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# The Role of ATP-Sensitive Inward Rectifier Potassium Channels In The Regulation of Reactive Oxygen Species In The Western Honey Bee, *Apis Mellifera* L.

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THE ROLE OF ATP-SENSITIVE INWARD RECTIFIER POTASSIUM  
CHANNELS IN THE REGULATION OF REACTIVE OXYGEN SPECIES IN  
THE WESTERN HONEY BEE, *APIS MELLIFERA* L.

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
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in

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by  
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## Table of Contents

Acknowledgements .....	ii
Abstract .....	iv
Chapter 1. Literature Review .....	1
1.1. Biology of the Western Honey Bee .....	1
1.2. Human Interactions with Honey Bees .....	3
1.3. Declines in Managed Honey Bee Populations, and Colony Loss.....	4
1.4. Threats to Honey Bee Health .....	5
1.5. Reactive Oxygen Species .....	11
1.6. Inward rectifier potassium channels (KIR).....	15
1.7. ATP-sensitive K <sub>IR</sub> (K <sub>ATP</sub> ) channels.....	19
1.8. K <sub>ATP</sub> Channels: Function in Regulation of ROS/Redox .....	22
1.9. Viruses and Antiviral Defense Mechanisms of Honey Bees .....	23
1.10. K <sub>ATP</sub> Channels as a Target for Enhancing Honey Bee Health .....	24
1.11. K <sub>ATP</sub> Agonists Increase Survivorship after Infection with Model Virus .....	25
1.12. Overarching Hypothesis to be Tested .....	27
1.13. Objectives of the Study .....	27
Chapter 2. Test The Hypothesis That Pharmacological Modulators of K <sub>ATP</sub> Channels Can Reduce Oxidative Stress in the Honey Bee, <i>Apis Mellifera</i> . .....	28
2.1. Introduction.....	28
2.2. Materials and Methods.....	31
2.3. Results.....	37
2.4. Discussion .....	46
2.5. Conclusion .....	49
References .....	51
Vita.....	68

## Abstract

Colonies of managed honey bees are annually being lost at an unsustainable rate, partly due to reduced immunocompetence that leads to acute viral outbreaks and mortality. To aid in restoring honey bee health despite the myriad of environmental stressors, this thesis focuses on identifying novel physiological pathways that can mitigate virus-mediated mortality through increased immune function. Previous work has demonstrated that a family of potassium ion channels, termed  $K_{ATP}$  channels, mediate the survival of honey bees during infection from a model virus, suggesting  $K_{ATP}$  channels may drive antiviral immunity. Interestingly, these channels have been linked to the regulation of reactive oxygen species (ROS), which are known to function as an immune system stimulator during virus infection. Thus, the overarching goal of this thesis study was to validate the linkage between  $K_{ATP}$  channels, ROS, and bee survivorship. Our findings in this thesis provide evidence that pinacidil, a  $K_{ATP}$  channel activator, is capable of dramatically reducing antioxidant levels in bees during chemically-induced ROS, suggesting  $K_{ATP}$  channels play a part in regulating levels of ROS. Further, mortality was significantly reduced in bees from colonies that had heavy mite infestations, which supports the notion that ROS is an intermediate molecule for immune health. While additional investigation is required to fully characterize the relationship between  $K_{ATP}$  channels, ROS, and antiviral immunity, this study has begun to fill significant gaps in knowledge pertaining to mechanisms honey bees use to regulate their antiviral immune response.

## Chapter 1. Literature Review

### 1.1. Biology of the Western Honey Bee

The Western honey bee, *Apis mellifera*, is a hymenopteran insect belonging to the superfamily Apoidea. This superfamily, of which all bees are members, evolved from sphecoid wasps <sup>1</sup> after the evolution of flowering angiosperm plants 100-125 million years ago <sup>2-4</sup>; the diversity of the superfamily then radiated as angiosperms diversified <sup>2</sup>. Angiosperms are believed to have evolved to use shape, color of flowers, and food, in the form of excess pollen and nectar, to attract bees to their reproductive structures. During visitation to the flower, bees become coated in pollen, containing the plants genes and a portion of the pollen would inadvertently be transferred as they visited the flowering structures of other individuals of the same species. Thus, bees facilitated fertilization of plants.

The name “honey bee” may be applied any of the members of the genus *Apis*, which is considered to consist between six and eleven species <sup>5</sup>. Two species of honey bee are commonly kept in artificially constructed hives: the Eastern honey bee, *Apis cerana*, and the Western honey bee, *Apis mellifera* <sup>6</sup>. While the keeping of Eastern honey bees is common throughout southeastern Asia, the management of the Western honey bee, *Apis mellifera*, is far more widespread <sup>7</sup>. The Western honey bee has now displaced the Eastern bee in many places where it was formerly kept, as it is a superior honey producer <sup>6</sup>, and has now been artificially introduced around the world <sup>5</sup>.

The Western honey bee is a eusocial insect, living in colonies containing an average of 20,000-60,000 individuals <sup>5</sup>. Within these colonies, there are three castes of bees; a single reproductive female (the queen), up to 2000 males (drones <sup>8</sup>), and tens of thousands of non-

reproductive females (workers). The queen lays eggs in a matrix of wax cells, called combs. The queen, herself, decides whether each egg laid will be fertilized, based on the dimensions of the cell <sup>9</sup>. Honey bees are a haplodiploid organism, meaning that the number of sets of chromosomes an offspring receives determines its sex. An individual receiving only one copy of genes (an unfertilized egg, haploid) will develop into a male (drone), while an individual receiving two copies of genes (a fertilized egg, diploid) will cause them to develop into a female (worker or queen) <sup>5,10</sup>. A female larva can mature into a worker or queen based on the diet received during the larval stage. Larvae destined to become queens are fed a diet of pure royal jelly until pupation. Royal jelly is a substance secreted from the hypopharyngeal gland, in the head of young worker bees <sup>5</sup>. Queen-destined larvae are fed this substance *ad libitum* throughout their time as larvae. Otherwise, larvae are fed only a rationed amount of this secretion, mixed with pollen and honey. On the third day of age, the diet-induced fate of the larvae becomes permanent, and cannot be reversed <sup>11</sup>. After spending 5-6 days in the larval stage (depending on caste), worker bees will cap the cell of the late stage larvae. After capping, the bee will pupate, then emerge from the cell as an adult 16-24 days (again, depending on caste) after hatching from the egg <sup>5,12</sup>.

As adults, worker bees progress through a series of behavioral duties as they age. This age-based division of labor among workers is known as temporal polyethism <sup>13</sup>, and is common among social insects <sup>14,15</sup>. Temporal polyethism in honey bees is regulated by titers of juvenile hormone <sup>15</sup>, among other factors <sup>12,16</sup>. Immediately following eclosion, the adult worker will feed on pollen, stored in the surrounding comb. This initial pollen feeding triggers the development of the hypopharyngeal glands, which will later be used to produce food for successive generations of larvae. While these glands are in development, the young worker will begin cleaning recently

vacated brood cells in order to prepare the cells for successive generations of brood. After the hypopharyngeal glands have developed, the worker assumes the role of “nurse” bee, feeding developing larvae the secretions of this gland. After completing her service as nurse, the worker is tasked with the ripening and processing of nectar into honey. This task is followed by storing and processing pollen, building and repairing wax combs, guarding the hive, and finally, foraging for nectar, pollen, water, and propolis (sap). The life span of the adult worker bee is variable; in warmer months, when foraging is prevalent, workers may live 35-42 days. During the winter months, however, when foraging activity is reduced, workers may live up to six months <sup>17</sup>.

## **1.2. Human Interactions with Honey Bees**

Bees and the honey they produce have been important parts of human history for many thousands of years. The earliest known record of human interaction with bees comes from cave paintings in Altamira, in the north of Spain. Here, ancient peoples are depicted climbing cliffs to rob honey from wild colonies of bees dwelling therein. These paintings are believed to have been created around 6000 B.C.E., but may be up to 2000 years older <sup>18</sup>. After some period of “honey hunting”, humans began managing bees in artificially constructed hives. The oldest known instance comes from hieroglyphs at Temple of the Sun of Ne-User-Re in Abusir, Egypt. These hieroglyphs, dated to around 2500 B.C.E., depict ancient beekeepers managing organized hives and extracting honey <sup>6</sup>.

Whereas humankind has interacted with bees in order to obtain honey and other hive products throughout recorded history, these commodities make up only a small portion of the value of honey bees today. The majority of the contemporary value of bees lies in their role as managed crop pollinators. Every year, 2.5 million colonies of honey bees are rented for the



pollination of almost 90 different crops <sup>5</sup>. Crop pollination currently has an annual estimated value of between 15-20 billion dollars in the United States <sup>19</sup>, and 213 billion dollars, globally <sup>20</sup>. While 35% of global food crops *benefit* from animal pollination <sup>21,22</sup>, it is critical for the production of crops that compose 10% of human diet. Among crops where animal pollination is required, honey bees provide the majority of this service <sup>21-24</sup>.

