesticides, such as those commonly used to treat for pests in the hive, can impact bee cardiac function and tolerance to viral infection. These results suggest that K_{ATP} channels provide a significant link between cellular metabolism and the antiviral immune response in bees, highlighting the significant impact of environmental stressors on pollinator health.

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CHAPTER 1

INTRODUCTION

1.1 THE HONEY BEE

Honey bees are insects belonging to the order Hymenoptera, which includes wasps, bees, ants, and sawflies, and are classified along with other bees in the family Apidae. The evolution of bees is thought to be associated with the diversification of flowering angiosperms, which evolved to produce excess nectar and pollen as a reward for bees and other pollinators to facilitate pollen dispersal (Winston, 1987). Honey bees are assigned to the genus *Apis*, the first representatives of which are thought to have originated around 30 to 40 million years ago (Culliney, 1983; Engel, 1998; Ruttner, 1988). *Apis* is currently comprised of 10 species (Arias and Sheppard, 2005), the most notable being the European honey bee, *Apis mellifera*, and the Asian honey bee, *Apis cerana*, due to their long history of association with humans (DeWeerd, 2015). Genotypic analyses suggest that *A. mellifera* originated in Africa and expanded into Eurasia at least twice, resulting in genetically distinct populations in eastern and western Europe (Whitfield et al., 2006). The divergence between *A. mellifera* and its closest relative *A. cerana* is thought to have occurred in western or central Asia sometime within the past 1 million years, and thanks to human intervention, *A. mellifera* is now globally distributed, found on all continents save Antarctica (Culliney, 1983; Ruttner, 1988). Evidence of the long-standing association between humans and honey bees can be observed in 7000 year old cave paintings that depict the collection of honey from wild bee hives (Crane, 1999). The honey for which honey bees are so famous, and the floral nectar from which it is processed, represent the carbohydrate portion of the honey bee diet, while the remainder of the nutrients required for survival, including proteins,

lipids, and most vitamins and minerals, are derived from the pollen that they collect (Snodgrass, 1910). These food resources are stored in the honey bee nest, or hive, which is made up of a beeswax comb that is housed in either a man-made or naturally-occurring container or crevice. The comb serves as the structural element of the hive and consists of a repeating series of hexagonal cells that are used both for storing resources and for rearing young, or brood.

1.2 HONEY BEE SOCIAL ORGANIZATION

Honey bees are eusocial insects with colonies typically comprised of a single reproductive queen, a variable number of reproductive males known as drones, and a number of sterile female workers that may range in number from the thousands to tens of thousands, depending on the size of the nesting site, season, and the availability of resources (Ruttner, 1988). The queen is the only reproductive female within the colony, as the ovaries of the workers are developmentally suppressed by the presence of a pheromone excreted by the queen (Winston, 1987). Honey bee workers typically perform all tasks not related to reproduction, with their behavior changing over time due to an age-dependent division of labor known as temporal polyethism (Winston, 1987). Young worker bees, known as nurse bees, primarily focus on tasks related to brood care and tend to remain within the broodnest, whereas middle-aged bees focus on nectar processing, hive maintenance, and guard duty, often moving throughout the hive to complete tasks, while older workers eventually become foragers, repeatedly leaving the hive in search of resources until death (Johnson, 2008). Although these roles usually progress in accordance with age, there is some level of plasticity to this division of labor, as workers can change tasks early, continue a task longer than usual, or even revert to a prior task in response to environmental conditions and the needs of the colony (Robinson, 1992). Drones serve no role other than mating with a queen,

as they perform no tasks within the hive and must be cared for by worker bees, resulting in seasonal fluctuations in the drone population of a colony (Winston, 1987).

1.3 HONEY BEE DEVELOPMENT

Honey bees are oviparous insects that undergo holometabolous development and are genetically haplodiploid, as the queens and workers develop from fertilized eggs while the drones develop from unfertilized eggs. The queen lays each egg individually at the bottom of an empty cell in the comb of the hive. Whether a fertilized egg develops into a queen or a worker is determined by the timing and content of the larval diet, with queens receiving more of the protein royalactin, which is delivered as part of a worker-produced secretion known as royal jelly (Flanders, 1960; Kamakura, 2011). Eggs typically hatch about three days after being laid and the larvae are cared for by nurse bees until the cell is capped, usually around 8 days after being laid. The developmental time for each caste varies, with queens requiring approximately 7 days to reach maturity after the cell has been capped, workers requiring 12 days, and drones requiring 15 days (Winston, 1987). Despite the fact that queens and workers develop from the same genome, the queen may live for years, making her lifespan considerably longer than that of the worker, which typically only lives for an average of 15 to 38 days during the spring and summer in temperate climates (Page Jr and Peng, 2001). During colder months, however, honey bee workers are not subject to the risks associated with foraging and have considerably greater fat body stores, allowing them to survive for 140 days or more (Page Jr and Peng, 2001). The average lifespan of a drone is estimated at anywhere from 20 to 40 days during the warmer seasons, but in colder climates the drones will be removed from the hive and left to die as winter approaches (Page Jr and Peng, 2001).

1.4 ECONOMIC AND AGRICULTURAL IMPORTANCE OF THE HONEY BEE

The honey bee is one of the most ubiquitous and familiar insects in the world, valued for its commercially and agriculturally important role as a pollinator of a wide variety of crops that provide both food and fiber, as well as for the natural resources such as honey and wax that it provides. In the United States alone, the annual value of pollination services provided by honey bees exceeds \$14 billion (Morse and Calderone, 2000), while globally, the contribution of pollinators to food production is estimated at more than \$200 billion (Gallai et al., 2009). In terms of worldwide food production, an estimated 35% of the food consumed by humans comes from crops that depend on pollinators, with 52 of the 115 leading global food commodities dependent on honey bee pollination for either fruit or seed set (Klein et al., 2007). Honey is also an important international commodity, with an estimated global production of 1.07 million metric tons and value of \$1.25 billion in 2007 (vanEngelsdorp and Meixner, 2010). With the world population currently exceeding 7 billion people, and predicted by the United Nations to reach almost 10 billion by 2050, honey bees and other pollinators will play an increasingly essential role in providing food security and contributing to the sustainability of agriculture.

1.5 HONEY BEES IN DECLINE

Estimates indicate that the global number of managed bee colonies has increased by roughly 45% during the last 50 years; unfortunately, this trend has fallen far short of the 300% increase in the production of crops that depend upon pollination by bees during the same time period (Aizen and Harder, 2009). Furthermore, this overall increase in the number of managed colonies does not reflect a uniform increase across all countries, as these increases have occurred primarily in countries such as China, Argentina, and Turkey while countries in North America and Europe

have seen steady declines (Aizen and Harder, 2009). In the United States, the number of managed colonies has decreased by approximately 50% during this same time period, despite increases in the acreage of crops requiring animal pollination (Council, 2007). According to the United States Department of Agriculture National Agriculture Statistics Service estimates, in 1980 there existed more than 4.1 million commercial honey-producing hives in the United States, 77,000 of which were located in Virginia (NASS, 1981). As of the 2016 reports, the number of honey producing hives in the United States has decreased to approximately 2.78 million with only 6,000 located in Virginia (NASS, 2017). Furthermore, annual surveys of beekeeping operations in the United States over the course of the last decade have consistently reported unacceptably high losses of managed colonies, with the most recent assessment describing a total average losses of 49% nationally, and over 50% in Virginia, during the 2014 to 2015 season (Seitz et al., 2016). Even more sensational, however, is the phenomenon known as Colony Collapse Disorder (CCD), which describes a set of symptoms related to large-scale, unexplained losses of managed honey bee colonies (vanEngelsdorp et al., 2009). These high levels of colony loss and concerns about CCD in the United States and elsewhere have increased public awareness of pollinator health issues and focused international research efforts on understanding why these losses occur.

1.6 FACTORS INFLUENCING HONEY BEE HEALTH

There exist a variety of factors that negatively impact the health and survival of both managed and wild bee populations, including the spread of parasites and pathogens, loss of habitat, reduced availability of forage or reduced quality of available food resources, climate change, poor queen quality, changing cultural and commercial beekeeping practices, as well as exposure to agrochemicals both in the field and in the hive. These various factors are often closely

intertwined, and no single stressor can be identified as the sole driver of colony loss. There is a growing consensus, however, that the increasing prevalence of parasites and pathogens, especially viruses, are among the most significant threats to managed bee colonies (Evans and Schwarz, 2011; Manley et al., 2015; Paxton et al., 2015). Viral infections appear to be closely associated with weakened or dying bee colonies, as well as with colonies believed to be affected by CCD, suggesting that reduced immunocompetence is a significant factor contributing to colony loss (Cox-Foster et al., 2007; Dainat et al., 2012; Genersch and Aubert, 2010; McMenamin and Genersch, 2015; vanEngelsdorp et al., 2009). It is not uncommon for bee colonies to be infected with multiple viruses concurrently (Chen et al., 2004; de Miranda et al., 2010; Runckel et al., 2011), and there exist multiple routes of transmission for bee viruses (Shen et al., 2005). Infections often remain asymptomatic, having no apparent impact on the overall health or productivity of a colony, until such time as the colony is subjected to factors that disrupt the balance between these pathogens and the immune response of the insect. There are many stressors that can reduce the effectiveness of the honey bee immune response, including exposure to pesticides (Di Prisco et al., 2013), as well as the presence of the ectoparasitic mite *Varroa destructor*, which both 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; Ryabov et al., 2014). Disruptions in this complex interplay between host, pathogen, and parasite can significantly alter the critical balance that exists between viral replication strategies and the host immune defenses and is thought to be a significant factor driving colony loss (Genersch and Aubert, 2010; Nazzi et al., 2012).

1.7 RESEARCH OVERVIEW

Despite the economic and agricultural importance of honey bees and the growing awareness of the role that viral pathogens play in their decline, the mechanisms involved in the honey bee antiviral immune response remain poorly characterized. This represents a critical gap in our knowledge of the complex relationship between host and pathogen that must be filled if we are to better understand how viruses and immune deficiencies affect pollinator health and to develop more effective strategies to mitigate managed colony loss. The overall goal of the research described here is to expand the body of knowledge related to honey bee physiology and the mechanisms by which honey bees regulate or tolerate viral infections. Much of this work is focused on the investigation of a specific type of ion channel known as the ATP-sensitive inwardly rectifying potassium (K_{ATP}) channel. K_{ATP} channels couple cellular metabolism to the membrane potential of the cell and play an important role in a variety of tissue types, including the insect heart, making them a subject of interest not only for understanding invertebrate cardiac physiology, but also as a potential target for novel insecticides. Most of what is known about these ion channels is the result of work performed in mammalian systems, with insect studies being limited to only a few species and physiological systems. Research using *Drosophila melanogaster* as a model to investigate the insect antiviral immune response has demonstrated that K_{ATP} channels appear to play an evolutionarily conserved role in mediating the survival of insects and mammals alike during viral infections (Croker et al., 2007). This finding has led to the further observation that insect coronary K_{ATP} channels have a function in modulating insect antiviral RNA interference (RNAi), presumably by facilitating tissue-specific regulation of innate immune response mechanisms by the cellular environment of the heart (Eleftherianos et al., 2011). The research presented here describes a role for K_{ATP} channels in the regulation of

honey bee cardiac activity and reveals an evolutionarily conserved relationship between K_{ATP} channels and the antiviral immune response of bees. This work also presents a protocol for the observation, measurement, and pharmacological manipulation of honey bee cardiac activity, in addition to demonstrating an efficient model virus system for the study of viral infections in honey bees. Finally, this work begins to link environmental stressors to changes in honey bee cardiac function by examining the effects of a common in-hive acaricide and its active metabolite on bee heart rate.

1.8 RESEARCH OBJECTIVES

Although honey bees are greatly valued as pollinators, as honey producers, and even as model social insects for a variety of research topics, they can also be a challenging organism to study. Much of what is known about honey bee physiology is inferred from research conducted in other model organisms such as *D. melanogaster*, which has many advantages, including ease of rearing, short generation time, lack of a harmful bite or sting, and above all, the ease with which mutant strains can be generated. Rearing honey bees requires considerably more space and resources than fruit flies, nor have they proven to be as easy to genetically manipulate. Furthermore, investigations of antiviral immune responses in bees must contend with several major obstacles. One such obstacle is that bee colonies are often covertly infected with one or more viruses at any given time, posing a challenge for researchers focused on the outcome of infection with a single virus. Another is that infectious clones for honey bee viruses have not been developed, making it difficult to accurately characterize infection dynamics and limiting the precision of such studies. Consequently, an important aspect of the honey bee research described here is the development of new protocols or models appropriate for the study of this important insect species.

- Chapter 2 of this dissertation describes a protocol for the visualization, measurement, and pharmacological manipulation of honey bee heart rate. This protocol is intended to provide a simple and effective method for assessing changes in bee cardiac function that has the added advantage of being inexpensive to perform, as it requires few resources. This protocol provides the groundwork for the studies described in Chapters 3 and 5.
- Chapter 3 describes an investigation of the role that K_{ATP} channels play in regulating honey bee cardiac function, utilizing the protocol described in Chapter 2 to examine the effects that modulators of these ion channels have on heart rate. The data presented here confirm a role for K_{ATP} channels in the regulation of honey bee dorsal vessel contractions and provide insight into the underlying physiology that governs the regulation of bee cardiac function.
- Chapter 4 describes the use of the entomopathogenic flock house virus (FHV) as a model system for the study of virus infections in honey bees and reveals an important role for the regulation of the bee antiviral immune response by K_{ATP} channels. The data presented here suggest that K_{ATP} channels provide a significant link between cellular metabolism and the antiviral immune response in bees.
- Chapter 5 describes an investigation of the role that the octopaminergic formamidine acaricide amitraz and its active metabolite *N*-(2,4-dimethylphenyl)-*N'*-methylformamidine (DPMF) play in the regulation of honey bee cardiac function, as well as their impact on the ability of honey bees to resist or tolerate viral infection. The data presented here confirm that amitraz and its metabolite DPMF alter honey bee cardiac activity by acting upon octopamine receptors and also demonstrate a possible negative synergistic interaction between exposure to these compounds and a viral pathogen. This work emphasized how exposure to environmental stressors can impact honey bee cardiac physiology and immunocompetence.

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CHAPTER 2

DISSECTION AND OBSERVATION OF HONEY BEE DORSAL VESSEL FOR STUDIES OF CARDIAC FUNCTION

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2.1 ABSTRACT

The European honey bee, *Apis mellifera* L., is a valuable agricultural and commercial resource noted for producing honey and providing crop pollination services, as well as an important model social insect used to study memory and learning, aging, and more. Here we describe a detailed protocol for the dissection of the dorsal abdominal wall of a bee in order to visualize its dorsal vessel, which serves the role of the heart in the insect. A successful dissection will expose a functional heart that, under the proper conditions, can maintain a steady heartbeat for an extended period of time. This allows the investigator to manipulate heart rate through the application of cardiomodulatory compounds to the dorsal vessel. By using either a digital microscope or a microscope equipped with a digital camera, the investigator can make video recordings of the dorsal vessel before and after treatment with test compounds. The videos can then be scored at a time convenient to the user in order to determine changes in heart rate, as well as changes in the pattern of heartbeats, following treatment. The advantages of this protocol are that it is relatively inexpensive to set up, easy to learn, requires little space or equipment, and takes very little time to conduct.

2.2 INTRODUCTION

The overall goal of this methodology is to allow the investigator to quickly and easily observe and quantify the effect that a pharmacological agent has on the heart rate of honey bees. Bees, like other insects, have an open circulatory system that disseminates hemolymph, the insect equivalent of blood, throughout the body cavity, known as the hemocoel. The circulation of hemolymph is essential for the transport of nutrients, immune factors, waste products, as well as neurohormones and other signaling molecules (Klowden, 2013). Circulation is facilitated by the dorsal vessel, which extends along the dorsal midline of the insect, as well as accessory pulsatile organs. The dorsal vessel is divided into two functionally distinct sections, designated the heart in the abdomen and the aorta in the thorax and head. Propagated contractions in the heart pump hemolymph towards the thorax and head, while accessory pulsatile organs ensure hemolymph flow to the extremities.

Insect cardiac function can be observed using a variety of methods, depending upon the size, physiology, or life stage of the insect. A common approach for observing heart rate in larvae or smaller insects is the use of intravital imaging (League et al., 2015). This method is less useful in adult bees, however, as it can be difficult to clearly view the dorsal vessel through the abdominal wall. An established approach for recording heart rate in a variety of insects, including bees, is the use of contact thermography, which utilizes thermistors applied to the exterior of the insect to detect cardiac pulsations (Wasserthal, 1980, 1996). Heart rate in adult bees has also been recorded using an electrophysiological technique to measure an electrical impedance signal. This technique requires the insertion of electrodes into the animal next to the heart and the use of an impedance converter to record heartbeats (Schwab et al., 1991; Wasserthal, 1996). Similarly, electrocardiograms have been used to detect electrical signals produced by the heart and

combined with video recording of the bee to assess changes in cardiac activity (Kaiser et al., 2014). A distinct advantage to these approaches is that heart rate is assessed in an intact, living bee, rather than in a dissected specimen, which helps to ensure the availability of the full range of physiological responses in the subject. The challenges of these approaches include accounting for immobilization or anesthetization of the subject, the need to limit outside variables and stimuli that might alter heart rate, as well as determining an appropriate delivery method when testing pharmacological agents.

Another approach that has been used for studying bee cardiac activity is to partially dissect the insect in order to expose the heart, then measure dorsal vessel contractions using a force displacement transducer (Papaefthimiou and Theophilidis, 2011). In this protocol, the heart is continually bathed with running physiological saline and test compounds can be dissolved in this solution for application to the subject. A significant difference between this method and those previously described is that the ventral nerve cord is removed, eliminating the role that the central nervous system has been shown to play in modulating heart rate (Schwab et al., 1991). The result is that the baseline heartbeat, which is usually quite erratic, stabilizes at a much lower frequency and amplitude than is typically observed in a living insect (Papaefthimiou and Theophilidis, 2011; Schwab et al., 1991). What all of these methods have in common is that they require highly specialized and often expensive equipment, in addition to a certain level of expertise, in order to be conducted. Perhaps the greatest disadvantage is that none of these approaches are particularly well suited to experiments that involve testing a large number of subjects, such as screening a library of potentially cardiomodulatory compounds.