### **1.3. Declines in Managed Honey Bee Populations, and Colony Loss**

Mass losses of honey bee colonies have been well documented in many periods throughout recorded history. In 1942, the United States Department of Agriculture's National Agricultural Statistics Service (NASS) began an annual survey of the number of managed colonies within the country <sup>25</sup>. The number reached an all-time high of 5.9 million honey producing colonies in 1947, but has since diminished to 2.3 million in 2008 <sup>25</sup>. It has been hypothesized that the decline in colonies between 1947 and the early 1970s may be partially attributed to the introduction for synthetic insecticides, as well as other factors <sup>26</sup>. The steep decline in the late 1980s, however, is likely attributable to the introduction of the ectoparasitic mite, *Varroa destructor*.

Overwintering, a period of relative inactivity for honey bees during cooler months, is a time often associated with colony loss. During this time, colonies slow or cease brood production, and the population of adult bees within the colony dwindles. Losses during this period have historically ranged from 5-10% <sup>27</sup>. In recent decades, the number of colonies lost during overwintering has increased dramatically. In 2018, overwintering colony losses were estimated to have risen to 30.7%, a level that is considered economically unacceptable <sup>27</sup>.

Closely associated with recent colony losses is the phenomenon known as *Colony Collapse Disorder*, or CCD <sup>25,28</sup>. First reported in 2006, the condition was originally defined by a number of concise symptoms, differentiating it from other diseases <sup>25</sup>. First, no significant

number of dead bees would be found within the colony or in apiary, though the loss of adult bees from the afflicted colonies would have occurred rapidly. Second, while the affected colony would have a fatally small population of workers, it would often still possess a viable queen, brood, and stored food indicating that the colony had not failed due to starvation or queen failure<sup>29-31</sup>. Lastly, the affected colonies would not be subject to “robbing”, a kleptoparasitic behavior in which bees from other, stronger colonies steal stored food from weakened or dead colonies<sup>32</sup>. It is believed that no single factor is responsible for CCD, or for the bulk of colony losses over the past decade; rather, a number of stress factors are thought to collectively reduce colony health, resulting in pathogen infection and subsequent colony loss<sup>25,32</sup>.

#### **1.4. Threats to Honey Bee Health**

Honey bees are currently subjected to a number of biological and physiological stressors, including, but not limited to pathogenic and parasitic infection, hive pests, exposure to pesticides, and lack of varied forage. These factors may affect adult bees, larvae, or both.

##### **1.4.1. Parasitic Infestations**

The ectoparasitic mite *Varroa destructor* is considered to be the single most severe threat to honey bee health<sup>33</sup>. The mite, introduced to the United States in 1987, inflicts damage upon honey bees by feeding on the fat body<sup>34-36</sup> and hemolymph<sup>33</sup> of adult and immature bees, simultaneously transmitting a multitude of viruses<sup>37,38</sup>. While feeding can result in physical deformities and reduced protein, lipids, and overall weight<sup>37</sup>, the viruses transmitted during feeding are far more damaging to honey bee health<sup>39</sup>. If untreated, *Varroa* infestation will likely lead to the demise of the afflicted colony<sup>40</sup>, an outcome which is uncommon among host-parasite relationships<sup>41</sup>. *Varroa* infestation is currently controlled by a number of chemical means including pyrethroid, organophosphate, and formamidine insecticides, as well as organic

acids, and essential oils <sup>40</sup>. It is notable that there is evidence that many previous and current chemical treatments of *Varroa* control may have intrinsically deleterious effects on colony health <sup>25</sup>.

In addition to chemical control, many notable bee breeding programs have demonstrated the ability to select for traits in bee populations that limit *Varroa* reproduction <sup>42</sup>. These include hygienic behavior <sup>43,44</sup>, grooming <sup>45</sup>, and the non-reproduction of mites in brood cells <sup>46,47</sup>. Notable examples of breeding programs include “pol-line” of bees, bred at the USDS-ARS facility in Baton Rouge, Louisiana. This effort aims to incorporate traits of *Varroa* sensitive hygienic behavior into commercially viable stock <sup>48</sup>. The Minnesota Hygienic line of bees selected colonies based on their ability to detect and remove brood that had been artificially killed by freezing, a proxy for detecting disease or mite infestation <sup>49</sup>. Efforts at Purdue University in Indiana produced ‘mite-biting’ or ‘ankle-biting’ strains of bees, which selected for grooming behavior where bees actively bite or chew at phoretic *Varroa* mite <sup>50</sup>.

The tracheal mite, *Acarapis woodii*, and closely related mites *Acarapis dorsalis* and *Acarapis externis*, also parasitize honey bees <sup>51</sup>. While the latter two species are considered harmless, *A. woodii* once represented a substantial threat to bee health, though they are of less concern today <sup>5</sup>. Some consider tracheal mites to have been the causative agent behind a series of mysterious colony losses, termed “Isle of Wight Disease”, during the early 20<sup>th</sup> century <sup>25,52</sup>, though this position is debated <sup>25,53</sup>. Tracheal mites, unlike *Varroa*, feed from inside the honey bee, infesting the passages of the tracheae. Heavy infestations have been correlated with reduced worker health as well as decreased honey production. Tracheal mite infestation may only be effectively diagnosed by microscopic examination of worker bee trachea <sup>54</sup>; as these mites are no longer considered a major threat to bee health, this is now rarely performed. Many treatments

that are effective for the control of the more damaging *Varroa* mite are thought to control tracheal mites, especially those utilizing formic acid and essential oils <sup>5</sup>.

#### 1.4.2. Pests of the Honey Bee Colony

A pest of increasing significance is the small hive beetle, *Aethina tumida*. This damaging beetle of the family Nitidulidae is native to Sub-Saharan Africa. The beetle was relatively unknown to the beekeeping community prior to the first discovery in the United States in 1998, though it has since established itself as a damaging pest <sup>55</sup>. Adult beetles enter honey bee colonies, where they lay eggs in cracks and crevices. After hatching, small hive beetle larvae mature rapidly and eat wax, pollen, brood, and stored honey, before exiting the hive to pupate in the soil <sup>5</sup>. While feeding on the colony food stores, the beetles inoculate stored honey with a yeast, *Kodamaea ohmeri* <sup>56</sup>, that causes honey to quickly ferment to ease digestion by the beetle larvae. If infestation of beetles is severe, the bee colony may abscond <sup>5</sup>. The body shape and sclerotization of the small hive beetle make them difficult for honey bees to control. The only chemical method of in-hive control available to beekeepers is the organophosphate coumaphos, though its tendency to persist in wax combs and deleterious effects on bee reproductive health have led to less use. Other mechanisms of control include entomopathogenic nematodes, in-hive traps, and movement of colonies to areas receiving increased sunlight. It may also be useful to reduce the amount of space inside of colonies unoccupied by bees, such as excessive supers (hive boxes containing combs for the production of honey) <sup>57</sup>.

#### 1.4.3. Bacterial Pathogens

While many species of bacteria are present within a colony of honey bees, two are known to cause significant damage. The first, and most serious, is the spore forming bacterium *Paenibacillus larvae* <sup>58</sup>. Infection with *P. larvae* causes the disease known as American

foulbrood, or AFB <sup>59</sup>. *P. larvae* exists in both active and vegetative states, and forms spores by which the bacterium spreads <sup>54</sup>. Honey bee larvae are infected when they consume these spores, which contaminate their food. The bacterium then moves from the bees gut to their hemolymph, where it reproduces. Larvae are most susceptible to infection during the first two days after hatching eggs. Larvae infected with bacteria will usually die after capping, but before pupation <sup>60</sup>. Concurrent with the death of the honey bee larvae is the formation of further *P. larvae* spores <sup>59,61</sup>. These spores have been documented to persist for up to 35 years <sup>62</sup>, but may persist much longer, even in the absence of a living colony of bees <sup>54,61</sup>. Low-level infections within a colony may persist for years <sup>63</sup>, especially if the colony is of a hygienic stock <sup>59,60</sup> though they will eventually prove fatal <sup>5</sup>. If detected, low-level infection may be treated with antibiotics, such as oxytetracycline, but many state apiary inspection programs recommend the destruction of the colony and any associated equipment. Some states have programs to sterilize contaminated hive components, including combs. These utilize a variety of technologies, including ethylene oxide fumigation, and gamma irradiation <sup>5</sup>.

Infection with another bacterium, *Mellisococcus plutonius*, causes a similar disease, known as European Foulbrood (EFB). This is considered less serious, but may result in the death of young larvae <sup>5</sup>. Unlike *P. larvae*, the bacteria *M. plutonius* does not form spores. Honey bee larvae infected with European Foulbrood often become twisted within their cells, and become corn-yellow in color. Colonies that are weak or stressed may be more susceptible to EFB, while stronger colonies remain resilient <sup>5,54</sup>. The disease appears most frequently in the spring. Infection with EFB may be treated with antibiotics, but will frequently clear with feeding of the colony, replacement of the queen, or the onset of a good nectar flow <sup>54</sup>.

#### 1.4.4. Fungal Pathogens

Several species of fungi may impact the health of honey bee colonies. *Ascosphaera apis*, a fungus in the family Ascosphaeraceae <sup>64</sup>, is the causative agent of the disease known as chalkbrood <sup>5,54</sup>. While this disease is commonly known to affect honey bees, it has also been shown to infect leafcutting bees (Apoidea: Megachilidae) <sup>54</sup> and, more recently, bumble bees (Apidae: Bombini) <sup>65</sup>. This disease causes the death of larvae, which become overgrown with fungal mycelium, giving them the appearance of “mummies”. The mass of overgrown mycelium takes on the cylindrical shape of the inside of the cell in which the larvae resides. Adult bees within the colony may discard these fungal masses on the floor or in front of the colony, where they resemble pieces of chalk <sup>5,54</sup>. Chalkbrood infestations are rarely fatal, even in severe cases, but may render the colony non-reproductive. The infection may be cleared by feeding, requeening, and increasing ventilation within the colony <sup>5</sup>.