The greatest strength of the approach described here is its simplicity. The protocol is relatively easy to master, the setup requires little space, and only minimal financial input is necessary. The

method requires little more than some bees, a few surgical instruments, an isotonic solution, and either a digital microscope or a traditional microscope with a digital camera. Bees are dissected to visualize the dorsal vessel and digital videos are used to record heart rate before and after treatment with pharmacological agents. Although video recording is not actually necessary to observe changes in heart rate, it will greatly increase throughput, i.e., the number of subjects that can be processed in a given amount of time. The investigator can maximize efficiency by recording a large number of videos at once and then later scoring these videos at a more convenient time. Another advantage of this approach is that videos allow the investigator to start over, should the scoring process be interrupted, make it easier for the viewer to be blinded to the treatment in order to reduce bias.

2.3 PROTOCOL

2.3.1 Collection and preparation of test subjects

2.3.1.1) Collect the appropriate number of bees from the colony.

Note: The number needed depends upon not only the size and scope of the experiment, but also the skill of the investigator. For example, if there are 2 treatment groups with a desired sample size of 10 bees per group, a reasonably skilled investigator might collect at least 30 bees to account for unsuccessful dissections and end up with 20 useful videos to score.

2.3.1.2) Minimize the amount of time that passes between collection and dissection.

Note: Although bees can be housed in the lab for days prior to dissection, the success rate of dissections (*i.e.*, likelihood of maintaining stable heart rate in a dissected dorsal vessel) has been observed to decrease relative to the amount of time that bees are housed away from the colony.

2.3.1.2.1) Provide bees with a source of water and food while housed in the lab. For example, at a minimum, provide access to a 50% (w/v) solution of sucrose in water (this is sufficient for durations of less than 6 h). For longer periods, provide bees access to honey.

2.3.1.2.2) House bees in the lab overnight at a temperature of approximately 32 °C and 60-80% relative humidity to reduce stress and avoid dehydration.

2.3.1.3) Prior to dissection, anesthetize bees briefly to aid in handling.

Note: This can decrease the success rate of dissections and reduce throughput.

2.3.1.3.1) Chill the bees either by placing them on ice or into a refrigerator for just long enough to reduce movement in order to aid in handling.

2.3.1.3.2) Alternatively, briefly expose bees to CO₂ in order to aid in handling.

Note: Extended exposure to cold can reduce the success rate of dissections. Extended or repeated exposure to CO₂ can also reduce the success rate of dissections.

2.3.2 Dissection of dorsal abdominal wall

Note: Bees should be alive at the time of dissection.

2.3.2.1) Using forceps and/or microdissection scissors, remove legs and wings to facilitate dissection of the abdomen. Keep a small beaker or similar container filled with distilled water nearby for the purpose of rinsing instruments between dissections.

2.3.2.2) While restraining the bee with forceps, utilize the microdissection scissors to cut laterally along the dorsal abdominal wall between the first and second tergites (see Figure 2.1).

2.3.2.3) While lightly gripping the posterior edge of the second tergite with the forceps, cut longitudinally along each side of the bee from the initial incision to the stinger (see Figure 2.2).

Use caution when cutting to avoid puncturing the gastrointestinal tract.

2.3.2.4) Exchange the scissors for a second set of fine forceps and utilize them to carefully separate the dorsal abdominal wall from the rest of the abdomen. Gently remove the stinger and any portion of the gastrointestinal tract that remains attached to the dorsal abdominal wall. Avoid rupturing the gut, as the contents can coat the abdominal wall and impede visualization of the dorsal vessel.

2.3.2.5) Arrange the dorsal abdominal wall in the desired orientation beneath the camera so that the dorsal vessel is visible (see Figure 2.3). Utilize the microdissection scissors to trim away any excess abdominal wall that impedes visualization of the dorsal vessel. The shape of the dorsal abdominal wall should resemble a shallow cup or bowl when properly situated.

2.3.2.5.1) Since the dorsal vessel does not extend into the hindmost abdominal segment of the bee, remove the final tergite in order to improve visualization of the dorsal vessel.

2.3.2.6) Utilizing an adjustable volume micropipette, cover the dorsal vessel with 10 μ L of an isotonic solution to maintain physiological conditions and facilitate a steady heartbeat.

Note: The recommended solution is quarter strength Ringer's solution (0.120 g/L calcium chloride, 0.105 g/L potassium chloride, 0.050 g/L sodium bicarbonate, and 2.250 g/L sodium chloride), which has been found to facilitate a stable, continuous heartbeat.

2.3.3 Observation and modulation of heart rate

2.3.3.1) Allow the dorsal vessel to sit undisturbed until a stable, continuous heartbeat is achieved (usually within 300 s).

Note: Heartbeat is visualized as rhythmic contractions of the dorsal vessel. Initially, there may appear to be no heartbeat, especially if the bee was anesthetized, but the heart will usually resume beating after resting in isotonic solution for several minutes and can continue beating for hours, provided it remains bathed in solution.

2.3.3.2) Measure the heart rate in terms of the number of beats per minute (bpm).

2.3.3.2.1) Record the number of contractions observed during a 60 s period. Use a hand tally counter and a timer to facilitate this process.

2.3.3.3) Measure the change in heart rate by recording the observed bpm before and after treatment with a cardiomodulatory compound.

Note: Although the time required to observe an effect on heart rate may vary depending upon the compound being tested, changes in heart rate can typically be observed within minutes.

2.3.3.3.1) Determine the baseline heart rate immediately prior to the addition of any test compound.

Note: Post-treatment heart rate can usually be determined after 90 to 120 s.

2.3.3.3.2) Prepare potential cardiomodulators (*e.g.*, octopamine) by dissolving the compound in the same isotonic solution used to bathe the dorsal vessel.

2.3.3.3.3) Add the test compounds to the solution surrounding the dorsal vessel by utilizing a micropipetter.

2.3.3.4) For greater accuracy and higher throughput, make a video recording of each test subject and then use the videos to score heart rate at a later time.

Note: This allows a single investigator to stagger dissections in order to facilitate almost continuous production of videos. When recording videos, the minimum recommended length is approximately 240 s with any test compound being added at the 60 s mark. This ensures that the investigator has a 60 s window for scoring baseline heart rate and then another 60 s window for scoring post-treatment heart rate 120 s after treatment.

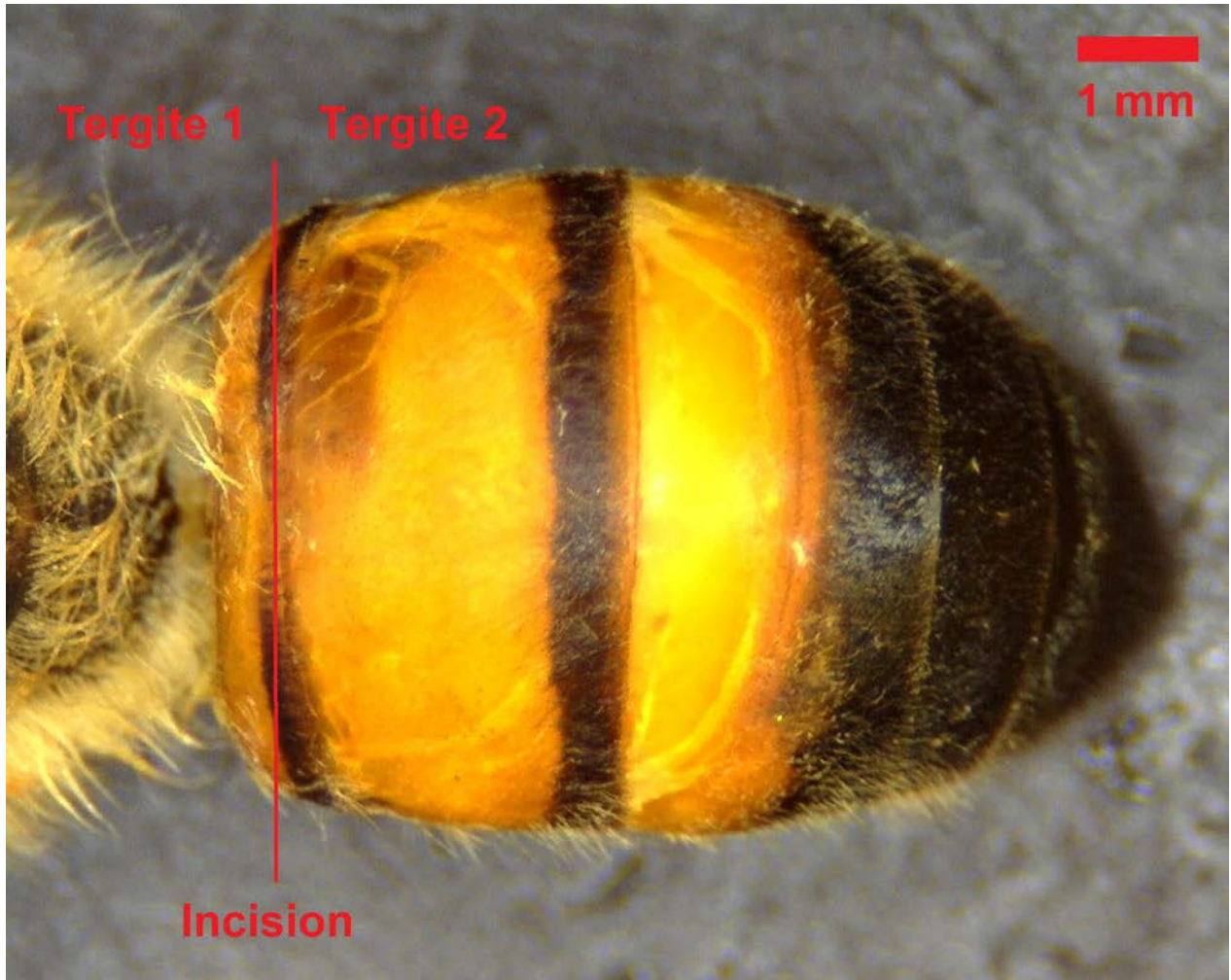


Figure 2.1: Dorsal view of bee abdomen. The initial incision should be made between the first and second tergites, as denoted by the red line.



Figure 2.2: Lateral view of bee abdomen. The second and third incisions should be made along either side of the abdomen, as denoted by the red line.

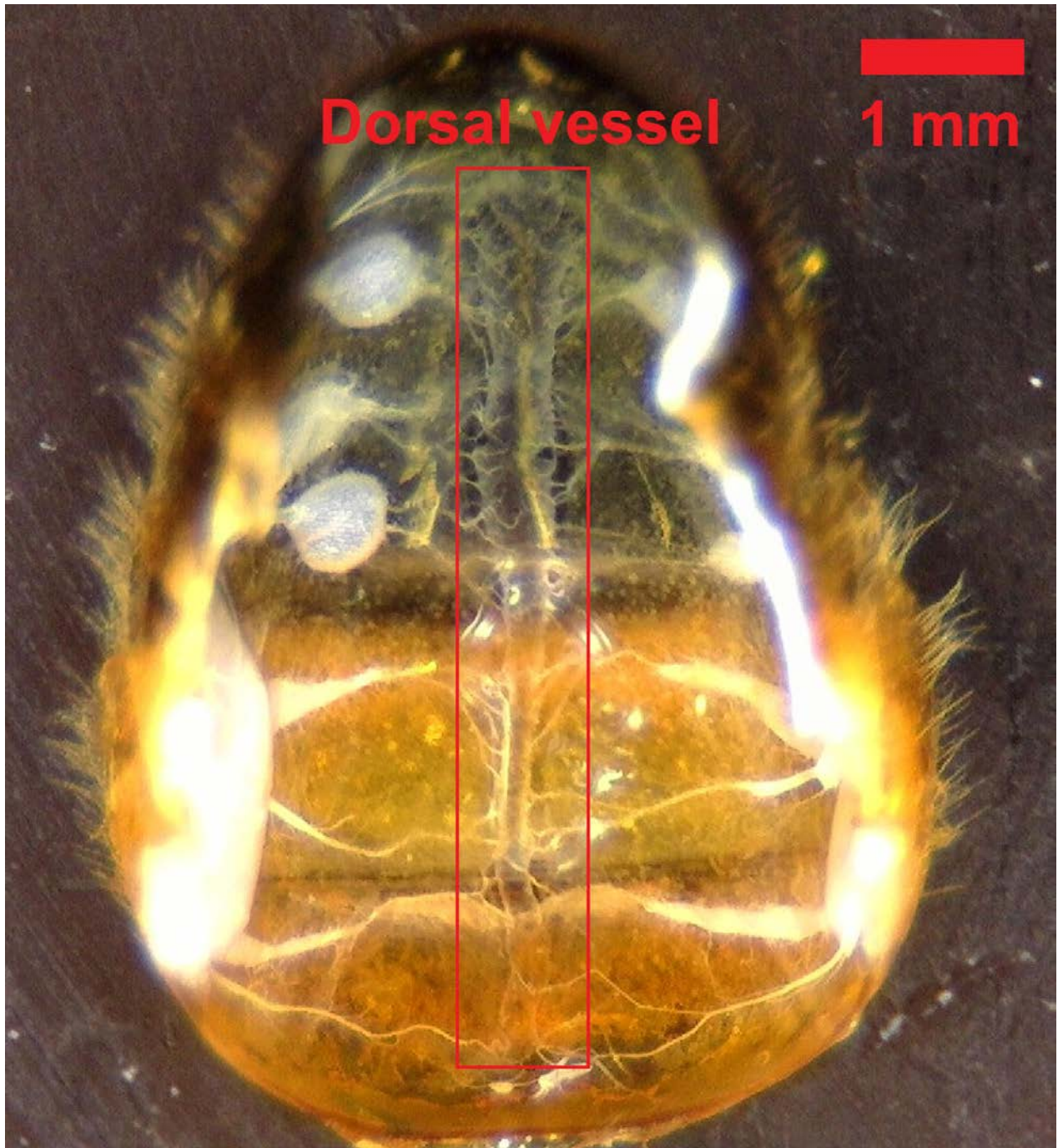


Figure 2.3: View of the dorsal vessel. Once the gut and stinger have been removed, the dorsal vessel is visible along the midline of the dissected dorsal abdominal wall.

2.4 REPRESENTATIVE RESULTS

Since many of the pharmacologically active compounds that might be tested using this protocol are not soluble in water, it is necessary to have a reliable solvent that will allow test compounds to be delivered via the isotonic solution used to bathe the dorsal vessel. Dimethyl sulfoxide (DMSO) is a solvent that is commonly used as a vehicle for delivering experimental drugs and other compounds in animals (Castro et al., 1995), and it has been used successfully for this purpose in studies examining the effect of pesticides on honey bee cardiac activity (Papaefthimiou et al., 2013). Consequently, DMSO was selected as a potential vehicle for delivering test compounds to the dorsal vessel, which required that it first be tested for cardiomodulatory activity. In this protocol, the dissected dorsal vessel is initially bathed in 10 μ l of quarter strength Ringer's solution and then heart rate is determined prior to adding test compounds. Test compounds are delivered at a known concentration in 10 μ l of DMSO dissolved in quarter strength Ringer's solution and then the dorsal vessel is observed for subsequent changes in heart rate.

Prior to testing any compounds, a concentration range of DMSO was tested for any effect on heart rate, relative to quarter strength Ringer's solution with 0% DMSO. DMSO was dissolved at 0.2%, 2%, 10%, and 20% v/v in quarter strength Ringer's solution in order to test a final concentration of 0.1%, 1%, 5%, and 10% when added in equal volume to the solution already bathing the dorsal vessel. This approach was used, as opposed to adding DMSO at the desired concentration in the initial 10 μ l used to bathe the dorsal vessel, because not every dissection yields a stable heartbeat. If DMSO has an adverse effect on heart rate, this effect might not be distinguishable from a failed dissection. Heart rate was assessed immediately prior to adding DMSO, and then again 120 s later. Figure 2.4 shows the results of this experiment,

demonstrating that 0.1% and 1% DMSO in solution had no significant effect on heart rate, relative to the quarter strength Ringer's solution without DMSO, whereas 5% and 10% DMSO significantly decreased heart rate.

In order for this assay to be relevant, it must be possible to observe changes in heart rate following treatment with cardiomodulatory compounds. The biogenic amine octopamine is known to act as a neuromodulator in invertebrates (Roeder, 1999) and has been shown to act as a cardioaccelerant in both fruit flies (Johnson et al., 1997) and honey bees (Papaefthimiou and Theophilidis, 2011). Octopamine was tested against vehicle (1% DMSO in quarter strength Ringer's solution) as described above at concentration of 100 nM, 10 μ M, and 100 μ M. Figure 2.5 shows the results of this experiment, demonstrating that concentrations of 10 μ M and 100 μ M octopamine significantly increased heart rate.