Similar to chalkbrood, stonebrood is caused by infection with the fungal pathogen *Aspergillus flavus*. While the occurrence of this disease is rare in comparison with chalkbrood, it similarly forms “mummies” from infected larvae, though they may be black in color. Stonebrood mummies become hardened, and may be more difficult to crush with the fingers upon examination <sup>5</sup>.

While chalkbrood and stonebrood affect honey bee larvae, the microsporidian fungi *Nosema apis* <sup>66</sup> and *Nosema ceranae* affect adults <sup>54,67</sup>. Members of the genus *Nosema* are commonly pathogenic among insects, infecting locusts, grasshoppers, and moths as well as bees.<sup>54</sup> The two aforementioned species, *N. apis* and *N. ceranae*, are known to infect Western honey bees, though *Nosema cerana* was initially known only to infect the Eastern honey bee, *Apis cerana* <sup>68</sup>. It has since been shown that *N. cerana* has spread to the Western honey bee <sup>68,69</sup>,

with the possibility of displacing *N. apis* in years to come <sup>70</sup>. *Nosema spp.* spread through the ingestion pathogenic spores, which then infect the epithelium of the bee's midgut <sup>71</sup>. *Nosema* infection may lead to reduced longevity in workers <sup>72</sup>, as well as queen supercedure and reduced honey yields. Infected colonies may also refuse to take supplemental feed syrup, and may or may not show fecal streaking (dysentery) on the combs and woodenware of the colony <sup>5</sup>. Infections were able to be treated with the antibiotic *Fumigilin-B* (previously called *Fumidil-B*) though this compound is no longer available. Some data also suggest that use of this compounds may worsen the severity of the infection if the pathogen responsible is *N. cerana* <sup>5</sup>.

#### 1.4.5. Pesticide Exposure

Contemporary farming practices often rely on synthetic chemicals to control weeds (herbicides), fungi (fungicides), and insecticides (insecticides), creating opportunities for the accidental exposure of individual foraging honey bees, as well as entire colonies. If exposed to a sufficient concentration of these compounds, bees may be killed immediately (acute toxicity), over time with repeated exposure (chronic toxicity), or may be otherwise affected physiologically without directly related mortality (sub-lethal effects). Historically, pesticide regulations have focused on protecting bees from exposures to a single compound that would lead to direct poisoning <sup>25</sup>. Sub-lethal effects, in contrast, are more difficult to study, and include changes in learning ability, communication, and behavior <sup>25</sup>. Recent studies have shown that so-called “tank-mixes” of insecticides and fungicides may be more toxic to bee than either chemical alone <sup>67</sup>. The interactions between different chemistries also are challenging to study, as many different combinations of compounds may be used at varying rates depending on crop needs.

An important route of pesticide exposure for honey bees that is often neglected is the chemical of *Varroa* mites. These compounds, called *varroacides*, often leave behind persistent residues that may accumulate within the colony. A common matrix in which these compounds are often found is the wax combs, where the bees raise brood, and store food. Recent studies detected residues of both varroacides coumaphos and fluvalinate together in wax samples from 100% of colonies tested <sup>25,73,74</sup>. Coumaphos has been shown to have deleterious effects on sperm viability in drones <sup>75</sup>, and both coumaphos and fluvalinate have been implicated in the development and overall health of queens <sup>76-78</sup>.

## **1.5. Reactive Oxygen Species**

### **1.5.1. Overview and Origins of ROS**

Reactive oxygen species (ROS) are highly reactive, oxygen containing biological molecules, derived from molecular oxygen ( $O_2$ ) <sup>79</sup>. ROS may be classified as *radical* (having one or more unpaired electrons, such as  $O_2^-$ ) or *non-radical* (all electrons are paired, as in  $H_2O_2$ ) <sup>80</sup>. Due to the unique ability of oxygen to readily accept free electrons and its relative abundance within the cells of aerobic organisms <sup>81,82</sup>, these molecules are often the byproducts of aerobic metabolism <sup>83</sup>. ROS may be generated as a result of a number of normal processes within the cell (endogenous ROS), or as a result of extracellular factors (exogenous ROS). Within the cell, ROS are produced by NADPH oxidases in the cell membrane, as well as by the mitochondria, the endoplasmic reticulum, peroxisomes <sup>84-86</sup>, and during the catalytic action of cellular enzymes (such as xanthine oxidase <sup>87,88</sup>). While excessive quantities of ROS lead to the damage of lipids, proteins and DNA <sup>89</sup>, biologically ‘normal’ quantities of ROS are crucial components of many biological processes because they provide a messenger for signaling pathways and defense against pathogens, such as bacteria and fungi <sup>90</sup>.



### 1.5.2. Roles of ROS in Normal Cellular Function

The generation of small quantities of reactive oxygen species play important roles in the biological processes of a healthy, functional organism; this concept is referred to as *Redox biology* <sup>83</sup>. ROS are utilized in number of signaling pathways, especially those related to cell growth. Cellular growth factors, such as platelet-derived-growth-factor, have been shown to increase ROS production via NADPH oxidases that lead to downstream transmission of growth factor signaling <sup>83,91,92</sup>. Cancer cells, which are known to grow rapidly, produce large quantities of ROS as a byproduct of their higher rate of metabolism <sup>93,94</sup>. These ROS subsequently drive increased cellular proliferation, survival and metabolic adaptation <sup>83</sup>.

Reactive oxygen species also play key roles in innate and adaptive immune systems, where they function as second messengers <sup>83,95,96</sup>. During infection and injury, pathogen associated molecular patterns and/or cell damage associated molecular patterns activate the innate immune system by binding to toll-like receptors. Toll-like receptors, in turn, upregulate the production of ROS by NADPH oxidases and the mitochondria <sup>97,98</sup>, which activate inflammatory cytokines, causing an inflammatory immune response.

### 1.5.3. Excessive Quantities of ROS Cause Oxidative Stress, Cellular Death

When the concentration of ROS within a cell is very high, damage to the cell and subsequent cell death can occur through a condition known as *oxidative stress* <sup>89</sup>. It is important to clearly distinguish the difference between ROS and oxidative stress, which are incorrectly used interchangeably. ROS are the oxygen-containing molecules that may have beneficial or deleterious effects on the cell, depending on concentration. Oxidative stress, however, refers only to the condition resulting from the formation of excessive quantities of ROS, which leads to damage of cellular components.

Proteins represent one potential target for ROS damage during oxidative stress; those having amino acids with unsaturated bonds, or those containing sulfur <sup>82</sup> may be particularly susceptible to ROS damage. Another cellular component affected by oxidative stress is the genetic material of the cell <sup>82</sup>. During exposure to ionizing radiation, for example, the majority of cell mutations and deaths are due to reactions of O<sub>2</sub> metabolites (specifically the hydroxyl radical) and DNA. Cytotoxicity often arises as a result of the reaction between ROS and the sugar-phosphate backbone of the DNA molecule, leading to base-pair modification and chromosomal damage <sup>82</sup>. Membrane lipids are an additional cellular component that may be damaged during oxidative stress. Unsaturated bonds on molecules such as cholesterol readily undergo peroxidation upon reacting with ROS <sup>99</sup>. This leads to the formation of lipid peroxides and lipid-peroxyradicals, which themselves may react with other cellular components in the same way as ROS. The transition from cholesterol embedded into a membrane to the formation of lipid peroxides has been utilized to develop biological assays capable of quantifying cell damage resulting from ROS. It is clear that, when present in excess, reactive oxygen species represent a serious threat to cellular integrity. If the damage inflicted is severe, it may lead to apoptosis, tissue damage, and eventually, impairment or death of the organism <sup>99</sup>.

#### 1.5.4. Oxidative Stress and Honey Bees

Many of the factors that threaten honey bee populations may also cause oxidative stress, such as exposure to pesticides and other xenobiotics <sup>100</sup>. Similarly, the bipyridyl herbicide paraquat is well-known to induce oxidative stress, as the compound undergoes a cyclic oxidation/reduction reaction <sup>99</sup>. The first step involves a one-electron reduction of paraquat by NADPH, in which free radicals are formed. These radicals then donate their electron to O<sub>2</sub>, which yields a superoxide radical. Once NADPH is exhausted, the superoxide radical self-reacts

to produce hydroxyl radicals <sup>101</sup>, which are known to readily react with lipids, including cell membranes <sup>99</sup>. Other herbicides, such as atrazine <sup>100</sup>, glyphosate, and metolachlor, have also been shown to induce oxidative stress in honey bees <sup>102</sup>. Pyrethroid insecticides <sup>103</sup>, as well as organophosphates <sup>104,105</sup>, and organochlorines <sup>106-109</sup> are all known induce oxidative stress in mammals <sup>99</sup> and may be routinely encountered during by honey bees while foraging for food. In addition to xenobiotics, human activities, such as the cross-country transportation of colonies by beekeepers have also been shown to cause oxidative stress in bees <sup>110</sup>. The consequences of oxidative stress on honey bees include physiological changes that reduce both bee longevity and colony health <sup>100,111</sup>.