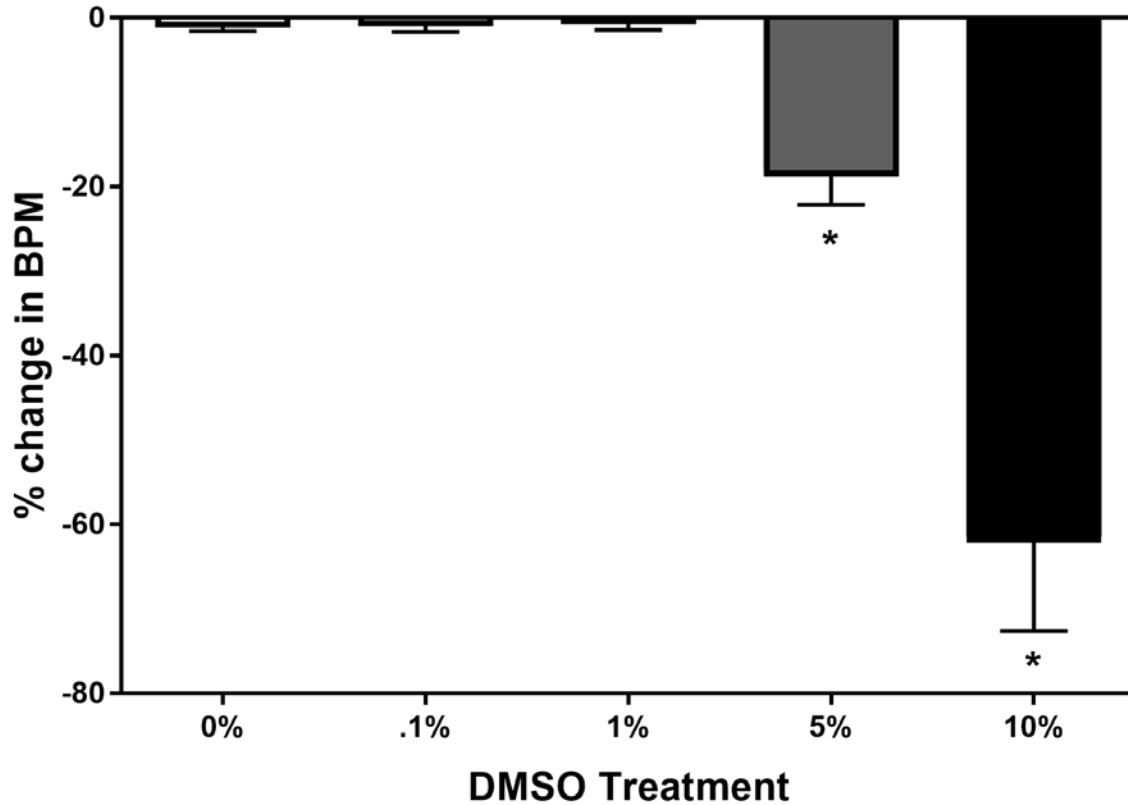


Figure 2.4: Effect of DMSO on bee heart rate. Graph shows the percentage change in basal heart rate, as measured in beats per minute. Treatment of honey bee dorsal vessel with 0.1% or 1% DMSO v/v in quarter strength Ringer’s solution does not significantly increase or decrease heart rate, relative to quarter strength Ringer’s solution without DMSO. Treatment with 5% or 10% DMSO in solution causes a significant decrease in heart rate. Results expressed as mean +/- SEM (n = 10). *P<.05 vs 0% DMSO control (Kruskal-Wallis test with Dunn’s multiple comparisons test).

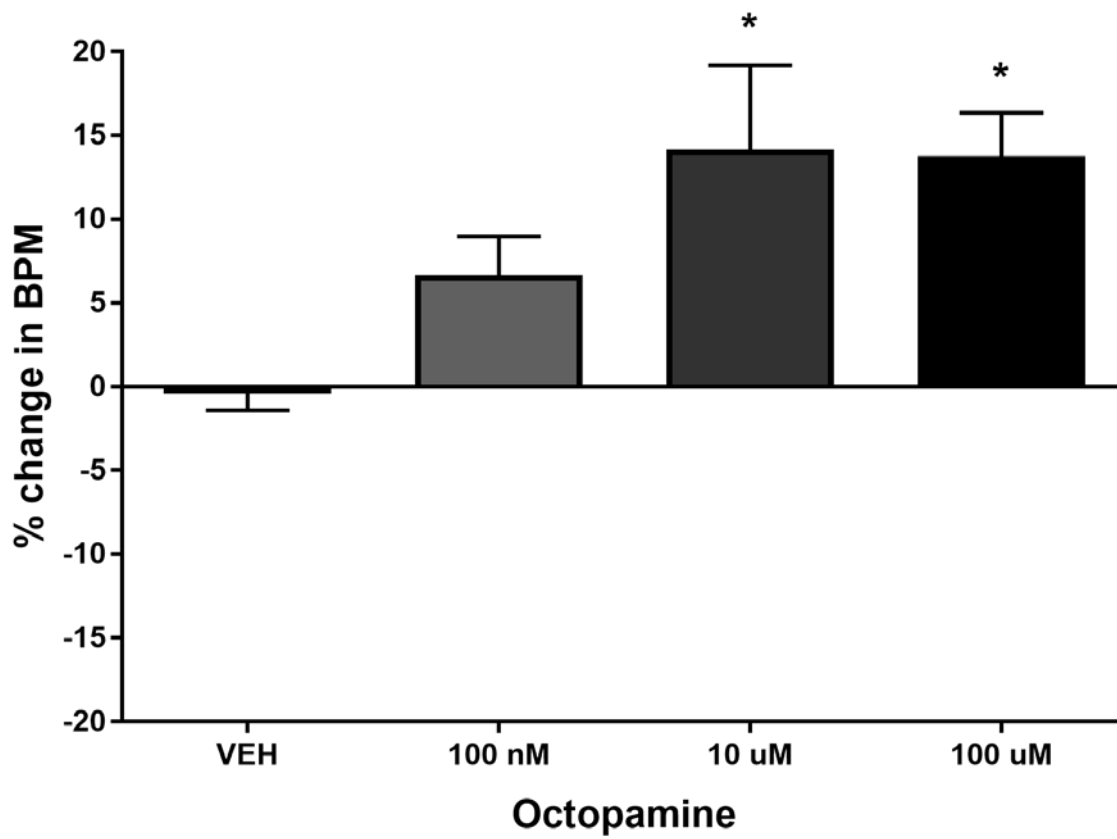


Figure 2.5: Effect of octopamine on bee heart rate. Graph shows the percentage change in basal heart rate, as measured in beats per minute. Treatment of honey bee dorsal vessel with 100 nM octopamine does not significantly increase or decrease heart rate relative to vehicle (1% DMSO v/v in quarter strength Ringer's solution). Treatment with 10 μ M and 100 μ M octopamine significantly increased heart rate relative to vehicle. Results expressed as mean \pm SEM (n = 10). *P<.05 vs vehicle control (Kruskal-Wallis test with Dunn's multiple comparisons test).

2.5 DISCUSSION

The protocol presented here provides a simple and effective approach to testing pharmacological compounds for their effects on honey bee heart rate. As observed in prior experiments that either transect the ventral nerve cord of a living insect (Schwab et al., 1991) or dissect out the ventral nerve cord when exposing the dorsal vessel (Papaefthimiou and Theophilidis, 2011), the loss of central nervous system regulation results in a stable, low frequency heartbeat. The low frequency of beats allows the investigator to visually assess heart rate without having to rely on delicate or expensive instrumentation. As has been demonstrated, the solvent DMSO can be used in low concentrations to dissolve pharmacological agents for delivery via the isotonic solution used to bath the dorsal vessel. Furthermore, heart rate can be accelerated in this method using the invertebrate neuromodulator octopamine, which has been previously shown to act as a cardioaccelerant in bees (Papaefthimiou and Theophilidis, 2011). Although this protocol was designed in order to measure changes in heart rate, it is possible to use it to observe other, more qualitative characteristics of heartbeat as well, such as changes in the pattern of contractions. Regardless of the end point in question, the protocol relies primarily upon the ability of the investigator to perform a clean, rapid dissection. The most common challenge that an investigator will face when using this protocol is the failed dissection, which results in a dissected abdominal wall that is not useful either because there is no heartbeat, or because the dorsal vessel is not visible.

The dorsal vessel can be easily obscured as the result of a poor dissection. Puncturing or tearing the gut can result in gut contents spilling out onto the abdominal wall, typically in the form of yellow/brown excrement that mixes with the vehicle and obscures the dorsal vessel. Although the abdominal wall can be rinsed with an isotonic solution to remove debris, doing so can

increase the chances of damaging the dorsal vessel. With practice, this can be easily avoided; however, if this remains an issue, an alternative is to remove the gut prior to dissection. This can be accomplished by first removing the stinger and then pulling the abdominal gastrointestinal tract out through the same hole. One noticeable difference between this approach and the dissection as described in the protocol is that removing the stinger and gut first will typically leave the dorsal abdominal wall covered by a translucent membrane that can also obscure the dorsal vessel. When dissecting the abdomen as described in the protocol, this membrane will usually be removed along with the gut. If the membrane, or some portion of it, is obstructing view, it can be removed carefully with fine forceps.

It is not uncommon for the dorsal vessel to display no sign of a heartbeat immediately following dissection. Bathing it in an isotonic solution and allowing it to rest for several minutes will usually result in a steady heartbeat resuming. A continued lack of a heartbeat can be the result of a number of different issues, including damage to the dorsal vessel caused by a clumsy dissection, excessive anesthesia, premature death of the bee, or a slow dissection. The most common cause, however, would seem to be related to the energy reserves of the bee prior to dissection. Bees that have been housed in the laboratory overnight, even when provided access to food and water, are far more likely to result in a failed dissection than bees just collected from the colony. Age may also be a factor, as the success rate appears to be greater with nurse bees than with foragers. This has not been tested in a controlled study, however, and would be an interesting line of inquiry to pursue.

Overall, the goal of this protocol is to provide a simple, inexpensive approach to assessing the effect of potential cardiomodulators on bee heart rate. This protocol could be adapted to observe differences in cardiac function as a result of other types of treatments or factors, or for use with

other insects. The greatest limitation of the protocol is that the dorsal vessel is not being observed in a living, intact insect. It is likely that some potentially cardiomodulatory compounds will not show an effect on heart rate if they function at the level of the central nervous system. In this case, an electrophysiological approach is likely necessary. The greatest strength of this protocol is its simplicity, as it costs little, requires little equipment, can be undertaken with little or no specialized training, and can be conducted quickly once mastered. As such, it can be a valuable tool for applications ranging from exploratory pilot work to large scale drug screening studies, and can also serve as a useful teaching tool in high school or university classrooms.

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CHAPTER 3

ATP-SENSITIVE INWARDLY RECTIFYING POTASSIUM CHANNEL MODULATORS ALTER CARDIAC FUNCTION IN HONEY BEES

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3.1 ABSTRACT

ATP-sensitive inwardly rectifying potassium (K_{ATP}) channels couple cellular metabolism to the membrane potential of the cell and play an important role in a variety of tissue types, including the insect dorsal vessel, making them a subject of interest not only for understanding invertebrate physiology, but also as a potential target for novel insecticides. Most of what is known about these ion channels is the result of work performed in mammalian systems, with insect studies being limited to only a few species and physiological systems. The goal of this study was to investigate the role that K_{ATP} channels play in regulating cardiac function in a model social insect, the honey bee (*Apis mellifera*), by examining the effects that modulators of these ion channels have on heart rate. Heart rate decreased in a concentration-dependent manner, relative to controls, with the application of the K_{ATP} channel antagonist tolbutamide and K_{ATP} channel blockers barium and magnesium, whereas heart rate increased with the application of a low concentration of the K_{ATP} channel agonist pinacidil, but decreased at higher concentrations. Furthermore, pretreatment with barium magnified the effects of tolbutamide treatment and eliminated the effects of pinacidil treatment at select concentrations. The data presented here confirm a role for K_{ATP} channels in the regulation of honey bee dorsal vessel contractions and provide insight into the physiology that governs the regulation of bee cardiac function.

3.2 INTRODUCTION

The honey bee (*Apis mellifera*), like other insects, relies upon an open circulatory system for the transport of nutrients, immune factors, waste products, and signaling molecules throughout the body cavity. The insect circulatory system also functions in thermoregulation, ventilation, and the maintenance of homeostasis. Furthermore, studies have shown that the insect immune and circulatory systems can closely interact to control infections (King and Hillyer, 2012; Sigle and Hillyer, 2016), highlighting the extensive level of integration between cardiac function and the insect immune response. The insect circulatory system is driven by a central pulsatile organ known as the dorsal vessel, which is situated along the dorsal midline and spans the length of the insect. The abdominal portion of the dorsal vessel serves as the functional equivalent of the vertebrate heart and uses peristaltic action to force hemolymph through the thoracic dorsal vessel, which is commonly referred to as the aorta (Jones, 1977). Openings known as ostia are located along the length of the heart, allowing hemolymph to be drawn into the vessel and pumped in an anterograde fashion through the aorta in the direction of the head (Miller, 1985). With the aid of accessory pulsatile organs, hemolymph is circulated throughout the extremities of the insect (Pass, 2000). The periodic reversal of heartbeat, and subsequent reversal of hemolymph flow, has been described in a number of different insects at varying life stages (Gerould, 1933; Wasserthal, 1996), but has not been found to occur in bees (Miller, 1997). Although historically a subject of much debate, a growing body of evidence supports the idea that the origin of insect heartbeat is myogenic and not neurogenic in nature, as the heart is able to generate contractions in the absence of neural input (Miller, 1985, 1997; Slama and Lukas, 2011). There is strong evidence, however, that demonstrates a role for a neural component in the regulation of heart rate and directionality of insect cardiac function (Heinrich, 1970; Miller,

1985), including bees (Schwab et al., 1991). A considerable amount of research has examined the role that various ion channels (Gu and Singh, 1995), classical neurotransmitters (Johnson et al., 1997; Zornik et al., 1999), and cardiomodulatory peptides (Cuthbert and Evans, 1989) play in regulating insect heart rate. The biogenic amine octopamine has been identified as one of the primary insect neuromodulators (Roeder, 1999) and is known to have a cardioacceleratory role in insects (Zornik et al., 1999), including bees (Papaefthimiou and Theophilidis, 2011). While examples of parallel studies can be found in a variety of other insect species, especially those of medical importance (Hillyer et al., 2015), very few studies have been conducted to explore the regulation of bee cardiac activity.

Cardiac muscle contractions are the direct result of action potentials, which are generated by voltage-gated ion channels located in the cellular plasma membrane. Inwardly-rectifying potassium (K_{ir}) channels play an important role in modulating this activity by stabilizing the resting membrane potential of the cell and mediating inward potassium transport across the membrane at potentials less than the potassium equilibrium rate constant (E_k) (Reimann and Ashcroft, 1999). One member of the family of K_{ir} channels, the ATP-sensitive inwardly rectifying potassium (K_{ATP}) channel, is regulated by the level of nucleotides present in the cytosol and consequently provides an important link between the metabolic state of the cell and its membrane excitability (Hibino et al., 2010). Mammalian K_{ATP} channels are heteromeric octamers composed of four pore-forming K_{ir} channel subunits (Kir6.1 or Kir6.2) and four regulatory sulfonylurea receptor (SUR) subunits (SUR1 or SUR2) (Ashcroft and Gribble, 1998) and have been described in a variety of different tissues, including heart, skeletal muscle, vascular smooth muscle, pancreas, as well as brain (Ashcroft, 1988). Relative to the extensive body of knowledge concerning mammalian K_{ir} channels, little is known about insect K_{ir}

channels, and even less about K_{ATP} channels. What is known about the insect K_{ir} channel gene family is due in large part to the versatility of *Drosophila* as a model organism, as fruit flies have proven a valuable tool in the identification and characterization of novel ion channels (Doring et al., 2002). Genes encoding insect K_{ir} channel subunits were first identified in the fruit fly (*Drosophila melanogaster*) (Doring et al., 2002) and this information was used to subsequently identify related genes in the yellow fever mosquito (*Aedes aegypti*) (Piermarini et al., 2013), the African malaria mosquito (*Anopheles gambiae*) (Raphemot et al., 2014a), and the bedbug (*Cimex lectularius*) (Mamidala et al., 2013). Although they are present in insects, K_{ir} channel genes do not appear to be as abundant or diverse as they are in mammals. The genome of the fruit fly, for example, has been found to encode three K_{ir} channel family genes (Doring et al., 2002), while five have been identified in the yellow fever mosquito genome (Piermarini et al., 2013), which is considerably fewer than the sixteen K_{ir} channel family genes belonging to seven subfamilies that have been identified in humans (Raphemot et al., 2014a). The characterization of K_{ir} channel genes in disease vector mosquitoes has focused almost entirely upon on their role in renal function, where they appear to be critical for urine production and potassium homeostasis (Raphemot et al., 2013), in order to evaluate their potential exploitation as a target for novel insecticides (Raphemot et al., 2014b; Rouhier et al., 2014; Swale et al., 2016). Research using fruit flies as a model to investigate the insect antiviral immune response has demonstrated that K_{ATP} channels appear to play an evolutionarily conserved role in mediating the survival of insects and mammals alike during viral infections (Crocker et al., 2007). This finding has led to the observation that insect coronary K_{ATP} channels have a function in modulating antiviral RNA interference (RNAi), presumably by facilitating tissue specific regulation of innate immune response mechanisms by the cellular environment of the heart (Eleftherianos et al., 2011).

While putative K_{ir} channel family genes can be found in the *Apis mellifera* genome, based on comparisons to *Drosophila melanogaster* or *Aedes aegypti* K_{ir} channel gene sequences, no data have been published that describe a functional characterization of the expressed proteins or explore their role in the honey bee or any other hymenopteran. This represents a major knowledge gap, given the significant interest in managed bee colony losses (Neumann and Carreck, 2010; Ratnieks and Carreck, 2010), concerns over the impact of pesticide usage on pollinator health (Mullin et al., 2010), and the growing understanding of the detrimental effects that viruses have on colony health and survival (Cox-Foster et al., 2007; Johnson et al., 2009; McMenamin and Genersch, 2015). The research described here will begin to address this gap by investigating the relationship between K_{ATP} channels and cardiac function in an agriculturally and economically important pollinator and model social insect. As a first step to exploring the physiological role of K_{ATP} channels in bee cardiac function, we demonstrated the effects of K_{ATP} channel modulators on bee heart rate, examining the K_{ATP} channel antagonist tolbutamide, agonist pinacidil, and known blockers barium (Ba^{2+}) and magnesium (Mg^{2+}). Tolbutamide is a sulfonamide drug known as a sulfonylurea that has been used in the treatment of type 2 diabetes due to its ability to stimulate insulin production in the beta cells of the pancreas (Ashcroft and Ashcroft, 1992). In mammalian systems, tolbutamide blocks K_{ATP} channels by binding to the SUR1 subunit, resulting in depolarization of the cell membrane (Kaubisch et al., 1982). Tolbutamide treatment has been found to increase mortality and viral RNA expression in virus infected fruit flies (Croker et al., 2007; Eleftherianos et al., 2011). Pinacidil is a cyanoguanidine drug that is used as an antihypertensive due to its vasodilatory properties through relaxation of vascular smooth muscle (Arrigoni-Martelli and Finucane, 1985). Pinacidil opens K_{ATP} channels by binding to the mammalian SUR2 subunit, resulting in hyperpolarization of the cell membrane

(Uhde et al., 1999). Treatment with pinacidil has been shown to confer protective effects against viral infections in fruit flies, increasing survival and decreasing viral load via modulation of the antiviral immune response through an as yet undetermined mechanism (Eleftherianos et al., 2011). The sulfonylureas and cyanoguanidines are thought to have distinct, but closely associated, binding sites in mammals (Uhde et al., 1999), supporting the idea that the sites are coupled in a negative allosteric fashion (Bray and Quast, 1992). Whether this is true for insects remains unknown, as the SUR selectivity of these compounds has not been examined in an insect model. Ba^{2+} and Mg^{2+} were selected since Ba^{2+} is known to selectively block K_{ir} channels at low- to mid-micromolar concentrations and Mg^{2+} is capable of physically blocking K_{ir} channels (Armstrong and Taylor, 1980; Armstrong et al., 1982; Horie et al., 1987; Matsuda et al., 1987). The results of this work demonstrate that K_{ATP} channel modulators have an observable effect on honey bee heart rate that supports their expected physiological role in bee cardiac function.

3.3 METHODS

Subjects

European honey bees (*Apis mellifera*) were used for all experiments. Bee colonies were maintained according to standard beekeeping practices using commercial hives that were housed in an apiary located at the Virginia Tech Price's Fork Research Facility (Blacksburg, VA). For all laboratory experiments, worker bees were collected from brood frames during typical foraging times to ensure collection of predominately nurse bees. Bees kept in the lab overnight were housed at 32 °C with a relative humidity of 50-80% and provided *ad libitum* access to honey and a 50% solution of sucrose in water.

Dissection and heart rate assays

Visualization of the honey bee heart and measurements of heart rate were conducted as previously described (O'Neal and Anderson, 2016). To summarize, each bee was dissected to remove the dorsal abdominal wall and expose the dorsal vessel. The dorsal vessel was bathed in an isotonic solution (¼ strength Ringer's solution; Sigma Aldrich) and the heartbeat was allowed to stabilize. Baseline heart rate was measured for 1 min prior to the addition of any test compounds or vehicle controls, then measured again 2 min later. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted in ¼ strength Ringer's solution to the desired concentration, 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).

Concentration response experiments

The aim of this experiment was to assess the effects of K_{ATP} channel modulators on bee heart rate by evaluating a range of concentrations for each modulator. The modulators that were tested include the K_{ATP} channel antagonist tolbutamide, the K_{ATP} channel agonist pinacidil, as well as the cations Ba^{2+} and Mg^{2+} . 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, from the low nanomolar to the high millimolar, in order to establish a profile for each compound. Each treatment group had a sample size of 10 individual bee dissections.

Barium pretreatment experiment

The aim of this experiment was to examine the hypothesis that tolbutamide and pinacidil modulate heart rate by acting upon cardiac K_{ATP} channels. Also studied was whether or not

pretreatment with a low concentration of the K_{ir} channel blocker Ba^{2+} would alter the effects of treatment with tolbutamide or pinacidil. Ba^{2+} was chosen over Mg^{2+} due to its effectiveness at lower concentrations and its increased specificity for K_{ir} channels. Based on the results of the previous experiment, 1 μM Ba^{2+} was selected to test against 100 μM tolbutamide and 100 nM pinacidil. The concentration of Ba^{2+} used was the highest concentration that did not produce a significant effect on heart rate. The concentrations of tolbutamide and pinacidil were selected due to their significant effect on heart rate and the relatively low variability of that concentration. The protocol for the dissection and heart rate assay was followed as described, with one exception. Following dissection and visualization, the heart was bathed in either vehicle or vehicle containing 1 μM Ba^{2+} . The protocol then continued as previously described, with the application of 100 μM tolbutamide, 100 nM pinacidil, or vehicle control following 1 min of baseline heart rate assessment. Each treatment group had a sample size of 12 individual bee dissections.

Statistical Analysis

All analyses and calculations were conducted using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). The half maximal effective concentration (EC_{50}) was determined using a four parameter nonlinear regression equation. All results are expressed as the mean \pm standard deviation (SD). Each 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, nonparametric tests were used for all analyses. For both the concentration-response experiments and the blocking experiment, each treatment group was compared to the vehicle control using a Mann-Whitney test ($P < 0.05$).

3.4 RESULTS

Concentration response experiments

Tolbutamide produced a concentration-dependent effect on heart rate, as the application of increasing concentrations of tolbutamide resulted in greater decreases in heart rate with no evidence of an increase at any concentration (Fig 3.1). A significant decrease in heart rate relative to the vehicle control was observed at concentrations of 3 μ M and above (Mann-Whitney test; $P < 0.05$) with maximal effect (complete cessation of heart beat) observed at 1 mM. Nonlinear regression estimated an EC_{50} of 0.18 mM (95% CI: 0.14 – 0.23 mM; Hill Slope: 0.99; R^2 : 0.79). Pinacidil, however, produced a biphasic effect on heart rate, as the application of a high nanomolar concentration increased heart rate, while the application of concentrations in the high micromolar range and above decreased heart rate (Fig 3.1). A significant increase in heart rate relative to the vehicle control was observed at 100 nM (Mann-Whitney test; $P < 0.0001$), while a significant decrease was observed at concentrations of 100 μ M and above (Mann-Whitney test; $P < 0.001$) with maximal effect observed at 1 mM. Due to the biphasic response of the heart to pinacidil treatment, the EC_{50} value was calculated using the 3 μ M and greater treatments. Nonlinear regression estimated an EC_{50} of 0.31 mM (95% CI: 0.24 – 0.39 mM; Hill Slope: 1.84; R^2 : 0.76). Mean baseline heart rate (\pm SD) across treatment groups measured 101.9 \pm 5.0 BPM for tolbutamide and 98.2 \pm 3.5 BPM for pinacidil.

The cations Ba^{2+} and Mg^{2+} had uniformly inhibitory effects on bee heart rate. Ba^{2+} produced a concentration-dependent effect on heart rate, as the application of increasing concentrations of Ba^{2+} resulted in greater decreases in heart rate (Fig 3.1). A significant decrease in heart rate relative to the vehicle control was observed at concentrations of 10 μ M and above (Mann-

Whitney test; $P < 0.0001$) with maximal effect observed at 1 mM. Nonlinear regression estimated an EC_{50} of 0.62 mM (95% CI: 0.39 – 0.96 mM; Hill Slope: 0.62; R^2 : 0.81). Mg^{2+} also produced a concentration-dependent effect on heart rate, as the application of increasing concentrations of Mg^{2+} resulted in greater decreases in heart rate (Fig 3.1). A significant decrease in heart rate relative to the vehicle control was observed at concentrations of 30 mM and above (Mann-Whitney test; $P < 0.0001$) with maximal effect observed at 100 mM. Nonlinear regression estimated an EC_{50} of 27.29 mM (95% CI: 23.04 – 31.39 mM; Hill Slope: 2.77; R^2 : 0.88). Mean baseline heart rate (+/- SD) across treatment groups measured 101.1 +/- 4.1 BPM for Ba^{2+} and 100.6 +/- 4.1 BPM for Mg^{2+} .

Barium pretreatment experiment

Pretreatment with Ba^{2+} magnified the effects of tolbutamide treatment and eliminated the effects of pinacidil treatment (Fig 3.2). Pretreatment with 1 μ M Ba^{2+} followed by treatment with vehicle resulted in no significant change in heart rate relative to the vehicle control. Pretreatment with vehicle followed by either 100 μ M tolbutamide or 100 nM pinacidil resulted in either a significant decrease or increase in heart rate, respectively, with effects comparable to those observed in the previous experiment (Mann-Whitney test; $P < 0.001$). Pretreatment with 1 μ M Ba^{2+} followed by treatment with 100 μ M tolbutamide resulted in a complete cessation of heart beat, comparable to the effect of 1 mM tolbutamide treatment, making the decrease in heart rate significant relative to both the vehicle control and the tolbutamide control (Mann-Whitney test; $P < 0.001$). Pretreatment with 1 μ M Ba^{2+} followed by treatment with 100 nM pinacidil resulted in no significant increase in heart rate relative to the vehicle control, but a significant difference relative to the pinacidil control (Mann-Whitney test; $P < 0.001$). Mean baseline heart rate (+/- SD) across treatment groups measured 98.6 +/- 5.7 BPM.

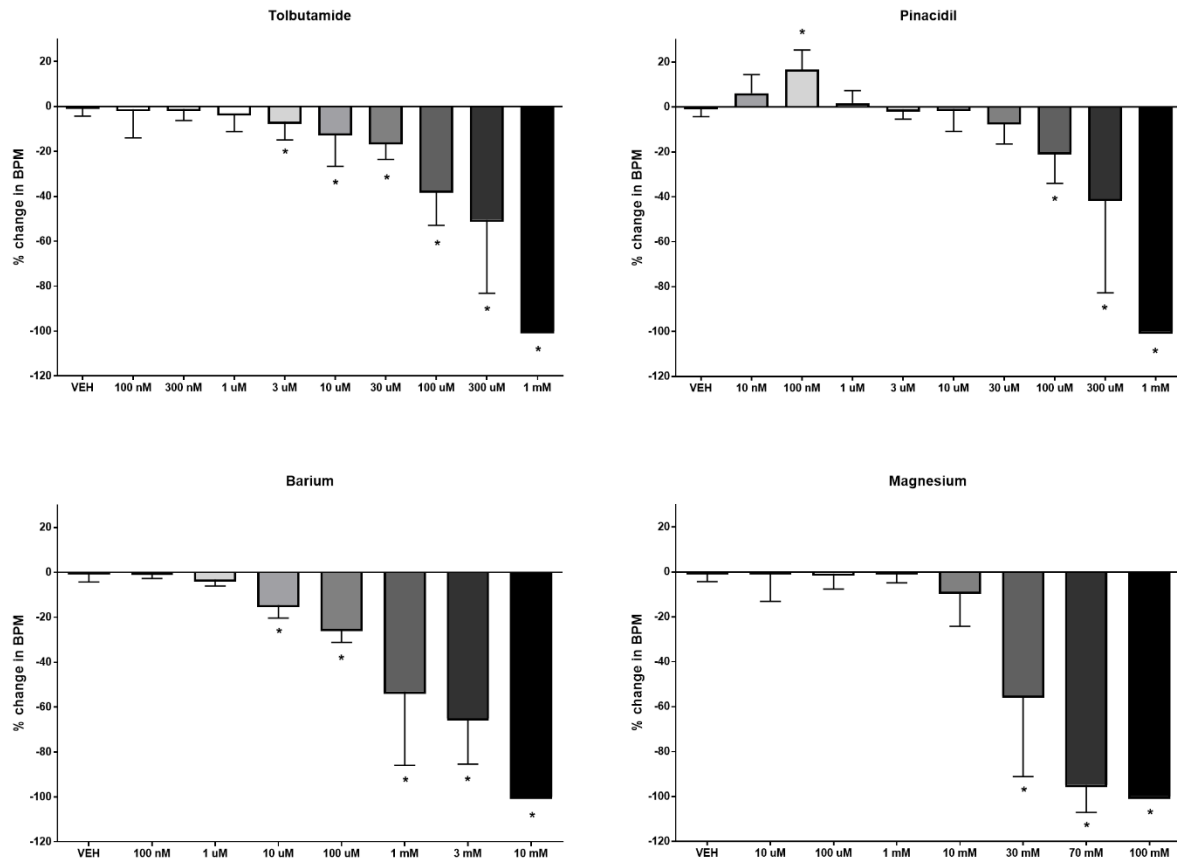


Figure 3.1. Bar graphs of the percent change in heart rate (beats per minute, BPM) resulting from the application of increasing concentrations of tolbutamide, pinacidil, barium, and magnesium. Relative to a vehicle control (VEH = 1% DMSO v/v in Ringer's solution), heart rate decreased in a concentration-dependent manner with the application of increasing concentrations of tolbutamide, barium, and magnesium, whereas heart rate increased with the application of a low concentration of pinacidil, but decreased at higher concentrations. The mean values were compared to a vehicle control using a Mann-Whitney nonparametric unpaired *t*-test where $P < 0.05$ was considered significant, as represented by an asterisk. Data presented as mean values \pm standard deviation ($n = 10$) and analyzed using GraphPad Prism 7 software.

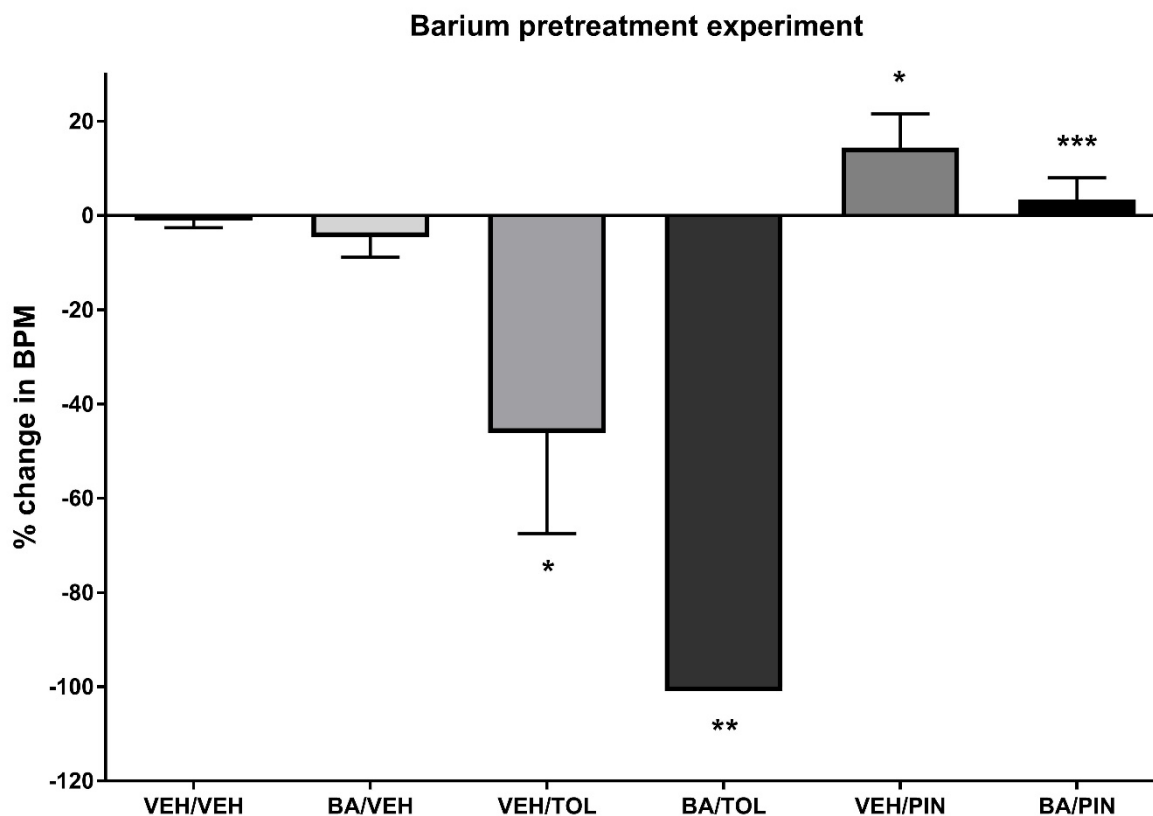


Figure 3.2. Bar graph of the percent change in heart rate (beats per minute, BPM) resulting from the application of barium followed by tolbutamide or pinacidil. Application of a low concentration of barium (BA = 1 μ M) followed by vehicle (VEH = 1% DMSO v/v in Ringer's solution) resulted in no significant change in heart rate, relative to a vehicle/vehicle control. Application of vehicle followed by tolbutamide (TOL = 100 μ M) decreased heart rate while vehicle followed by pinacidil (PIN = 100 nM) increased heart rate. Application of barium followed by tolbutamide resulted in a greater decrease in heart rate than observed with tolbutamide alone. Application of barium followed by pinacidil resulted in no significant change in heart rate, relative to a vehicle control, which was significantly lower than vehicle/pinacidil group. Mean values were compared using a Mann-Whitney nonparametric unpaired *t*-test where

$P < 0.05$ was considered significant, with significant difference from vehicle control group represented by an asterisk. Double asterisk indicates that mean value is significantly different from both vehicle control and vehicle/tolbutamide groups. Triple asterisk indicates that mean value is not significantly different from vehicle control group, but is significantly different from vehicle/pinacidil group. Data presented as mean values \pm standard deviation ($n = 12$) and analyzed using GraphPad Prism 7 software.

3.5 DISCUSSION

Here we have demonstrated that tolbutamide and pinacidil significantly alter cardiac activity in honey bees, most likely through their interaction with K_{ATP} channels expressed in the plasma membrane of the dorsal vessel, though interaction with K_{ATP} channels in the mitochondrial membrane are also possible and cannot be ruled out by this work. Direct application of the K_{ATP} channel antagonist tolbutamide to the exposed bee heart resulted in a concentration dependent decrease in heart rate, whereas the agonist pinacidil produced a biphasic response with an increase in heart rate at low concentrations and a decrease at higher concentrations. Direct comparison of these results to other insect systems is not possible due to the lack of comparable studies using invertebrate models. Interestingly enough, however, comparisons can be made with vertebrate systems. The biphasic response of the changes in bee heart rate that was observed following treatment with pinacidil supports earlier findings that pinacidil inhibited both the amplitude and velocity of isolated rabbit heart contractions in a biphasic manner (Nielsen et al., 1989). This not only suggests that the drug may have more than one site of action, but also supports the idea of an evolutionarily conserved role for K_{ATP} channels in cardiac function. Despite the obvious differences between the tubular invertebrate heart and the compact mammalian heart, both have been shown to originate from a similar set of progenitor cells and

subsequently develop under the regulation of a similar suite of genes (Bodmer, 1995; Ocorr et al., 2007). Furthermore, both hearts function in a myogenic fashion with insect hearts having been shown to possess a pacemaker regulatory node with an analogous function to the sinoatrial or atrioventricular nodes (Bodmer et al., 2005; Slama, 2006). Thus, the idea that K_{ATP} channels might function in such a conserved fashion between insects and mammals should not be surprising, especially given that treatment with the K_{ir} channel blockers Ba^{2+} and Mg^{2+} also resulted in a concentration dependent decrease in heart rate. When applied in combination, pretreatment with a low concentration of Ba^{2+} had a significant effect on subsequent treatments with synthetic modulators, amplifying the effect of tolbutamide and eliminating the effect of pinacidil on heart rate. The amplified effect of the combined Ba^{2+} and tolbutamide treatment is suggestive of blocking activity at more than one site. The loss of effect with the combined Ba^{2+} and pinacidil treatment suggests that Ba^{2+} is preventing pinacidil induced potassium flow. This is in keeping with patch clamp studies conducted in canine ventricular myocytes that found pinacidil induced current was reduced by Ba^{2+} treatment (Arena and Kass, 1989). This evidence suggests that the observed effects are the result of interactions with cardiac K_{ATP} channels, rather than off-target effects on heart rate, and supports an evolutionarily conserved role for K_{ATP} channels in the cardiac function of bees.