#### 1.5.5. ROS in balance: A Crucial Component in Aerobic Life

While some level of ROS is crucial for the function of an organism, excessive ROS leads to cellular damage, and, eventually, death. As such, aerobic organisms have evolved systems to keep ROS levels in balance; levels must be sufficiently high so as to perform functions crucial to homeostasis, yet below the threshold at which oxidative damage occurs. While these defenses are present in all cells, they are well illustrated in cancer cells. While producing relatively large quantities of ROS, cancer cells carefully counterbalance these levels by producing reciprocally high concentrations of antioxidants and enzymes, so as to avoid oxidative damage and cell death <sup>112</sup>. The presence of ROS activates the transcription of antioxidant genes <sup>113</sup>, and the antioxidants become sacrificially oxidized by ROS molecules, preventing ROS from reacting with other molecules within the cell, thereby preventing damage to crucial cellular systems. Other defenses include enzymes such as catalase that convert the non-radical ROS hydrogen peroxide into non-reactive byproducts. These, and other similar mechanisms prevent the cancer cell from experiencing oxidative stress <sup>83,114,115</sup>. If the capacity of the cell's antioxidant and

enzymatic defensive systems is exceeded, however, ROS will begin to cause damage to the cancer cell, leading cell death from oxidative stress.

Balancing levels of ROS is also crucial during the immune responses occurring during injury and infection. The appropriate level of ROS is determined by the oxidative state of the cell. For example, a cell experiencing infection with an already low level of ROS is likely to benefit from a slight increase of ROS, as this would lead to an enhanced immune response. If the ROS levels in the infected cell were to increase dramatically, however, they may promote a pathological response, such as autoimmunity and lead to cell death. The value of ROS, then, is hormetic, in that a moderate of quantity of ROS is crucial to the health of the organism, while an excess of ROS leads to cell damage and death.

#### **1.6. Inward rectifier potassium channels (KIR)**

Ion channels represent a crucial component of all life forms, as they allow the passage of charged particles, called ions, to move across membranes, down their respective concentration gradients, into and out of the cell. Potassium channels generally pass potassium from the inside of the cell to the outside of the cell, as is seen in the voltage-gated potassium ( $K_v$ ) channels utilized in the propagation of an action potential in nerve cells <sup>116</sup>. However, a novel type of potassium channel that was originally discovered in human skeletal muscle does not possess a voltage-sensing domain. Electrophysiological studies have shown that these channels alter the current amplitude based on membrane potential. Further analysis showed a greater  $K^+$  flux into the cell, rather than outward, as predicted by the Nerst equation <sup>117,118</sup>. As this behavior was unexpected, these channels were first known as ‘anomalous rectifier’ potassium channels, but were later renamed to “potassium inward rectifier” to reflect their biophysical properties <sup>117</sup>.

By convention, the movement of  $K^+$  ions into a cell generates an inward current, whereas an efflux of  $K^+$  ions generates an outward current. The voltage-dependent decrease in outward current is called “rectification” and is resultant of an intracellular blockade of the  $K_{IR}$  channel pore by divalent cations, such as magnesium and polyamines (e.g. putrescine, spermine and spermidine) that occurs at membrane potentials greater than the Nernst potential for potassium,  $E_K$ . The blockade of the pore that leads to a change in current is termed “inward rectification”, which allows  $K_{IR}$  channels to exhibit larger inward currents at hyperpolarized potentials ( $E_M < E_K$ ) as compared to outward currents <sup>117</sup>. Further, rectification has been shown to be dependent upon the charge of the ‘rectification residue’ (N171,  $K_{IR}$  1.1, human nomenclature) with negative charged amino acids, enabling strong rectification and neutral or positive charged amino acids leading to weak rectification <sup>119</sup>.

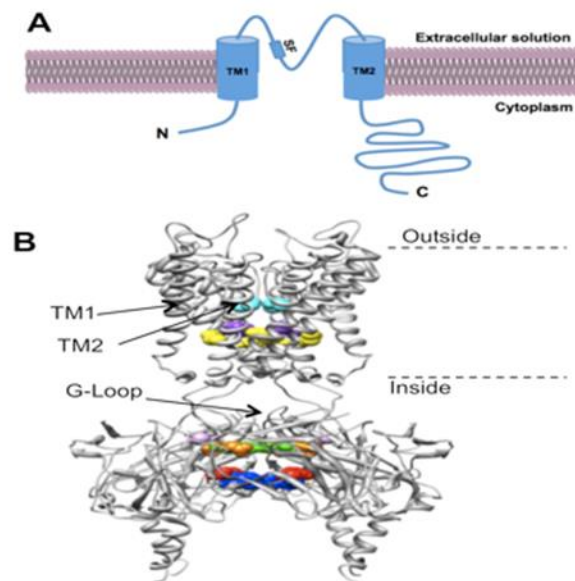
$K_{IR}$  channels are expressed in variety of excitable and non-excitable cells and serve essential roles in the regulation of various physiological processes, ranging from maintaining the resting membrane potential, cardiac and neuronal excitability, epithelial transport, metabolic homeostasis, muscle contraction, and cellular signaling <sup>117,118</sup>. On a molecular level,  $K_{IR}$  channels are structurally simple ion channels that consist of 4 subunits assembled around a central, water-filled pore, through which  $K^+$  ions move down their electrochemical gradient to traverse the plasma membrane. Each subunit consists of a central transmembrane domain, a re-entrant pore-forming loop, and a cytoplasmic domain comprised of amino and carboxyl termini <sup>117</sup> (Figure 1.1A). A molecular homology model of a  $K_{IR}$  channel is shown in Figure 1.1B <sup>119</sup>.

$K_{IR}$  channels are known to be a critical conductance pathway for human tissue function, as evidenced by the fact that genetic mutations of  $K_{IR}$  channels result in numerous diseases, such as Andersen's syndrome, hypertension, and diabetes <sup>120,121</sup>. As such,  $K_{IR}$  channel subtypes have

been the focus of significant drug discovery campaigns; the development of novel pharmaceuticals to mitigate morbidity from diseases resulting from K<sub>IR</sub> channel mutations has been a major focus <sup>119,122</sup>. Further, K<sub>IR</sub> channels have been proposed to represent an important ion channel in arthropods. They have recently been shown to represent a putative insecticide target in mosquitoes <sup>123,124</sup>, a critical ion conductance pathway in *Drosophila* salivary gland function <sup>124</sup>, essential for the proper function of *Aedes aegypti* and *Drosophila* Malpighian tubules <sup>125,126</sup> and innate antiviral immunity <sup>127,128</sup>, and cardiac function in honey bees <sup>129</sup>.

In mammals, K<sub>IR</sub> channels are encoded by the *KCNJx* genes, which are comprised of sixteen members that play essential physiological roles in modulating the functions of most organ systems <sup>117</sup>. The *KCNJx* family is divided into seven groups based on amino acid sequence homology <sup>119,130</sup>. Human K<sub>IR</sub> channels are subdivided into seven subfamilies based on different gene families (K<sub>IR</sub> 1.x-7.x) <sup>118</sup>. These subfamilies are further divided into four functional groups: classical K<sub>IR</sub> channels (K<sub>IR</sub> 2.x), G-Protein gated channels (K<sub>IR</sub> 3.x), ATP- sensitive K<sub>IR</sub> channels (K<sub>IR</sub> 6.x), and potassium transport channels (K<sub>IR</sub> 1.x, 4.x, 5.x, 7.x) <sup>117</sup>. The channels may also be grouped by their regulatory and gating mechanisms; K<sub>IR</sub>1, 2, 4, 5, and 7 are considered “constitutively active”, while K<sub>IR</sub> 3.x channels are G-protein coupled receptors (GPCR’s), and K<sub>IR</sub> 6.x channels are regulated by the presence or absence of nucleotides, such as adenosine triphosphate (ATP). For arthropods, significantly less is understood regarding gene families, tissue expression patterns, and gating properties with nearly all of the information coming from mosquitoes and *Drosophila*. *Aedes aegypti* has been shown to possess 5 genes encoding K<sub>IR</sub> channels (K<sub>IR</sub>1, K<sub>IR</sub>2A, K<sub>IR</sub>2B, K<sub>IR</sub>2B’, and K<sub>IR</sub>3) <sup>131</sup> with splice variants existing for the genes K<sub>IR</sub>2A and K<sub>IR</sub>2B <sup>132</sup>. *Drosophila melanogaster* is known to possess 3 K<sub>IR</sub> channel encoding genes, termed *ir1*, *irk2*, and *irk3* that encodes K<sub>IR</sub>1, K<sub>IR</sub>2, and K<sub>IR</sub>3, respectively <sup>133</sup>. K<sub>IR</sub>

channels are ubiquitously expressed in *Drosophila* tissue systems and their expression patterns have recently been reviewed <sup>134</sup>. In *Drosophila*, the tissue expression patterns of K<sub>IR</sub> channels are highly variable and are described in Luan and Li (2012) <sup>134</sup>, but it is predicted that the gating of the K<sub>IR</sub> channel is dependent upon the tissue expression versus the encoding gene as it is in mammals. Unfortunately, the genes encoding K<sub>IR</sub> channels or tissue expression of K<sub>IR</sub> channels in honey bees are not currently known. However, previous work has shown significant influence of highly specific K<sub>IR</sub> channel modulators to honey bee biology and physiology, which indicates they are expressed and functional <sup>127,129</sup>.