These findings are significant as they highlight the important role that K_{ATP} channels serve in regulating and maintaining proper cardiac function. It is important to point out that this work was conducted using dissected heart preparations that disconnected the dorsal vessel from the central nervous system and other inputs that typically influence heart physiology *in vivo*. Nevertheless, such studies are important for evaluating many aspects of cardiac function, and offer certain advantages over a whole animal approach (O'Neal and Anderson, 2016). K_{ir} channels modulate

action potentials by stabilizing the resting membrane potential of the cell, and K_{ATP} channels do so in response to changes in the metabolic state of the cellular environment. In the heart, this can significantly change the heart rate of the insect, subsequently having an effect on physiological processes such as thermoregulation and circulation (Miller, 1997). This also raises intriguing questions regarding the role that K_{ATP} channels play in maintaining homeostasis and the regulation of the antiviral innate immune response (Crocker et al., 2007). The treatment of fruit flies with the K_{ATP} channel antagonist tolbutamide has been demonstrated to have a significant effect on the outcome of a viral infection, resulting in more rapid mortality and greater accumulation of virus in the insect, whereas treatment with the agonist pinacidil conferred some protective benefits to infected flies (Eleftherianos et al., 2011). Is the modulation of the insect's heart rate, or the blocking of the insect's ability to modulate heart rate in response to changes in the cellular environment, responsible in whole or in part for this observation, or is there some other connection between K_{ATP} channel regulation and the innate immune response? This question represents a valuable area of future research in the honey bee, given the serious impact that viral infections have on the health of managed bee colonies. A better understanding of this relationship is also important given the potential use of K_{ATP} channels as novel targets for insecticides (Raphemot et al., 2014b; Rouhier et al., 2014; Swale et al., 2016), as the effects of pesticide exposure on pollinator health remains a topic of considerable discussion. Even if K_{ATP} channel modulators do not prove to be immediately toxic to beneficial insects, there is certainly room for concern that interactions with the innate immune response would result in sublethal effects that could impact the overall health of the colony in the long term. In conclusion, the data presented here provide evidence for the role of K_{ATP} channels in the regulation of honey bee cardiac activity and demonstrate the effect that K_{ATP} channel modulators have on heart rate. This

work sets the stage for continuing investigation of the physiological role of K_{ATP} channels in bees. This work also raises questions about the possibility of evolutionarily conserved interactions between K_{ATP} channels and the bee antiviral immune response and provides cautionary insight into the impact that future insecticides targeting K_{ATP} channels might have on pollinator health.

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CHAPTER 4

ATP-SENSITIVE INWARDLY RECTIFYING POTASSIUM CHANNEL REGULATION OF VIRAL INFECTIONS IN HONEY BEES

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4.1 ABSTRACT

Honey bees are economically important pollinators of a wide variety of crops that have attracted the attention of both researchers and the public alike due to unusual declines in the numbers of managed colonies in some parts of the world. Viral infections are thought to be a significant factor contributing to these declines, but viruses have proven a challenging pathogen to study in a bee model and interactions between viruses and the bee antiviral immune response remain poorly understood. In the work described here, we have demonstrated the use of flock house virus (FHV) as a model system for virus infection in bees and revealed an important role for the regulation of the bee antiviral immune response by ATP-sensitive inwardly rectifying potassium (K_{ATP}) channels. We have shown that treatment with the K_{ATP} channel agonist pinacidil increases survival of bees while decreasing viral replication following infection with FHV, whereas treatment with the K_{ATP} channel antagonist tolbutamide decreases survival and increases viral replication. Our results suggest that K_{ATP} channels provide a significant link between cellular metabolism and the antiviral immune response in bees.

4.2 INTRODUCTION

Honey bees (*Apis mellifera*) play an economically vital role in global agriculture as pollinators of a wide variety of crops, in addition to being valued for the honey and other natural products that they provide. Declines in the numbers of both managed and wild pollinators have served to increase public awareness of bee health issues and prompted researchers to increase efforts to understand the factors driving these declines. While there exist a variety of factors that negatively impact the health and survival of both managed and wild bee populations, there is a growing consensus that the prevalence of parasites and pathogens, especially viruses, are among the most significant threats to pollinator health (Evans and Schwarz, 2011; Manley et al., 2015; Paxton et al., 2015). Viral infections have been found to be closely associated with weakened or dying bee colonies, as well as with colonies believed to be affected by Colony Collapse Disorder (CCD) (Dainat et al., 2012; Genersch and Aubert, 2010; McMenemy and Genersch, 2015; vanEngelsdorp et al., 2009). The viruses that most commonly infect bees are nonenveloped, positive sense, single-stranded RNA viruses belonging to either the family *Dicistroviridae* or *Iflaviridae* in the order *Picornavirales* (Chen and Siede, 2007). Bee colonies are commonly infected with multiple viruses concurrently (Chen et al., 2004; de Miranda et al., 2010; Runckel et al., 2011), and those infections may range from asymptomatic to producing overt deformities, paralysis, and even death. There exist multiple routes of transmission for bee viruses (Shen et al., 2005), the most damaging being the ectoparasitic mite *Varroa destructor*, which can suppress overall immune responsiveness and cause seemingly harmless chronic infections to become acute and devastating (Nazzi et al., 2012; Ryabov et al., 2014). Evidence suggests that this complex interplay between host, pathogen, and parasite significantly alters the critical balance

that exists between viral replication strategies and the host immune defenses and is thought to be a significant factor driving colony loss (Genersch and Aubert, 2010; Nazzi et al., 2012).

One of the primary invertebrate antiviral defense mechanisms is an evolutionarily-conserved, post-transcriptional gene silencing mechanism known as RNA interference (RNAi), which recognizes the presence of double-stranded RNA to initiate targeted RNA degradation (Ding, 2010; Ding and Voinnet, 2007). Insects also employ the Janus kinase and Signal Transducer and Activator of Transcription (Jak/STAT), Toll, and Immune deficiency (Imd) innate immune response pathways in response to viral infections, though the importance of these seems to vary (Avadhanula et al., 2009; Sabin et al., 2010; Zambon et al., 2005). The bee genome has been demonstrated to encode the major proteins associated with these immune pathways (Brutscher et al., 2015), and bees have been shown to utilize an antiviral RNAi response (Chejanovsky et al., 2014; Desai et al., 2012; Maori et al., 2009; Wang et al., 2013). Interestingly, however, bees have been found to possess only about one third as many genes associated with insect immunity as fruit flies and mosquitoes (Evans et al., 2006), possibly suggesting a reduced reliance on the immune response of the individual. This discrepancy may be explained by the effectiveness of social immune barriers to infection that function at the colony level or it is possible that bees also rely on homeostatic mechanisms to control damage at the level of the cell or tissue to simply tolerate infection (Schneider and Ayres, 2008). Studies in the fruit fly *Drosophila melanogaster*, for example, have shown that Jak/STAT deficient flies are still able to resist infection with high titers of virus, despite a reduced transcriptional response to infection and an increased viral load (Dostert et al., 2005). Conversely, RNAi deficient flies have been observed to experience rapid mortality in response to viral infections, despite experiencing viral loads no greater than those observed in wild type controls (Galiana-Arnoux et al., 2006). Similar findings have demonstrated

the importance of the heart in maintaining homeostasis during the innate immune response of both mammals and insects during a viral infection and suggest that ATP-sensitive inwardly rectifying potassium (K_{ATP}) channels play an evolutionarily conserved role in mediating this interaction between the host and the virus (Crocker et al., 2007).

K_{ATP} channels belong to the family of inwardly rectifying potassium (K_{ir}) channels, which are a class of potassium (K^+) selective ion channels that conduct larger inward currents at membrane potentials negative to the equilibrium potential of K^+ than outward currents at potentials positive to it under physiological conditions (Hibino et al., 2010). K_{ir} channels mediate K^+ transport across the membrane and are responsible for stabilizing the resting membrane potential of the cell and for regulating action potential duration in electrically excitable cells such as cardiac muscle (Hibino et al., 2010). K_{ATP} channels are heteromeric octamers composed of four pore-forming K_{ir} channel subunits and four regulatory sulfonylurea receptor (SUR) subunits (Ashcroft and Gribble, 1998) that are regulated by the relative level of ATP present in the cytosol and consequently provide an important link between the metabolic state of the cell and its membrane potential (Hibino et al., 2010). In mammals, roles for K_{ATP} channels have been described in a variety of different tissues, including heart, skeletal muscle, vascular smooth muscle, pancreas, and brain (Ashcroft, 1988). While much of the research investigating K_{ATP} channels focuses on their role in the sarcolemma and the cytoplasmic membrane, it is worth noting that K_{ATP} channels have also been identified in the mitochondrial membrane (Inoue et al., 1991). Evidence suggests that mammalian K_{ATP} channels facilitate cardiovascular tolerance to endotoxic shock by linking vasoreactivity to metabolic demand, allowing coronary smooth muscle cells to adapt to the systemic stress caused by the innate immune response to infections (Kane et al., 2006). Relative to mammals, considerably less is known about the physiological role of insect K_{ir}

channels and K_{ATP} channels. The majority of research focused on insect K_{ir} channels has been conducted in fruit flies (Doring et al., 2002) and mosquitoes (Piermarini et al., 2013), though a recent study has investigated bedbugs (Mamidala et al., 2013). Much of this research has focused on the role of K_{ir} channels in Malpighian tubule function and their potential for exploitation as a novel insecticide target in *Aedes* and *Anopheles* mosquitoes (Raphemot et al., 2014; Rouhier et al., 2014; Swale et al., 2016). Research related to the insect innate immune response has found that K_{ATP} channels appear to play a role in mediating the survival of *Drosophila* during viral infections similar to that observed in mammals (Crocker et al., 2007). This finding has led to the further observation that insect K_{ATP} channels have a function in modulating antiviral RNAi, presumably by facilitating tissue-specific regulation of innate immune response mechanisms by the cellular environment of the heart (Eleftherianos et al., 2011).

At this time, very little is known about the role of K_{ir} channels in the honey bee or any other hymenopteran. Recently published findings have shown that K_{ATP} channel modulators can significantly alter heart rate in the honey bee (O'Neal et al., 2017), but there is no information available about the relationship between K_{ATP} channels and the antiviral immune response of bees. Unfortunately, investigations of bee antiviral immune responses must contend with several major obstacles. One of those obstacles is that bee colonies are often covertly infected with one or more viruses at any given time, posing a challenge for researchers focused on the outcome of infection with a single virus. Another is that infectious clones for honey bee viruses have not been developed, despite the fact that numerous bee viruses have been sequenced (de Miranda et al., 2004; Fujiyuki et al., 2004; Ghosh et al., 1999; Govan et al., 2000; Lanzi et al., 2006; Leat et al., 2000; Maori et al., 2007). Some research has been conducted using semi-purified virus preparations (Chen and Siede, 2007), but complete removal of contaminating viruses is often

impossible, making it difficult to accurately characterize infection dynamics. Recent work has proposed and tested Sindbis virus expressing enhanced green fluorescent protein as an experimental model of honey bee virus infection, using it to show that nonspecific double-stranded RNA triggers an antiviral response that controls viral infection in bees (Flenniken and Andino, 2013). In this study, we demonstrate the use of a different virus, flock house virus (FHV), as another experimental model for the study of viral infections in bees. FHV belongs to the family *Nodaviridae*, whose members are nonenveloped, icosahedral viruses with genomes composed of two single-stranded, positive sense RNAs (Schneemann et al., 1998). FHV has been well characterized structurally and genetically and has served as a model system for the study of host-virus interactions in a variety of arthropod species (Venter and Schneemann, 2008). Here we report that FHV infects adult bees, causing rapid onset of mortality and accumulation of viral RNA. Furthermore, infection-mediated mortality can be altered by pre-exposure to K_{ATP} channel modulators. Pharmacological activation of K_{ATP} channels reduced the onset of mortality and the level of viral RNA production following FHV infection, whereas inhibition of K_{ATP} channels increased both the rate of mortality and viral replication.

4.3 METHODS

Subjects

European honey bees (*Apis mellifera*) were used for all experiments. Bee colonies were maintained as previously described (O'Neal and Anderson, 2016) according to standard beekeeping practices using commercial hives that were housed in an apiary located at the Virginia Tech Price's Fork Research Facility (Blacksburg, VA). For all laboratory experiments, frames of emerging worker brood were removed from the hive and housed in an incubator at 32

°C with a relative humidity of 50-80%. Newly emerged bees were collected from these frames over the course of 48 h and sorted into cages in groups of approximately 25 per cage. All cages were also provided with ¼ portions of a queen mandibular pheromone (QMP)-impregnated strip (Mann Lake Ltd.) to reduce stress by simulating the presence of an egg-laying queen. Bees remained housed in incubators under these conditions for the duration of all experiments.

Drug treatment

Stock solutions of tolbutamide and pinacidil (Sigma Aldrich) were initially prepared in dimethyl sulfoxide (DMSO). Cages of newly emerged bees were provided *ad libitum* access to a 50% solution of sucrose in water. Test groups received sucrose solution supplemented with 1% DMSO and either tolbutamide or pinacidil (2 mM final concentration), while untreated control groups received sucrose solution supplemented only with 1% DMSO. Preliminary testing demonstrated no significant effects of DMSO on either bee mortality or viral replication. For all experiments, bees were provided access to the same sucrose solution for the duration of the test. For all experiments lasting more than 48 h, bees were also provided *ad libitum* access to honey starting at 72 h.

Viral infection

All experiments involving viral infections utilized FHV, kindly provided by Dr. Anette Schneemann (The Scripps Research Institute, La Jolla, California), that was purified as previously described (Marshall and Schneemann, 2001). 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 viral suspension into the thorax of each bee. Injection of the same volume of 10 mM Tris-HCl, pH 7.5, was used as an injection control. For all experiments

involving drug treatments, bees were injected following 24 h of exposure to drug-supplemented sucrose solution as the only source of food and water.

Survival

Preliminary testing compared 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 significantly greater mortality than uninjected bees over a 7 d period. For all survival experiments, six replicates of approximately 25 bees were used for each treatment group. Survival was observed 12 h following injection and then every 24 h for 7 d. In order to assess the effect of infection with increasing titers of FHV on bee mortality, groups of bees were injected with approximately 1.5×10^7 pfu of FHV/bee (50.6 nl of 3×10^8 pfu/ μ l viral suspension), 1.5×10^6 pfu of FHV/bee, 1.5×10^5 pfu of FHV/bee, or 1.5×10^4 pfu of FHV/bee and survival was compared to injection controls. In order to assess the effect of tolbutamide and pinacidil treatment on survival following infection with FHV, bees were treated with either drug-supplemented or unsupplemented sucrose solution and then injected with either virus or a vehicle control. Pinacidil was tested against 1.5×10^6 pfu of FHV/bee and 1.5×10^7 pfu of FHV/bee. Tolbutamide was tested against a viral challenge of 1.5×10^6 pfu of FHV/bee and 1.5×10^5 pfu of FHV/bee.

Viral RNA expression

In order to assess the level of viral replication in bees, a group of otherwise untreated bees was injected with 1.5×10^6 pfu of FHV/bee. In order to assess the effect of tolbutamide and pinacidil treatment on FHV RNA expression following infection, groups of bees were fed either unsupplemented or drug-supplemented sucrose solution, as described for survival studies, and

then injected with 1.5×10^6 pfu of FHV/bee. For all treatments, 6 bees were collected at each time point, dissected, and then frozen together in liquid nitrogen for pooled analysis of RNA expression. Frozen samples were maintained at $-80\text{ }^{\circ}\text{C}$ until RNA isolation was performed. The head and abdomen were removed from each insect, as PCR inhibitors have been noted in the eye (Boncristiani et al., 2011) and preliminary experiments found that inclusion of the gut also inhibited PCR. The first time point for each treatment (0 h) was collected immediately following injection to be used as a calibrator, or reference sample, and subsequent time points were collected at 6, 12, and 24 h following infection.

Primers and probes

The following primers and probe were designed for the detection of FHV RNA1: forward 5'-GGACCGAAGTGCGGTGATG-3', reverse 5'-CAGTTTTGCGGGTGGGGGG-3', and probe FAM-5'-TGCCGCAATGAAGGATGTCT-3'-TAMRA. Primers and probe used for the detection of β -actin have been previously described (Chen et al., 2005): forward 5'-AGGAATGGAAGCTTGCGGTA-3', reverse 5'-AATTTTCATGGTGGATGGTGC-3', and probe FAM-5'-ATGCCAACACTGTCCTTTCTGGAGGTA-3'-TAMRA.

Quantitative PCR

Total RNA was isolated from each sample using TRI Reagent RT (Molecular Research Center, Inc.) and then $1\text{ }\mu\text{g}$ was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems). The thermal cycling parameters used for reverse transcription were $25\text{ }^{\circ}\text{C}$ for 10 min, $37\text{ }^{\circ}\text{C}$ for 120 min, and then $85\text{ }^{\circ}\text{C}$ for 5 min. Gene expression was determined using qPCR on a StepOne Real-Time PCR System (Applied Biosystems). Each PCR reaction ($20\text{ }\mu\text{l}$) contained TaqMan Fast Advanced Master Mix (Applied Biosystems), 100 ng cDNA template,

1 μ M forward and reverse FHV RNA1 primers, and 250 nM FHV RNA1 TaqMan probe. The thermal cycling parameters used for qPCR were 95 °C for 20 sec for enzyme activation followed by 40 cycles of denaturation at 95 °C for 1 s and annealing/extension at 60 °C for 20 s.

The comparative C_T ($\Delta\Delta C_T$) method, which determines the quantity of target in a sample relative to the quantity of target in a reference sample (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), was used to assess the change in FHV RNA1 expression at each time point relative to the 0 h sample. Measurements of target gene expression were normalized using *Apis mellifera* β -actin as the endogenous control. Three technical replicates were analyzed for untreated bees injected with 1.5×10^6 pfu of FHV/bee and four technical replicates were analyzed for all other samples. Serial dilutions of different inputs of target and reference genes were performed to verify that efficiencies of target and reference are approximately equal. Viral RNA expression is reported as the fold change \pm standard deviation relative to the calibrator sample for each treatment. Because the relative quantitation is exponentially related to the threshold value (C_T), calculation of the standard deviation results in an asymmetric distribution of the upper and lower limits, precluding direct statistical comparisons between expression levels in different treatment groups. Data analysis was performed using StepOne Software v2.3 (Applied Biosystems).

Statistical Analysis

All data analysis and graph construction was performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). Survival experiment results are reported as Kaplan-Meier survival curves, displaying mean values \pm standard error, with significant differences between the survival curves determined by the log-rank (Mantel-Cox) test.

4.4 RESULTS

FHV can infect honey bees

Intrathoracic injection of gradient-purified FHV into honey bees resulted in high mortality. The time to death was concentration-dependent, with increasing viral titers resulting in more rapid mortality (Figure 4.1). Bees injected with the highest titer of virus, receiving approximately 1.5×10^7 plaque-forming units (pfu) of FHV/bee, experienced 70% mortality within the first 24 h and 100% mortality by 72 h post injection. Bees injected with 1.5×10^6 pfu of FHV/bee experienced 30% mortality within the first 24 h and 100% mortality was not observed until 144 h post injection. Bees injected with 1.5×10^5 pfu of FHV/bee or 1.5×10^4 pfu of FHV/bee were indistinguishable from vehicle controls at 24 h post injection and 100% mortality was not observed within the time period monitored during this study. Log-rank tests of the Kaplan-Meier survival curves indicated a significant difference in survival between bees challenged with 1.5×10^5 pfu of FHV/bee or more and bees challenged with vehicle (Kaplan-Meier log-rank test; $P < 0.0001$). Increasing amounts of FHV RNA1 were detected in infected bees using quantitative real-time PCR (qPCR) (Figure 4.2), indicating productive FHV infection. These data demonstrate that FHV is capable of infecting bees and suggest that FHV can serve as a model virus in bees.

K_{ATP} channel modulators affect the outcome of FHV infection in bees

Treatment with the K_{ATP} channel agonist pinacidil increased survival of honey bees following infection with 1.5×10^6 pfu of FHV/bee, whereas treatment with the K_{ATP} channel antagonist tolbutamide decreased survival, relative to a untreated controls (Figure 4.3). Untreated bees experienced 65% mortality within the first 48 h and 100% mortality by 144 h post injection.

Pinacidil-treated bees experienced 5% mortality within the first 48 h and 100% mortality was not observed within the time period monitored during this study. Tolbutamide-treated bees experienced 87% mortality within the first 48 h and 100% mortality by 96 h post injection. Log-rank tests of the Kaplan-Meier survival curves indicated a significant difference in survival between untreated bees and both bees treated with pinacidil (Kaplan-Meier log-rank test; $P < 0.0001$) and bees treated with tolbutamide (Kaplan-Meier log-rank test; $P < 0.001$). When infected with a higher titer of virus (1.5×10^7 pfu of FHV/bee), survival in pinacidil-treated bees was somewhat less pronounced, but was still significantly increased relative to untreated bees (Kaplan-Meier log-rank test; $P < 0.0001$) (Figure 4.4). When infected with a lower titer of virus (1.5×10^5 pfu of FHV/bee), mortality in tolbutamide-treated bees became more pronounced and survival was significantly decreased relative to untreated bees (Kaplan-Meier log-rank test; $P < 0.0001$) (Figure 4.5).

In order to confirm that K_{ATP} channel modulators are affecting viral replication, relative changes in the amount of FHV RNA1 were assessed using qPCR following treatment with pinacidil or tolbutamide (Figure 4.6). Pinacidil treatment led to decreased accumulation of viral RNAs within 24 h post injection, with a one-log lower fold change in expression when compared to untreated controls. Tolbutamide treatment led to increased accumulation of viral RNAs within the first 24 h post injection, with a two-log higher fold change in expression when compared to untreated controls.

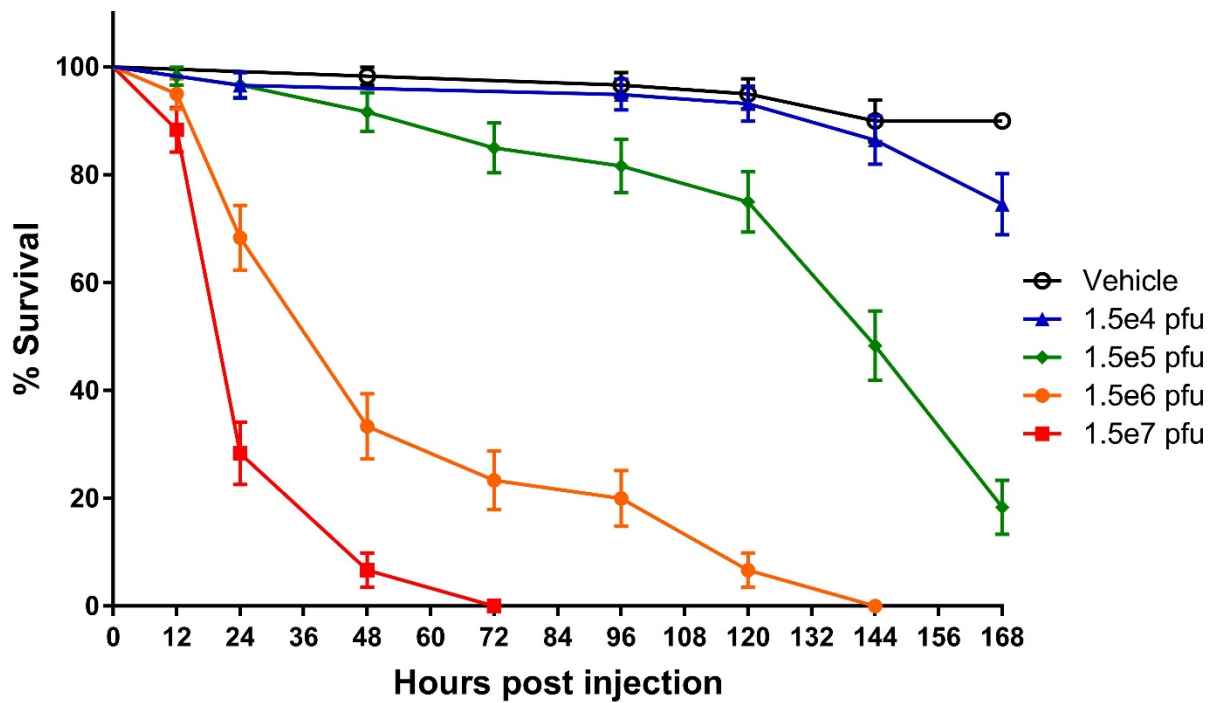


Figure 4.1. Honey bee survival following infection with increasing titers of FHV. Data presented as Kaplan-Meier survival curves with points representing mean values \pm standard error for 150 bees (6 replicate groups of 25 adult bees). Groups receiving 1.5×10^5 pfu of FHV/bee or more experienced significantly higher mortality than vehicle control group (Kaplan-Meier log-rank test; $P < 0.0001$). Data analyzed using GraphPad Prism 7 software.

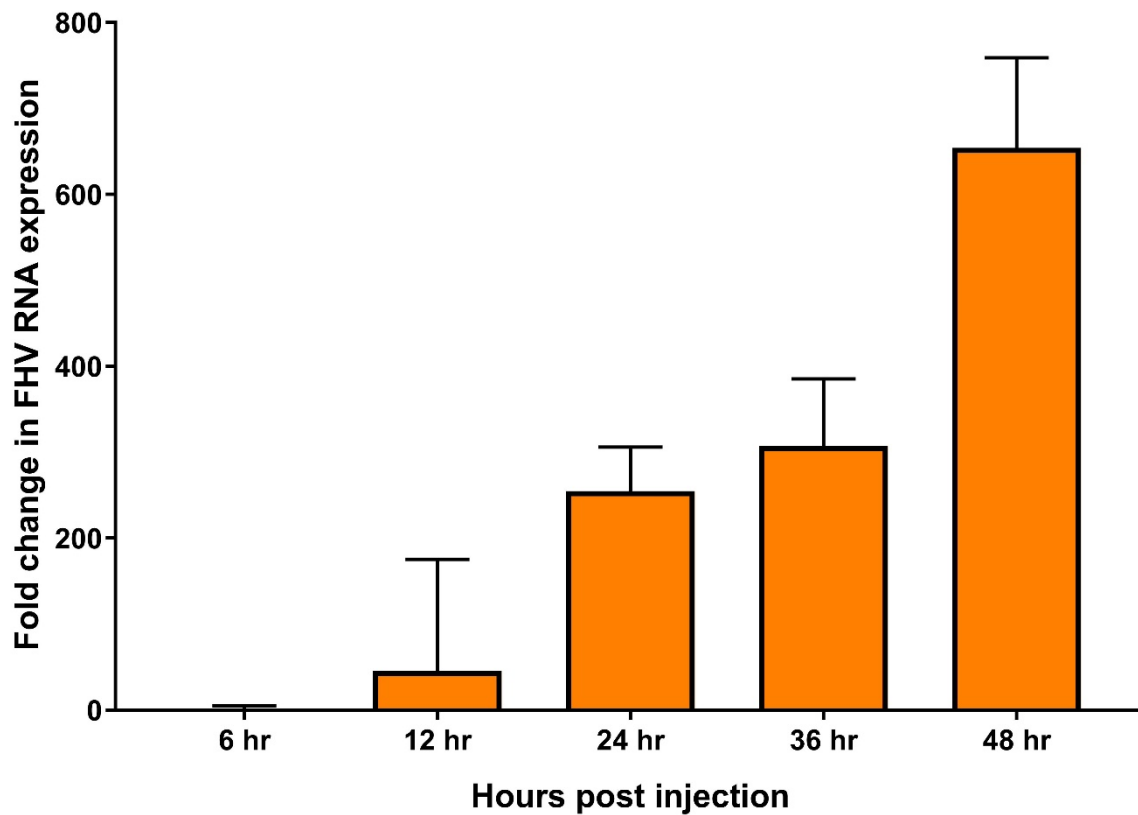


Figure 4.2. Fold change in FHV RNA expression over time following infection with 1.5×10^6 pfu of FHV /bee. Expression of FHV RNA1 was measured and presented as the mean fold change \pm standard deviation, relative to the amount of virus present at the time of infection. Time points represent 3 technical replicates of a pooled sample of 6 bees.

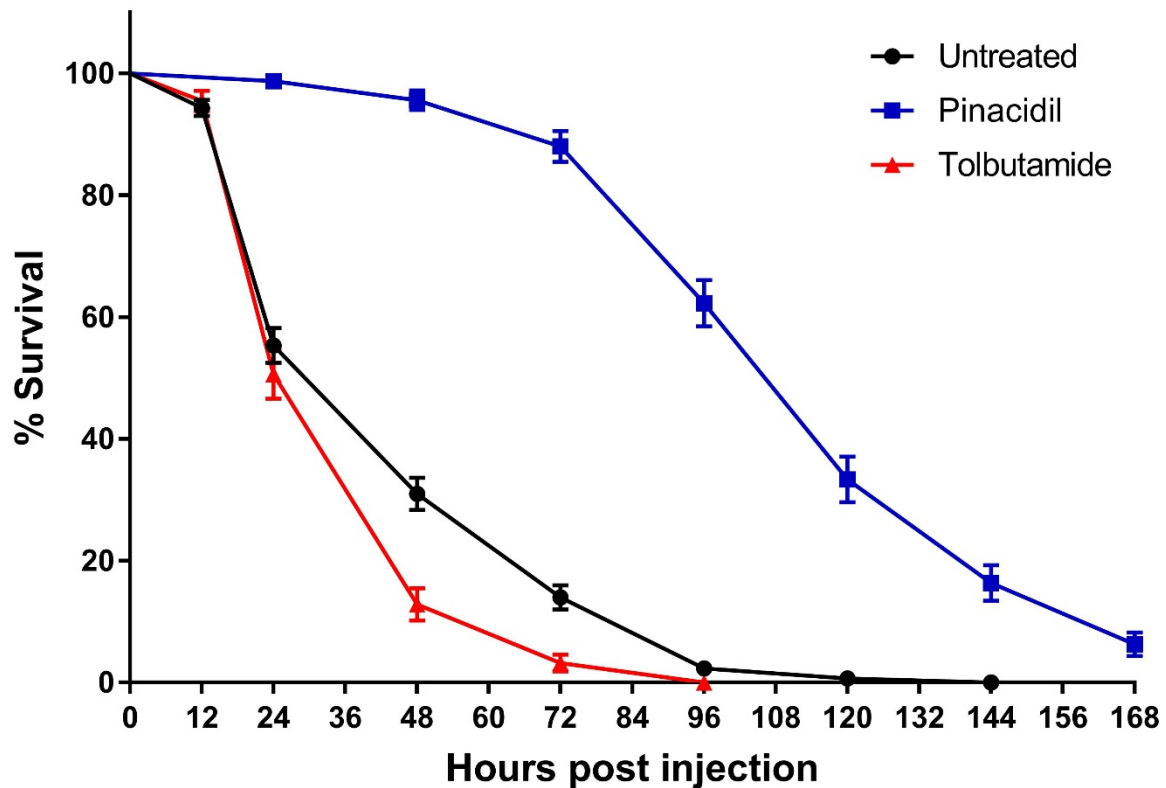


Figure 4.3. Honey bee survival following pinacidil or tolbutamide treatment and infection with 1.5×10^6 pfu of FHV/bee. Data presented as Kaplan-Meier survival curves with points representing mean values \pm standard error for 150 bees (6 replicate groups of 25 adult bees). Bees receiving pinacidil experienced significantly lower mortality than untreated bees (Kaplan-Meier log-rank test; $P < 0.0001$). Bees receiving tolbutamide experienced significantly higher mortality than untreated bees (Kaplan-Meier log-rank test; $P < 0.001$). Data analyzed using GraphPad Prism 7 software.

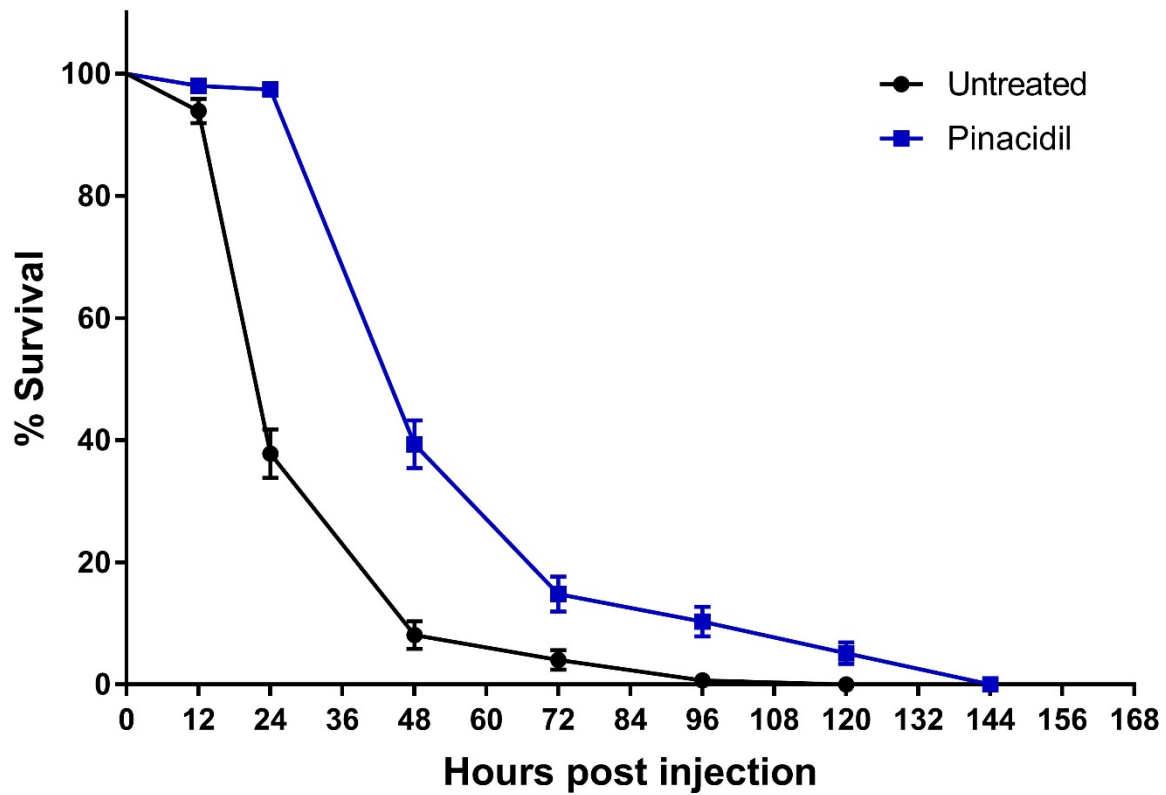


Figure 4.4. Honey bee survival following pinacidil treatment and subsequent infection with 1.5×10^7 pfu of FHV/bee. Data presented as Kaplan-Meier survival curves with points representing mean values \pm standard error for 150 bees (6 replicate groups of 25 adult bees). Bees receiving pinacidil experienced significantly lower mortality than untreated bees (Kaplan-Meier log-rank test; $P < 0.0001$). Data analyzed using GraphPad Prism 7 software.