**Figure 1.2. Molecular structure of K<sub>IR</sub> channels.** (A). Schematic of a K<sub>IR</sub> channel subunit indicating the cytoplasmic N- and C-terminus domains, two transmembrane-spanning domains (TM1 and TM2) and the pore-forming loop with selectivity filter (SF). (B). Homology model of the K<sub>IR</sub> 1.1 channel. The colored regions are the rectification residues of mammalian K<sub>IR</sub> channel families and small-molecule binding sites. Published in Swale, et al., *Curr. Opin. in Pharmacology*, 2014.

### 1.7. ATP-sensitive K<sub>IR</sub> (K<sub>ATP</sub>) channels

ATP sensitive K<sub>IR</sub> channels (K<sub>ATP</sub>) are one of the subgroups of K<sub>IR</sub> channels that are not yet fully characterized in arthropods, but have been thoroughly investigated and reviewed in mammals<sup>135-138</sup>. In mammals, the K<sub>IR</sub> 6.x family form heterooctamers with the high-affinity sulfonylurea receptors (SUR)<sup>139</sup> at a stoichiometric ratio of 4:4 (K<sub>IR</sub>:SUR)<sup>117</sup>. In this heteromeric channel complex, K<sub>IR</sub>6.x is responsible for pore formation and the obligate heteromer, SUR, is required for activation and regulation of the K<sup>+</sup> conductance<sup>139</sup>.

The presence of ATP inhibits the opening of the channel, while the presence of magnesium bound nucleotides and ADP has been shown to activate the K<sub>ATP</sub> channels<sup>140,141</sup>. Due to this activation property, K<sub>ATP</sub> channels sit at the crossroads of cell metabolism and membrane excitability<sup>137,142</sup>. Thus, K<sub>ATP</sub> channels are open during states of low metabolic activity, resulting in hyperpolarization of the membrane, which has cytoprotective effects<sup>143,144</sup>. Conversely, during times of high metabolism, K<sub>ATP</sub> channel activity decreases and the resulting membrane depolarization triggers cellular responses specific to that cell type.

K<sub>ATP</sub> channels are well-exploited target sites in mammalian pharmacology and as such, the pharmacological library of these targets is well developed with activators and inhibitors (Table 1.1). K<sub>ATP</sub> channel inhibitors, including chlorpropamide, tolbutamide, acetohexamide, (first generation sulfonylurea drugs), glibenclamide and glicazide, (second generation) and glimepiride (third generation) are important human pharmaceuticals as they are used in the treatment of type two diabetes.

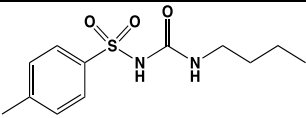
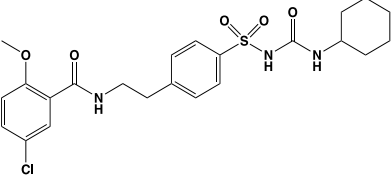
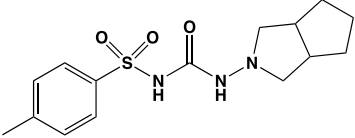
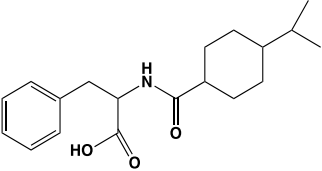
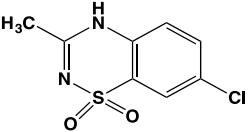
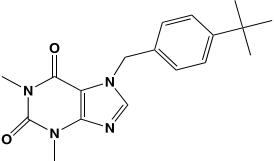
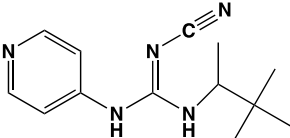
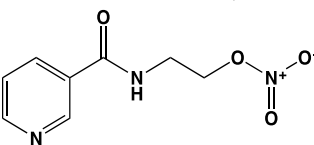
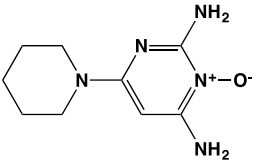
Pharmacological activation, or opening, of K<sub>ATP</sub> channels hyperpolarize the cell membrane potential that dampens cell excitability by reducing calcium entry into the cells. Channel activators include pinacidil, nicorandil, and diazoxide and minoxidil<sup>117,140,141 145 146</sup>.



Pinacidil and diazoxide both function as vascular vasodilators in humans <sup>147,148</sup>. Diazoxide preferentially targets SUR<sub>1</sub> subunits of K<sub>ATP</sub> channels, while pinacidil, minoxidil, and nicorandil target SUR<sub>2</sub> subunits. Minoxidil is used to treat hypertension and androgenic alopecia (male pattern baldness) <sup>117</sup>, while nicorandil is used in the treatment of angina, stemming from transient ischemia <sup>149</sup>. An additional K<sub>ATP</sub> activator, termed VU063, was serendipitously discovered during a high-throughput screen against mosquito K<sub>IR</sub>1 channels <sup>130</sup>. Subsequent characterization with fluorescent biochemical assays and voltage clamp electrophysiology demonstrated that VU063 is approximately 12-fold more potent than diazoxide, activates K<sub>IR</sub>6.2/SUR1 with faster kinetics than diazoxide, acts directly on K<sub>IR</sub>6.2/SUR1 to open the channels, and is specific for K<sub>ATP</sub> channels containing SUR1 <sup>130</sup>.

However, all of the available chemical modulators have been designed to treat human pathologies and no studies have been performed to identify specific modulators of arthropod K<sub>ATP</sub> channels. The molecular structure, potency, and selectivity of drugs targeting the major vertebrate K<sub>ATP</sub> channel subtypes are summarized in Table 1.1 and are thoroughly reviewed in Kharade et al (2016) <sup>122</sup>.

Table 1.1. Described small-molecule modulators of human K<sub>ATP</sub> channels

Compound	Structure	Selectivity	IC <sub>50</sub>	References
<b>Inhibitors</b>				
Tolbutamide		SUR1>SUR2	2-7 μM	Gribble et al., 1998
Glibenclamide (glyburide)		SUR1>SUR2	SUR1: 4 nM SUR2A: 27 nM	Gribble et al., 1998
Gliclazide		SUR1	50 nM	Gribble and Ashcroft., 1999
Nateglinide		SUR1	100 nM	Sunaga et al., 2001
<b>Activators</b>				
Diazoxide		SUR1>SUR2	60-80 μM	Dabrowski et al., 2003
VU0071063		SUR1	7 μM	Raphemot et al., 2014b
Pinacidil		SUR2	1 μM	Lefebvre and Horacek ,1992
Nicorandil		SUR2	100 μM	Sato et al., 2000
Minoxidil		SUR2	182 μM	Sato et al., 2004

### **1.8. K<sub>ATP</sub> Channels: Function in Regulation of ROS/Redox**

The initial linkage between K<sub>ATP</sub> channels and regulation of ROS levels was observed in the human heart. Cardiomyocyte death after ischemia/reperfusion was shown to correlate with oxidant stress and interestingly, it was shown that exposure to K<sub>ATP</sub> channel openers, such as diazoxide, are capable of increasing levels of ROS. This increase of ROS led to protection of the heart where there was no cellular damage in subsequent ischemic injuries<sup>150</sup>. The mechanism by which diazoxide induces cardioprotection is not fully elucidated, but recent work has shown that diazoxide can act as a trigger of preconditioning by a mechanism involving mitochondrial swelling and the generation of ROS<sup>151</sup>. These data provide evidence that in humans, ROS are beneficial when the time and concentration are appropriate and further, there is a functional linkage between K<sub>ATP</sub> channels and ROS generation.

As discussed in section 1.5, ROS are also critical to animal survival as they function as signal messengers for the immune system after virus infection and thus, a moderate increase in ROS benefits animal health by enhancing immune function<sup>152</sup>. In mammals, it has been shown that ROS production is initiated as a consequence of pathogen infection, and if ROS are generated at moderate levels, it induces activation of multiple immune pathways that lead to inhibition of virus replication. Importantly, ROS have been shown to be beneficial to mosquito survival by enhancing immune responses of mosquitoes after infection with bacterial or viral pathogens<sup>153,154</sup>, indicating a conserved function between mammals and insects. Considering this, ROS likely represent a potential physiological product capable of reducing pathogen infection and enhancing the overall health of managed honey bee colonies through increased innate antiviral immune function. However, there are significant gaps in our knowledge with respect to the functional linkage between K<sub>ATP</sub> channels and ROS levels in honey bees, and thus

this thesis project aimed to fill these gaps in knowledge.

## **1.9. Viruses and Antiviral Defense Mechanisms of Honey Bees**

The honey bee is host to more than 25 viruses that are usually non-enveloped, positive sense, single stranded RNA viruses belonging to either the family *Dicistroviridae* or *Iflaviridae*<sup>155</sup>. Most bee viruses manifest themselves by persisting via vertical and horizontal transmission, and exist in a latent state without perturbing bee immunity. However, increased stresses induced by chemical xenobiotics or heavy infestations of *Varroa* mites cause bee immunosuppression that results in increased pathogenicity of the virus<sup>156</sup>, leading to the death of hosts and possible collapse of the colony<sup>157-159</sup>. The increasing prevalence of bee viruses highlights the importance of identifying mechanisms to enhance immune function to mitigate virus mortality, which is a goal of this thesis work.

Bees are known to utilize an antiviral RNAi response<sup>160,161</sup>, along with other immune pathways (*e.g.* Toll, Jak), to protect from virus infection, but there is little known regarding the mechanisms honey bees use to initiate these responses. Previous work has demonstrated a correlation between ATP-sensitive inwardly rectifying potassium ( $K_{ATP}$ ) channels, virus mRNA replication, and honey bee survivorship<sup>127,128,162</sup>. These data showed that reduced expression or function of  $K_{ATP}$  channels leads to a strong increase of virus that results in an accelerated death of Flock House Virus-infected flies<sup>128,163</sup> and bees<sup>127</sup>. Similarly, viral-mediated mortality is alleviated through activation of  $K_{ATP}$  channels with pinacidil, suggesting that  $K_{ATP}$  channels play an evolutionarily conserved role in mediating this interaction between the host and the virus. However, the functional linkage between the  $K_{ATP}$  channels and the mechanism to drive antiviral immunity in bees is unknown and this gap in knowledge must be filled to enable the development of novel tools to enhance been immunity and overall health.

### 1.10. K<sub>ATP</sub> Channels as a Target for Enhancing Honey Bee Health

The coupling of K<sub>ATP</sub> channels to the metabolic state of the cell suggests that these channels may be involved in the regulation of ROS for cellular protection <sup>164</sup>. Indeed, studies on mammalian K<sub>ATP</sub> channels have shown that pharmacological activation of K<sub>ATP</sub> channels with diazoxide results in functional uncoupling of the mitochondria, yielding an increase in ROS production <sup>165,166</sup>. On the contrary, pharmacological inhibition of K<sub>ATP</sub> channels results in a decrease in ROS concentration within the animal <sup>167,168</sup>. Importantly, the role of K<sub>ATP</sub> channels and ROS modulation is conserved between mammals and insects as it has been shown that K<sub>ATP</sub> channels provide a protective role against hypoxic stress in *Drosophila* <sup>162</sup>. Similarly, a functional linkage between K<sub>ATP</sub> channels and oxidative stress has been shown in cockroaches <sup>169</sup>. These data provide evidence that K<sub>ATP</sub> channels are involved in regulating the titer of ROS, raising the intriguing possibility that viral-mediated mortality in honey bees may be mitigated by regulating ROS levels through K<sub>ATP</sub> channel modulation (Figure 1.2).

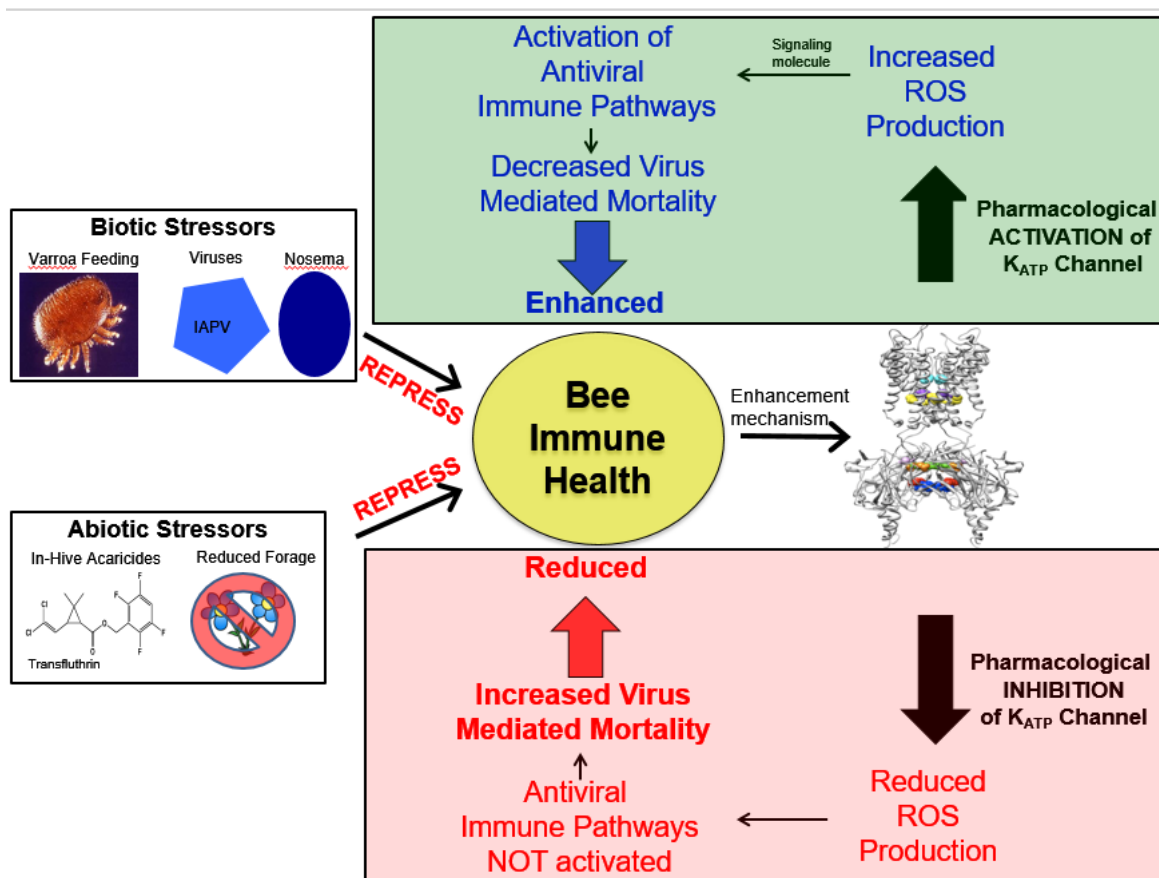
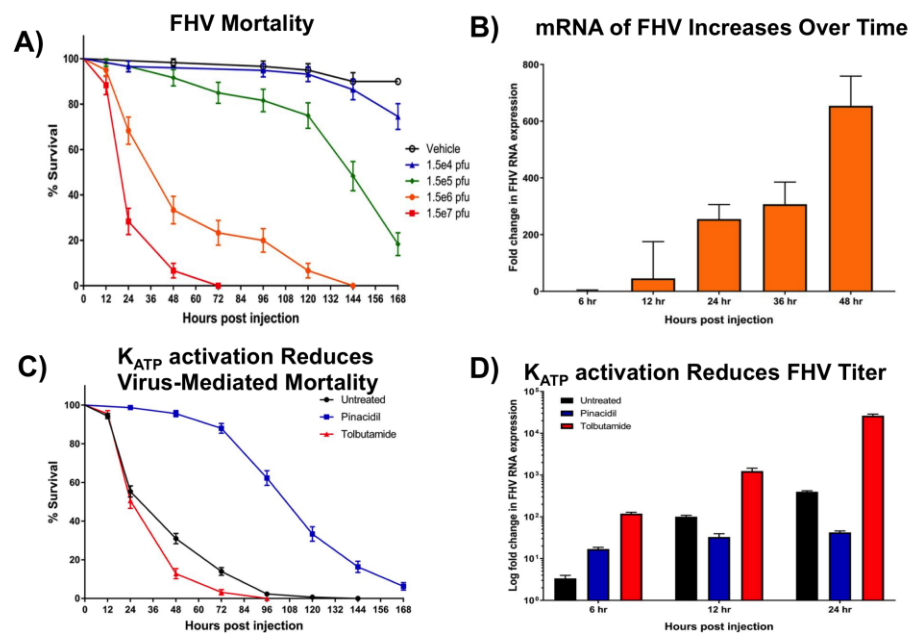


Figure 1.2, **Schematic diagram describing the overarching premise of this thesis.** Known correlations are activation and inhibition of K<sub>ATP</sub> channels reduces and increases virus mediated mortality, respectively. The aims of this thesis will address a portion of this correlation through ROS.

### 1.11. K<sub>ATP</sub> Agonists Increase Survivorship after Infection with Model Virus

As a proof-of-concept, previous work developed a laboratory assay for assessing the impact of drug exposure on the survival of virus infected honey bees, as well as changes in viral replication as measured through changes in the expression of viral mRNA<sup>127</sup>. In this assay, age-matched adult honey bees were infected via intrathoracic microinjection with an entomopathogenic model virus, flock house virus (FHV), then monitored for survival over a five to ten day period in caged groups. Testing demonstrated that honey bee mortality directly

increased relative to the titer of FHV administered (Figure 1.3A) and quantitative PCR (qPCR) analysis demonstrated that viral RNA expression increased over time (Figure 1.3B)<sup>127</sup>. Importantly, treatment with the  $K_{ATP}$  channel agonist pinacidil increased survival of bees while decreasing viral replication following infection with FHV, whereas treatment with the  $K_{ATP}$  channel antagonist tolbutamide decreased survival and increased viral replication (Figure 1.3C-D). These results suggest that  $K_{ATP}$  channels provide a significant link between cellular metabolism and the antiviral immune response in bees, indicating a likely linkage between ROS signaling, antiviral immune responses, and  $K_{ATP}$  channels in honey bees. Considering this, this thesis work aimed to further elucidate this connection by describing the influence of  $K_{ATP}$  channels to ROS and survivorship.