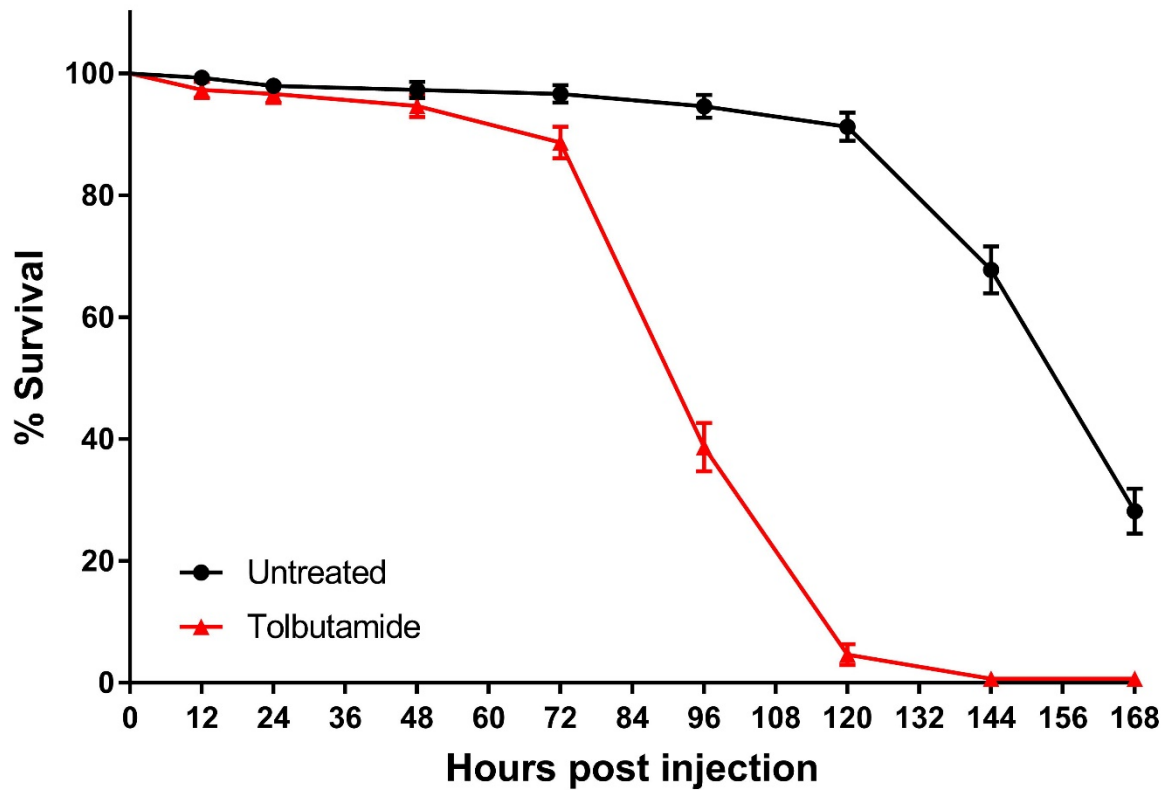


Figure 4.5. Honey bee survival following tolbutamide treatment and subsequent infection with 1.5×10^5 pfu of FHV/bee. Data presented as Kaplan-Meier survival curves with points representing mean values \pm standard error for 150 bees (6 replicate groups of 25 adult bees). Bees receiving tolbutamide experienced significantly higher mortality than untreated bees (Kaplan-Meier log-rank test; $P < 0.0001$). Data analyzed using GraphPad Prism 7 software.

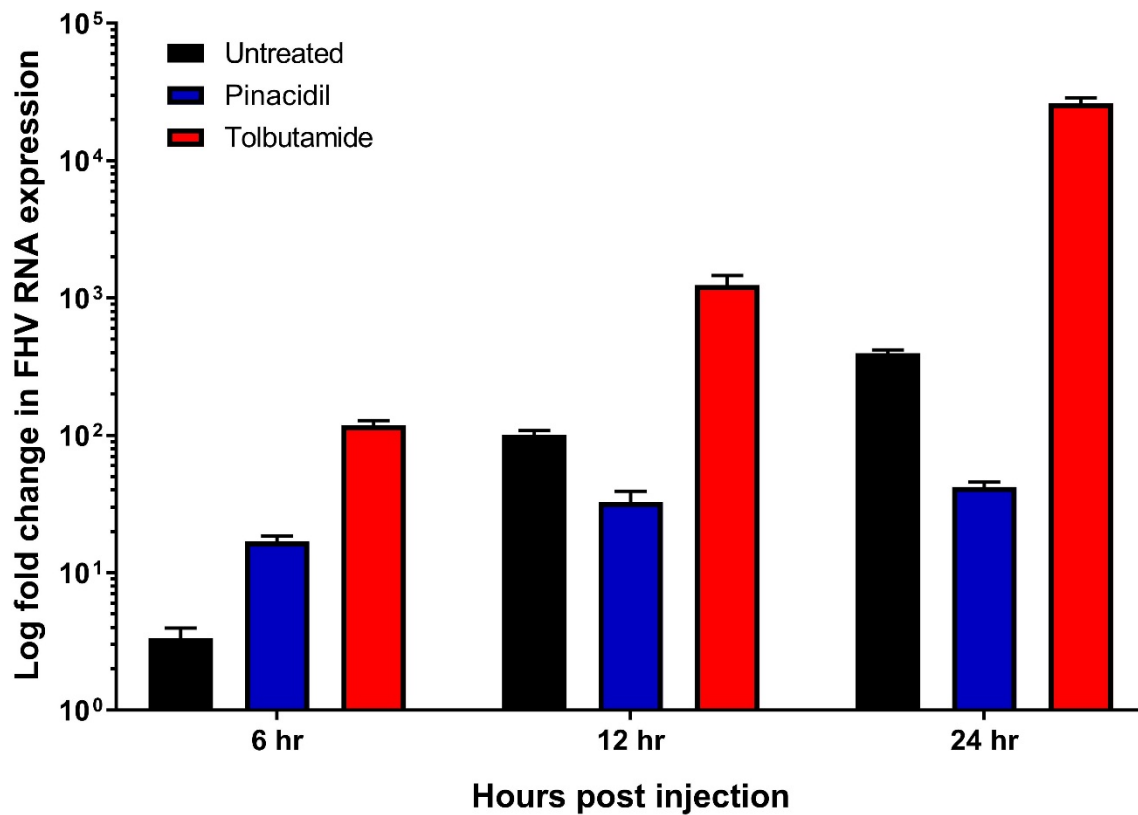


Figure 4.6. Log₁₀ fold change in FHV RNA expression over time following pinacidil or tolbutamide treatment and infection with 1.5×10^6 pfu of FHV/bee. Expression of FHV RNA1 was measured and presented as the log₁₀ mean fold change \pm standard deviation, relative to the amount of virus present at the time of infection. Time points represent 4 technical replicates of a pooled sample of 6 bees.

4.5 DISCUSSION

Our findings demonstrate the use of FHV as an experimental model for the study of viral infections in honey bees and reveal an evolutionarily conserved relationship between K_{ATP} channels and the antiviral immune response of bees. Despite the economic and agricultural importance of this domesticated pollinator species and the growing awareness of the role of viral pathogens in bee declines, the mechanisms involved in the antiviral immune response of bees remain poorly characterized. This represents a critical gap in our knowledge of the complex relationship between host and pathogen that must be filled if we are to better understand how viruses and immune deficiencies affect bee health and to develop more effective strategies to mitigate colony loss.

The establishment of FHV as a model system for virus infection in bees helps to avoid some of the major challenges facing investigations of the bee antiviral immune response, which include the lack of infectious clones of bee viruses and the prevalence of covert viral infections already present in many colonies. The use of FHV as a model virus should ensure that the bees used in an experiment will not be previously infected, as FHV is not a pathogen normally found in bees. Despite not being a naturally occurring bee virus, FHV bears much similarity to bee viruses in general, as it is a nonenveloped, positive sense, single-stranded RNA virus of insect origin (Scotti et al., 1983). FHV is a well-studied and extensively characterized virus that has an established history of use in studies investigating antiviral immunity in other insect species (Venter and Schneemann, 2008). FHV can be readily produced and purified in the lab, allowing investigators to precisely deliver a known quantity of a single virus during infection, which is not necessarily the case when using a semi-purified inoculum of a bee virus. Precision is further enhanced by infection via intrathoracic injection, rather than infection via an oral route, which

ensures that the same amount of infectious material is administered to each test subject. FHV also offers the opportunity to observe how the bee antiviral immune response copes with a novel viral challenge, rather than a virus that may be specifically adapted to persistently infect a bee host, thus simulating the challenges that would be faced by the introduction of either a novel virus or an established virus that has gained increased virulence. FHV possesses a known and well-studied suppressor of RNAi, the B2 protein, which inhibits processing of viral RNA by Dicer nuclease, rather than suppression of immune response genes or action through some unknown mechanism, as could be the case with other viruses that have not been as thoroughly characterized. Although it would be preferable in many cases to study the effects of a virus that has co-evolved with bees as a host, there are distinct advantages at this time to the use of FHV as an experimental model for the study of viral infections in bees.

Using FHV as a model system in bees, we have shown that K_{ATP} channels appear to have an important function in the tolerance of bees to viral infection. In *Drosophila*, this function has been shown to rely on modulation of RNAi (Eleftherianos et al., 2011), which serves as one of the primary invertebrate antiviral defense pathways (Ding, 2010). What this finding describes is a similar role for ion channels in the regulation of the honey bee antiviral immune response, in particular, ion channels that are sensitive to changes in the cellular environment. In mammals, K_{ATP} channels are understood to have an important role in the maintenance of cardiac cellular homeostasis and functional K_{ATP} channels have been demonstrated as a requirement for successful adaptation to stress (Zingman et al., 2002). Due to their presence in metabolically active tissues and integration with both cellular and systemic metabolic processes, K_{ATP} channels have been described as a unifying molecular coordinator of metabolic well-being under stress (Zingman et al., 2003). Evidence suggests that this evolutionarily conserved function is present

in insects as well (Crocker et al., 2007; Eleftherianos et al., 2011), leading us to hypothesize that K_{ATP} channels provide an important link between stress and reduced immunocompetence in bees. Exposure to chronic stress is understood to gradually weaken the immune response and reduce metabolic activity of an organism until it is no longer capable of survival (McEwen, 2000). Bees are no exception, as physiological stress can have a range of detrimental consequences for bee health and survival (Even et al., 2012). A variety of studies have demonstrated harmful synergistic interactions between simultaneous exposure to pesticides, dietary toxins, and pathogens in bees, but the mechanisms that explain these interactions remain unknown (Alaux et al., 2010; Aufauvre et al., 2012; Kohler et al., 2012; Vidau et al., 2011). K_{ATP} channels effectively serve as a sensor or feedback mechanism that responds to changes in the metabolic state of a cell, as one of their defining characteristics is that they are gated by the relative levels of intracellular nucleotides, primarily ATP and ADP (Hibino et al., 2010). Exposure to a wide range of environmental stressors can effectively depress or downregulate the metabolic activity of an organism, which includes ATP production and turnover (Hand and Hardewig, 1996). Consequently, K_{ATP} channels are in a unique position to link stress-induced changes in bee metabolism to the antiviral immune response.

In summary, we have demonstrated the effectiveness of FHV as a model system for virus infection in honey bees, but more importantly, we have revealed an important role for ion channel regulation of the honey bee antiviral immune response. We have shown that treatment with the K_{ATP} channel agonist pinacidil increases survival of honey bees while decreasing viral replication following infection with FHV, whereas treatment with the K_{ATP} channel antagonist tolbutamide decreases survival and increases viral replication. Based on our findings and what is known about the role of the evolutionarily conserved K_{ATP} channel in other systems, we propose

an important role for this ion channel in connecting the antiviral immune response of bees to changes in the cellular environment induced by environmental stressors. Additional work is necessary to confirm this hypothesized role of K_{ATP} channels and to expose the mechanisms of their relationship to stress and the immune response, but this represents a promising area for future research designed to enhance our understanding of honey bee immune responses and the factors that negatively impact pollinator health.

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CHAPTER 5

AMITRAZ AND ITS METABOLITE MODULATE HONEY BEE CARDIAC FUNCTION AND TOLERANCE TO VIRAL INFECTION

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5.1 ABSTRACT

The health and survival of managed honey bee (*Apis mellifera*) colonies are affected by a wide range of 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 a model virus system, 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.

5.2 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 Tactic®, 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 Christopher 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.

5.3 METHODS

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 using 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.

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).

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.

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 μ M 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 μ M 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.

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 μ M 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 μ M) 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.

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)/ μ l 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.

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.

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.

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 \pm 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.

5.4 RESULTS

Concentration response experiment

Phentolamine treatment decreased honey bee heart rate, whereas octopamine, amitraz, and DPMF treatment increased heart rate (Figure 5.1). 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 (\pm 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 increase 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.

Phentolamine pretreatment experiment

Pretreatment with phentolamine uniformly blocked the cardioacceleratory effects of octopamine, amitraz, and DPMF (Figure 5.2). Pretreatment with 100 nM phentolamine followed by treatment with vehicle resulted in no significant change in heart rate relative to the vehicle 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 (Figure 5.1). 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.

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 (Figure 5.3). 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 post-injection 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 post-injection. 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 that were treated with DPMF and infected bees that were treated with vehicle ($\chi^2 = 41.45$; $df = 1$; $P < 0.0001$). 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.

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 (Figure 5.4). Infected bees exposed to amitraz experienced 85% mortality just 1 d after infection, compared to only 10% for the infected control group. Surprisingly, 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 ($F = 1997.39$; $df = 1, 13$; $P < 0.0001$).

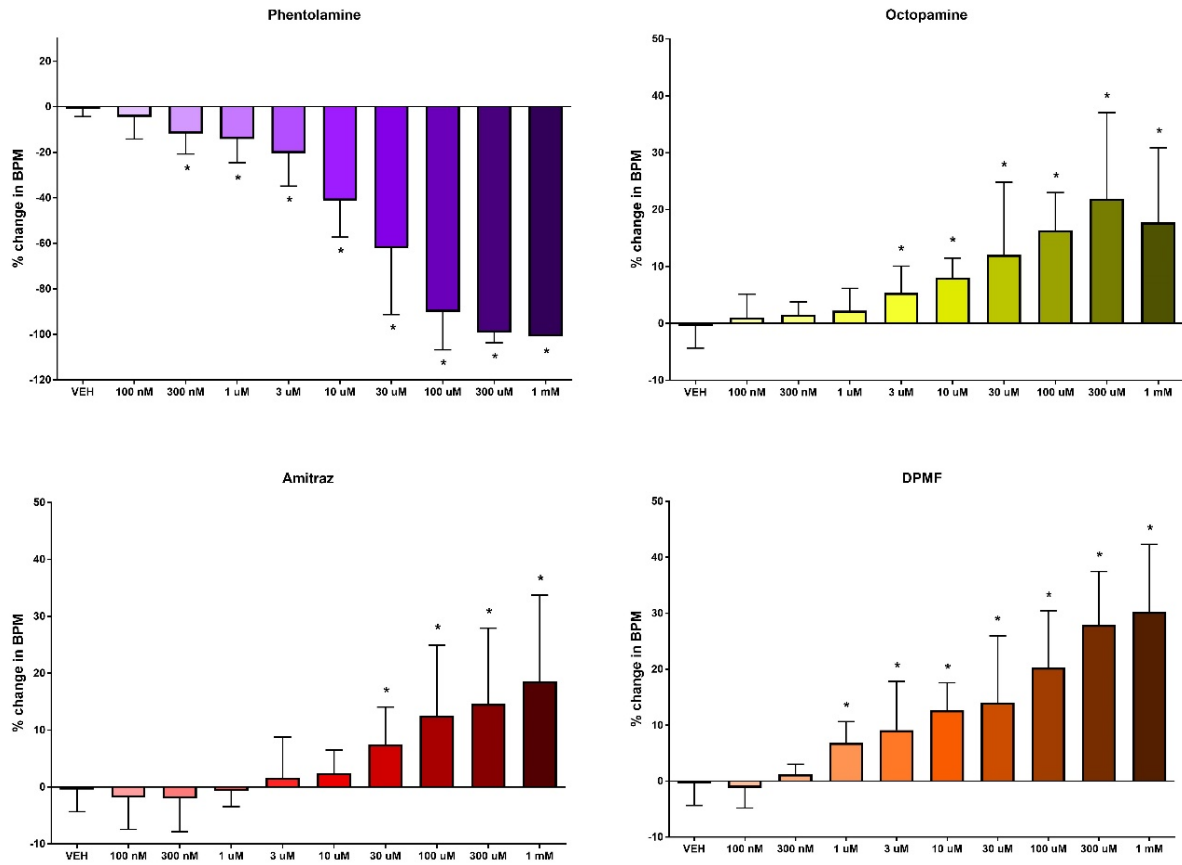


Figure 5.1. Percent change in heart rate (beats per minute, BPM) resulting from the application of increasing concentrations of amitraz, DPMF, octopamine, and phentolamine. Bars represent mean change in heartbeat frequency \pm standard deviation relative to baseline heart rate ($n = 10$). The mean treatment 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.