**Figure 1.3. Honey bee immunity and survival bioassays for infections with flock house virus.** (A) Survival of virus-infected adult bees. (B) Increasing viral RNA expression measured using qPCR. (C)  $K_{ATP}$  Activation (pinacidil) and inhibition (tolbutamide) reduced and increased virus mediated mortality, respectively. (D) Altered viral RNA expression after  $K_{ATP}$  modulation compared to untreated (black bars). Published in O'Neal et al., *Sci. Rep.*, 2017.

### **1.12. Overarching Hypothesis to be Tested**

Honey bees are exposed to a variety of different stressors, such as parasites, pesticides, and transcontinental travel, which have reduced the immunocompetence of the individual bee and increased their susceptibility to pathogens<sup>170-172</sup>. This increase in susceptibility to pathogens has resulted in a downward spiral of bee health and population decline. Sick and weakened bees diminish colony resiliency, ultimately leading to a breakdown in the social structure, production, immunity, and survivorship of the colony<sup>173</sup>. Unfortunately, removing the chemical stressors or eliminating mite-transmitted pathogens is impractical at the present time and therefore, methods to increase immunocompetence are needed to increase honey bee health. Previous work suggests a putative connection between K<sub>ATP</sub> channels and antiviral immunity in managed honey bees, and taken in context with work in mammalian systems, we speculate that the functional linkage between these factors is through ROS. Therefore, this thesis aimed to directly test the hypothesis that K<sub>ATP</sub> channel modulators are capable of regulating levels of ROS in individual honey bees. The data generated in this study will bridge the fundamental knowledge gap and result in an understanding of honey bee immune system regulation as well as reveal novel intervention points. To test this hypothesis, we propose the following objectives:

### **1.13. Objectives of the Study**

1. Identify K<sub>ATP</sub> modulators capable of affecting paraquat-induced ROS in honey bees
2. Determine if K<sub>ATP</sub> modulators increase survivorship from paraquat-induced ROS
3. Provide proof-of-concept that ROS induction reduces mortality stemming from mite infestation



## **Chapter 2. Test The Hypothesis That Pharmacological Modulators of K<sub>ATP</sub> Channels Can Reduce Oxidative Stress in the Honey Bee, *Apis Mellifera*.**

### **2.1. Introduction**

The Western honey bee, *Apis mellifera* L., is the most widely managed crop pollinator in the United States, and significantly contributes to the economic viability of our agricultural industry and the overall sustainability of food and fiber production. Declines in the numbers of both managed and wild pollinators have served to increase public awareness of bee health issues, and prompted researchers to intensify efforts to understand the factors driving these declines<sup>174-176</sup>. While a variety of factors negatively impact bee populations, there is a growing belief that the prevalence of viruses poses the most significant threat to honey bee health<sup>177</sup>. Managed honey bee colonies are commonly infected with multiple viruses concurrently<sup>178,179</sup>, though such infections may remain asymptomatic, and persist only at low levels in healthy colonies. Importantly, exposure to environmental stressors can weaken colony immunity and provoke acute outbreaks that result in overt deformities, paralysis, and death of individual bees, leading to colony loss. Taken together, colony losses appear to be closely associated with reduced immunocompetence<sup>175,180,181</sup>, and therefore, mechanisms to enhance immune function are likely to reduce infection rates and increase colony viability. However, much of the research occurring currently is focused on describing the factors causing mortality and not the identification of physiological mechanisms that can mitigate the stress-induced damages to restore honey bee health. Identification of novel physiological pathways within the individual honey bee that can increase colony sustainability is needed and is therefore the premise of this study

One of the primary bee antiviral defense mechanisms is an evolutionarily conserved gene silencing mechanism known as RNA interference (RNAi), which recognizes the presence of

double-stranded RNA to initiate targeted degradation of viruses <sup>182</sup>. Little information exists regarding mechanisms to enhance RNAi pathways in bees, but previous work has demonstrated that a family of potassium ion channels, termed ATP-sensitive inward rectifier potassium ( $K_{ATP}$ ) channels, play a significant role in mediating the survival of both mammals and insects during a viral infection <sup>128,163</sup>. These data suggest  $K_{ATP}$  channels may drive RNAi pathways and thus, antiviral immunity, highlighting the importance of defining the interaction between these two systems. Interestingly,  $K_{ATP}$  channels are coupled to the metabolic state of the cell, enabling these channels to regulate levels of reactive oxygen species (ROS) through mitochondrial function <sup>164</sup>. ROS are used in mammals as a signaling mechanism for immune system enhancement after virus infection <sup>128,162,163,169,183</sup>, indicating that a functional linkage between  $K_{ATP}$  channels, ROS, and antiviral defense mechanisms may also exist in honey bees.

ROS are well-documented to cause a number of deleterious events that lead to acute mortality of honey bees <sup>184,185</sup>, resulting from increased cellular damage <sup>186</sup>. Considering this, it is understandable that the majority of ROS research has focused on the deleterious consequences ROS have to honey bee health. However, ROS are also critical to animal survival, as they function as signal messengers for the immune system during virus infection. A moderate increase in ROS are also known to benefit human health by enhancing immune function<sup>152</sup>. In mammals, it has been shown that ROS production is initiated as a consequence of pathogen infection, and if ROS are generated at moderate levels, it induces activation of multiple immune pathways that lead to inhibition of virus replication. Importantly, ROS have been shown to be beneficial to mosquito survival by enhancing immune responses of mosquitoes after infection with bacterial or viral pathogens <sup>153,154</sup>, indicating a conserved function between mammals and insects. Therefore, the overarching premise of this thesis study is that ROS represent a physiological

product capable of reducing pathogen infection, and that chemical modulation of ROS levels can enhance the overall health of managed honey bee colonies through increased innate antiviral immune function. As such, this thesis work aims to fill significant gaps in our knowledge with respect to mechanisms that can modulate levels of ROS in honey bees by investigating the functional interplay between ROS, virus infection, and bee survivorship. This must be addressed for increased sustainability of managed bee colonies, and is thus the premise of this thesis.

Potassium ion channels are a fundamental component of many physiological mechanisms since they are responsible for establishing and maintaining the membrane potential of animal cells and serve crucial roles in cellular regulation<sup>187</sup>. A superfamily of potassium ion channels, termed inward rectifier potassium ( $K_{IR}$ ) channels, function as biological diodes due to the unique biophysical property that allows the flow of potassium ions in the inward direction more easily than the outward direction at hyperpolarizing potentials<sup>117</sup>.  $K_{IR}$  channels are known to be critical for proper function of multiple mammalian<sup>188-191</sup> and insect<sup>123,192-195</sup> physiological systems. All  $K_{IR}$  channels share similar molecular architecture; they are tetramers, assembled around an aqueous membrane-spanning pore that are gated by polyvalent cations that occlude the pore at cell potentials more positive than the  $K^+$  equilibrium potential<sup>196,197</sup>.  $K_{ATP}$  channels are a subfamily of  $K_{IR}$  channels, and are heterooctomeric complexes of four pore-forming  $K_{IR}$  channel subunits and four regulatory (SUR) subunits<sup>198,199</sup>. Contrary to “classic”  $K_{IR}$  channels,  $K_{ATP}$  channels are regulated by the ratio of intracellular ATP: ADP and thus, they couple the membrane potential to the metabolic state of the cell<sup>200</sup>.

The coupling of  $K_{ATP}$  channels to the metabolic state of the cell suggests that these channels may be involved in the regulation of ROS for cellular protection<sup>164</sup>. Indeed, studies on mammalian  $K_{ATP}$  channels have shown that pharmacological activation of  $K_{ATP}$  channels with

diazoxide results in functional uncoupling of the mitochondria, yielding an increase in ROS production <sup>165,166</sup>. On the contrary, pharmacological inhibition of K<sub>ATP</sub> channels results in a decrease in ROS concentration within the animal <sup>167,168</sup>. Importantly, the role of K<sub>ATP</sub> channels and ROS modulation is conserved between mammals and insects as it has been shown that K<sub>ATP</sub> channels provide a protective role against hypoxic stress in *Drosophila* <sup>162</sup>. Similarly, a functional linkage between K<sub>ATP</sub> channels and oxidative stress has been shown in cockroaches <sup>169</sup>. These data provide evidence that K<sub>ATP</sub> channels are involved in regulating the titer of ROS, raising the intriguing possibility that viral-mediated mortality in honey bees may be mitigated by regulating ROS levels through K<sub>ATP</sub> channel modulation.

## **2.2. Materials and Methods**

### **2.2.1. Solvents, Reagents, and Compounds**

The ROS inducing compound paraquat (PESTANAL, analytical grade), the K<sub>ATP</sub> channel modulator pinacidil, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were designated to be >98% pure. Chemical structure of the K<sub>ATP</sub> modulator pinacidil used in this study is shown in Table 1.3.