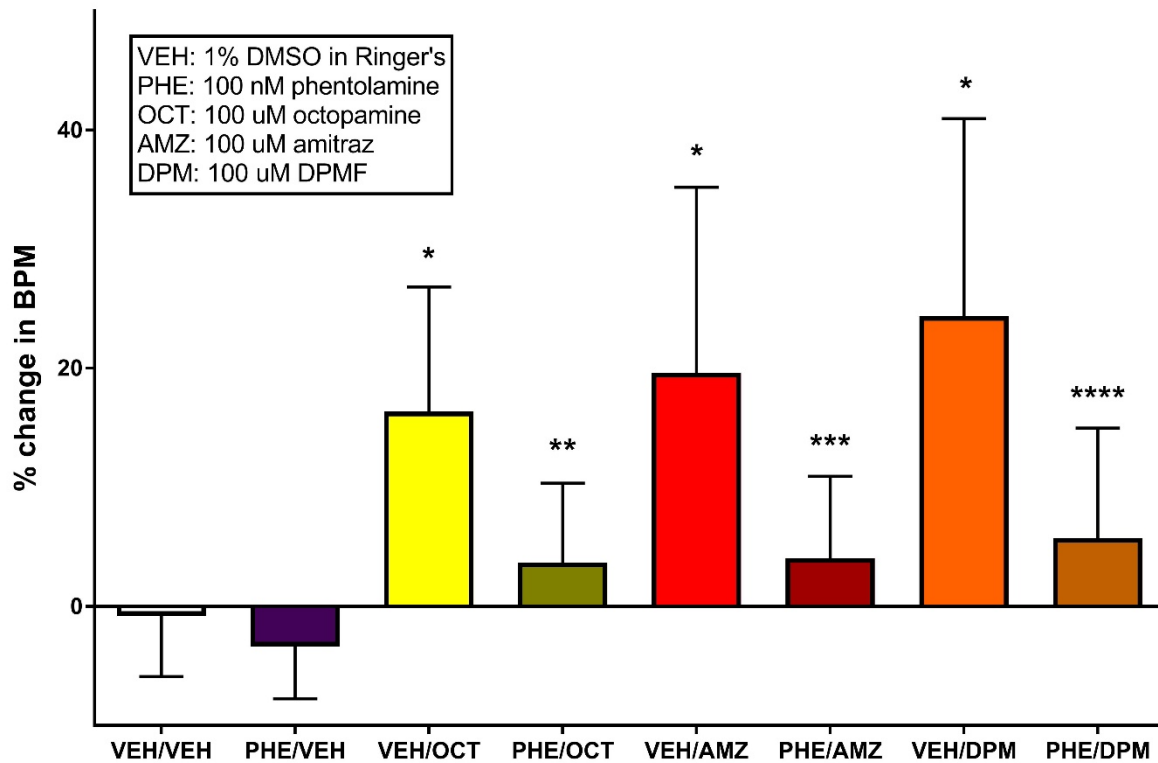


Figure 5.2. Percent change in heart rate (beats per minute, BPM) resulting from the application of phentolamine followed by octopamine, amitraz, or DPMF. Bars represent mean change in heartbeat frequency \pm standard deviation relative to baseline heart rate ($n = 12$). The mean values were compared 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.

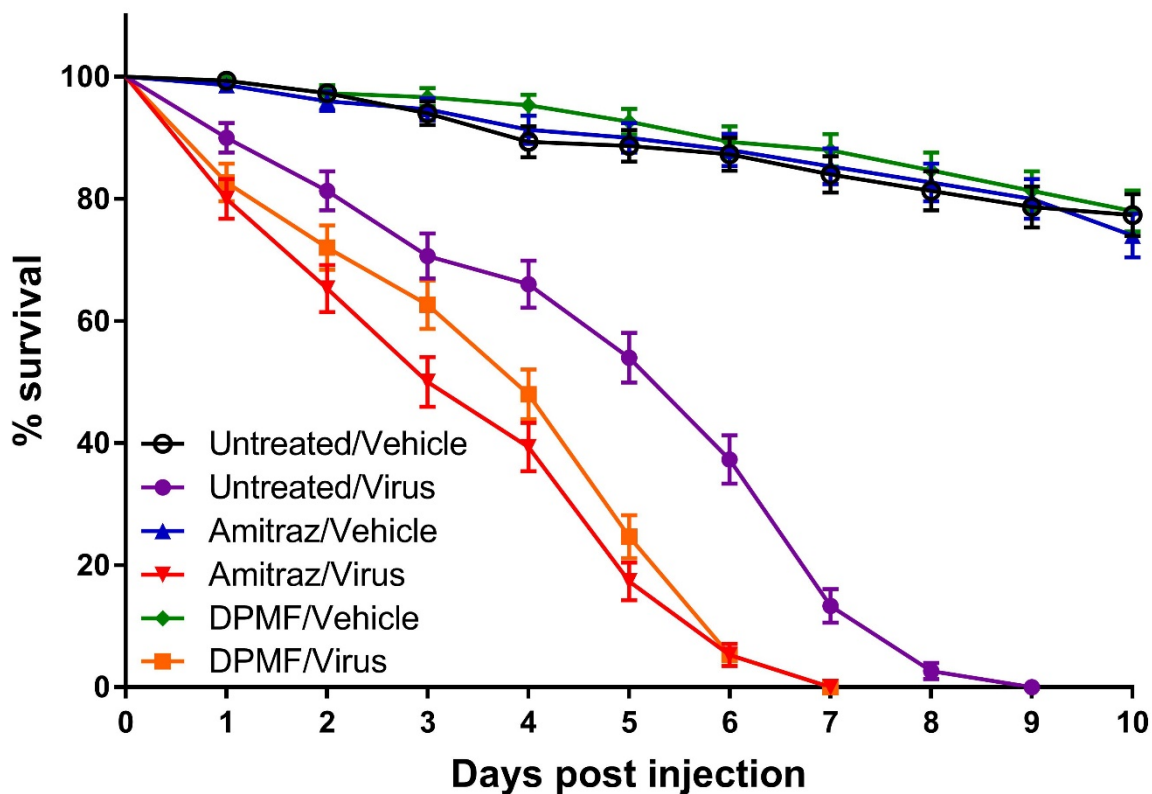


Figure 5.3. Effect of orally-dosed amitraz and its metabolite DPMF on honey bee survival following a virus challenge. Data presented as Kaplan-Meier survival curves with points representing mean values \pm standard error for 150 bees (6 replicate groups of 25 adult bees each per treatment). Amitraz/Virus 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.

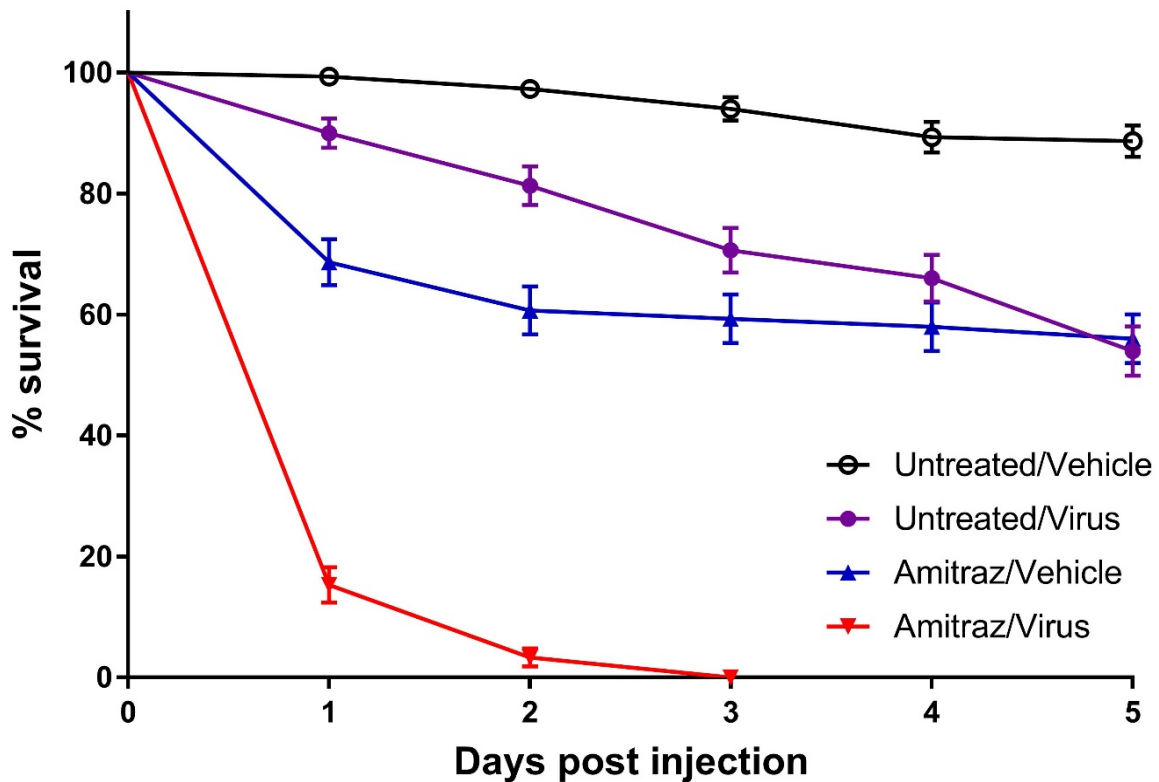


Figure 5.4. Effect of exposure to Apivar® (amitraz) strips on honey bee survival following a virus challenge. Data presented as Kaplan-Meier survival curves with points representing mean values \pm standard error for 150 bees (6 replicate groups of 25 adult bees each per treatment). The Amitraz/Virus 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.

5.5 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 and Theophilidis, 2011; Papaefthimiou et al., 2013). 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 observed to also 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.

The insect circulatory system is known 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. One of the primary invertebrate antiviral defense mechanisms 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), an idea that is supported by recent work demonstrating a role for ion channel regulation of viral infections in bees (O'Neal et al., 2017a).

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 also important to note, given that the findings reveal 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. 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.

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CHAPTER 6

SUMMARY

6.1 OVERVIEW

The economic and agricultural importance of honey bees as pollinators, as well as their wide appeal as producers of honey, have helped to facilitate the growing public awareness of the many factors that negatively influence pollinator health. Managed colony decline and unacceptably high annual colony loss in the United States and Europe have fostered a greater appreciation for the delicate balance that exists between the immunocompetence of a honey bee colony, the viruses and other pathogens that infect that colony, and the parasites that infest the colony. Perhaps the most significant of these parasites is the ectoparasitic mite *Varroa destructor*, which both weakens the bee immune response and facilitates the spread of viral pathogens. In order to develop more effective strategies to mitigate managed bee colony loss, we must work to improve our understanding of this complex interaction and how environmental stressors disrupt this balance. The overall goal of the research summarized here was to expand the body of knowledge related to bee cardiac physiology and to improve our understanding of the mechanisms by which bees regulate or tolerate viral infections. This work largely focused on the investigation of the role that ATP-sensitive inwardly rectifying potassium (K_{ATP}) channels play both in regulating cardiac function and in influencing the outcome of viral infections in bees. This work has also described novel methods and model systems that can be used to facilitate future research related to the investigation of bee health and immunity. Finally, this work has identified important questions that remain unanswered about the relationship between cardiac physiology and the ability of an insect to resist or tolerate viral pathogens.

6.2 CHAPTER 2 REVIEW

A wide variety of techniques have been employed to answer questions related to honey bee cardiac physiology. The primary goal of this work was to describe a simple, inexpensive method for assessing the effects of potential cardiomodulators on bee heart rate. This consisted of a detailed protocol for the dissection of the dorsal abdominal wall of a bee in order to visualize its dorsal vessel, allowing the investigator to manipulate heart rate through the application of cardiomodulatory compounds and then observe and measure changes in heart rate. The greatest limitation of this protocol is that the dorsal vessel is not being observed in a living, intact insect, so it is likely that some potentially cardiomodulatory compounds will not show an effect on heart rate if they function at the level of the central nervous system, necessitating an *in vivo* approach. The greatest strength of this protocol is its simplicity, as it costs little, requires minimal equipment, can be undertaken with little or no specialized training, and can be conducted quickly once mastered. As such, it can be a valuable tool for a wide range of applications, and can also serve as a useful teaching tool, especially in resource-limited classrooms or labs.

6.3 CHAPTER 3 REVIEW

K_{ATP} channels play a number of important roles in both mammals and invertebrates due to their ability to couple cellular metabolism to the membrane potential of the cell. Unfortunately, most of what is known about these ion channels is the result of work performed in mammalian systems, with invertebrate studies being limited to only a few species and physiological systems. The primary goal of this work was to investigate the role that K_{ATP} channels play in regulating honey bee cardiac function by observing the effects that K_{ATP} channel modulators have on the

heart rate of the insect. Application of the K_{ATP} channel antagonist tolbutamide and the K_{ATP} channel blockers barium and magnesium decreased bee heart rate in a concentration-dependent manner, relative to vehicle-treated controls. The application of a low concentration of the K_{ATP} channel agonist pinacidil increased heart rate, while higher concentrations decreased heart rate, resulting in a biphasic effect overall. An experiment involving pretreatment with barium showed that barium magnified the effects of tolbutamide treatment and eliminated the effects of pinacidil treatment at select concentrations. This work confirms a role for K_{ATP} channels in the regulation of bee dorsal vessel contractions and provides important insight into the physiology governing the regulation of bee cardiac function.

6.4 CHAPTER 4 REVIEW

Viruses are an important factor in the decline of managed honey bee colonies, but bee-specific viruses have proven a challenge to study and interactions between viruses and the bee antiviral immune response remain poorly understood. The primary goal of this work was to investigate the role of K_{ATP} channels in the regulation of viral infections in bees by testing how K_{ATP} channel modulators effect the outcome of a viral infection. Due to the lack of infectious clones for bee-specific viruses, this work demonstrated the use of the entomopathogenic flock house virus (FHV) as a model system for the study of virus infections in bees. Treatment with the K_{ATP} channel agonist pinacidil was then shown to increase the survival of bees and decrease viral replication following infection with FHV, whereas treatment with the K_{ATP} channel antagonist tolbutamide was shown to decrease survival and increase viral replication. Based on these findings and what is known about the role of the evolutionarily conserved K_{ATP} channel in other systems, an important role was proposed for this ion channel in connecting the antiviral immune response of bees to changes in the cellular environment induced by environmental stressors.

6.5 CHAPTER 5 REVIEW

When considering the multitude of factors that negatively impact managed honey bee colony health, it is difficult to assess the impact of viral pathogens without taking into account the interaction between viruses and infestations of the ectoparasitic mite *Varroa destructor*. At this time, the most effective strategy available for mitigating the detrimental effects of viral infections at the colony level is the chemical control of mite populations. The primary goal of this work was to investigate the physiological and immunological effects of the formamidine acaricide amitraz, commonly used as an in-hive *Varroa* treatment, as well as its primary metabolite in bees. Using FHV as a model virus system, as described in Chapter 4, this work found that exposure to a formamidine acaricide may have a negative impact on the ability of bees to tolerate viral infection. Furthermore, using the protocol described in Chapter 2, it was demonstrated that amitraz and its metabolite significantly alter 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.

6.6 CONCLUSIONS

This series of experiments grew from a single seed, which was the idea that K_{ATP} channels may play a role in the regulation of viral infections in insects. Much of the broader understanding of K_{ATP} channels is derived from work conducted in mammalian systems, in part from the exploration of mammalian physiology, but also because K_{ATP} channels serve as pharmacological targets in the treatment of human disease. As is frequently the case, the model insect species *Drosophila melanogaster* has served as the bridge through which this information was ported

into the body of knowledge related to insect physiology. The study of insect K_{ATP} channels has been expanded to include medically important species, namely the disease vector mosquitoes *Aedes aegypti* and *Anopheles gambiae*, as the ion channel came under consideration as a potential target for novel chemical controls, but it has not expanded much farther. This research was designed to explore the role of this particular ion channels in an agriculturally important beneficial and model social insect, the honey bee. The work presented here describes an evolutionarily-conserved role for K_{ATP} channels in bee cardiac physiology, as well as another conserved, but poorly-understood, role in the regulation of viral infections in bees. This work was accomplished by the design, testing, and implementation of novel methodology and model systems to study cardiac function and the dynamics of an acute viral infection. Using these new tools, this work was expanded to begin asking similar questions about the role of a biogenic amine receptor that also has a known role in insect cardiac function, the octopamine receptor. This relates on a more practical level to managed beekeeping as this receptor is the target of a class of commonly-used apicultural acaricides known as the formamidines.

Overall, the results presented in this series of experiments raises some immediate, practical concerns related to modern beekeeping practices, as well as concerns about the development of future chemical control agents. This work suggests that there is likely a trade-off to be considered when the decision is being made to treat a mite-infested bee hive with amitraz, and supports a policy of reducing chemical interventions in managed bee colonies. Although amitraz itself appears to have significant effects on bee cardiac physiology and a negative synergistic interaction with viral pathogens, it is perhaps more important to note that the metabolite DPMF acts in the same fashion, as this metabolite remains present in the hive significantly longer than amitraz. With regards to the development of future arthropod chemical interventions that target

insect K_{ATP} channels, it is important to note that such compounds may well have unintended, harmful effects on bees, and potentially other pollinator species. Even if K_{ATP} channel modulators do not prove to be immediately toxic to beneficial insects, there is certainly room for concern that interactions with the innate immune response would result in sublethal effects that could impact the overall health of the colony in the long term.

Finally, this work raises a number of questions about the broader understanding of the relationship between insect cardiac physiology and the ability of the insect to resist or tolerate viral pathogens. The results presented here and elsewhere describe a relationship between K_{ATP} channel modulation and the outcome of a viral infection, though the actual mechanics of this relationship remain unknown. Is the modulation of the insect's heart rate via K_{ATP} channels, or the blocking of the insect's ability to modulate heart rate in response to changes in the cellular environment, responsible in whole or in part for this observation, or is there some other connection between K_{ATP} channel regulation and the innate immune response? Intriguingly, this work also describes a similar relationship between cardiomodulatory compounds that target octopamine receptors and the outcome of a viral infection. Is there a shared relationship between these independent observations, or is this merely coincidence? It is possible that interference with cardiac function, whether by blocking K_{ATP} channels or octopamine receptors, results in a loss of homeostasis that reduces or limits the ability of the insect to tolerate infection. It is also possible that these compounds act through some unknown mechanism, or mechanisms, to regulate the innate immune response of the insect. 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 bee antiviral immunity, disease tolerance, and their relationship to factors that negatively impact pollinator health.