### **2.2.2. Honey Bee Colonies**

Colonies of Cordovan Italian bees were sourced from the USDA-ARS Honey Bee Breeding, Genetics and Research Facility (Baton Rouge, Louisiana). Queens were acquired from C.F. Koehnen and Sons (Ord Bend, California). Queens were open mated on location by the breeder. No brood was taken from the colonies for a period of 8 weeks after queen installation, so as to ensure all bees used were from the installed Koehnen queens, and not the preceding queen. *Varroa* mite levels were assayed twice per month by alcohol wash. Any colony having mite levels above the economic treatment threshold of 3.0 mites per 100 bees was excluded from

the study, unless specifically dictated by the methods (section 2.2.7). No *Varroa* mite treatments were made during the conduct of the study.

### 2.2.3. Hoarding Cages

Standard bee study cages were purchased from Small Life Supplies (Whittlesey, Peterborough, Cambridgeshire, Great Britain). The cages (figure 2.2.4) were machined from stainless steel, with glass closures. Cages were washed with acetone, then dried and autoclaved before each use. Segments of 3/8<sup>th</sup> inch PVC pipe (Ace Hardware (Oak Brook, IL) were cut to measure 1.5 inches in length, so as to form adapters to fit 5mL BAXTER oral syringes (Thermofisher Scientific (Waltham, MA)), which were filled with 10% sucrose solution for bee feeding. The protruding tip of each syringe was trimmed with a razor blade before filling with feeding solution and inserting into hoarding cages, so as to ensure that the diet solutions were accessible to the bees.

### 2.2.4. Modulation of Paraquat-Induced Oxidative Stress in Honey Bees

#### 2.2.4.1. Expose Bees to K<sub>ATP</sub> Activator Pinacidil during Paraquat Induced Stress.

Frames of near-emerging brood were removed from colonies and placed into purpose-made emerging cages courtesy of the USDA-ARS lab (Baton Rouge, LA). Cages were placed into a humidified incubator and were maintained at 32.5°C and 60-70 % percent relative humidity<sup>201</sup>. All K<sub>ATP</sub> modulators were made into 200 mM stock solutions in DMSO, to aid in solubility in aqueous solution. 10 uL of stock solution were then added to 990 uL of 1:1 sucrose, so that the final concentration of modulators in the diet was 2mM, and the final DMSO concentration was 1%. Newly emerged bees (less than 24 hours old) were then pre-fed with K<sub>ATP</sub> modulator or untreated 1:1 sucrose solution, containing 1% DMSO for 24 hours. After 24

hours of exposure to  $K_{ATP}$  modulators, 0.5 ug/uL of paraquat was added to the diet of treatment groups as well as positive controls, while untreated controls continued to receive normal sucrose/vehicle. Bees fed throughout the incubation period, with feeding solutions being remade every 24 hours. Whole bees were sampled beginning 24 hours from the onset of paraquat exposure, at the intervals of 24, 48, 72, and 96 hours. The fluorophore, Rhodamine B, was added to the sucrose to ensure the bees imbibe the chemical, similar to our other studies <sup>195</sup> and as shown in Figure 2.1 B-C. This compound fluoresces under 510 nm light; as such, bees that have fed on the compound visibly fluoresce under this wavelength. Bees that were not fluorescent were not included in the data analysis. Dead bees were not used in biochemical assays because melanization after death would have skewed biochemical data. Bees were stored at -80° C until the conclusion of the experiment.

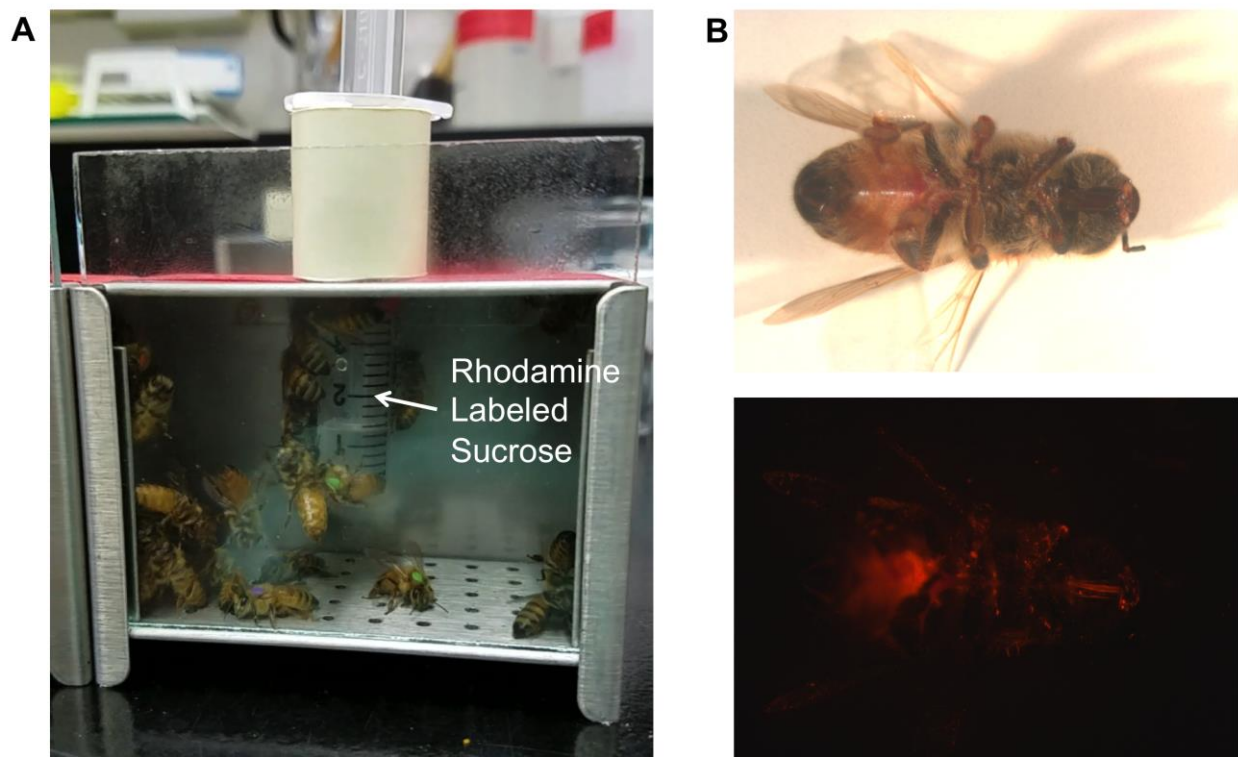


Figure 2.1. **Treatment chamber for honey bee exposure to chemicals.** **A)** Photo showing bees feeding on Rhodamine B labeled sucrose that would contain modulators. **B)** Image of a bee fed on Rhodamine labeled sucrose under white light and fluorescent lamps.

#### 2.2.4.2. Processing of Samples

To generate sample extract for analysis, whole bees were individually processed by first removing the sting gland, and were then be placed individually into 2.0 mL screw-top microcentrifuge tubes, along with 1.8 mg of 2.0 mm zirconium beads. Samples were returned to the -80° C freezer and, while still frozen, the bees were homogenized by dry milling using a Mini-Beadbeater 96 bead mill (Bio Spec Products, Bartlesville, OK) for 20 seconds at 3500 RPM. Samples were then reconstituted using 1.0 mL (per bee) (equivalent to 10 uL per mg of mass, as adult bees are ~ 100mg, each) of ice-cold 5mM potassium phosphate buffer, pH 7.4, containing 0.9% sodium chloride (Cayman Chemical, Ann Arbor, MI) with 0.3% Triton X-100 (Sigma-Aldrich, City, State) and lightly swirled. Reconstituted samples were then centrifuged for

15 minutes at 12,000xG at 4° C. The resulting supernatant was collected and divided into 120 uL aliquots, and stored at -80° C.

#### 2.2.4.3 Analysis of Samples

To biochemically assess the capability of  $K_{ATP}$  modulators to reduce ROS, two assays were performed. The first assay, the Total Antioxidant Capacity Assay (Cayman Chemical, Ann Arbor MI) evaluated the difference in ROS generated in each bee by measuring their total antioxidant capacity in a 96-well plate based colorimetric assay. The assay functions by measuring the ability of any and all antioxidants present in each sample to inhibit the oxidation of the chromogenic compound 2,2'-Azino-di-3-ethylbenzthiazoline sulphonate (ABTS®) by the compound metmyoglobin. If no antioxidants are present in the sample, metmyoglobin will reduce ABTS, producing a color change, which may be read by a spectrophotometer. An increase of antioxidants causes less color change and resultant absorption of light at 750 nm decreases, allowing for measurement the antioxidant content of the sample. This absorption value is then quantified by comparing the absorbance of the samples against a standard curve of Trolox (an analogue of tocopherol). The resultant values ascertained by this method are representative of millimolar Trolox equivalents, which are then standardized by dividing by the protein content of the sample, as determined using the Pierce™ 660nm protein quantification assay, as detailed in the following paragraph. Thus, the antioxidant kit indirectly measures the concentration of ROS; an increase ROS would deplete the total antioxidant capacity of the bee, while a decrease in ROS would result in a higher total antioxidant capacity. The assay was read using a BioTek™ Epoch® spectrophotometer (Fisher Scientific (Waltham, MA)), at 750 nm, per the kit instructions.



The second assay, the Pierce 660nm protein assay, is a dye-metal-complex based method of total protein quantification. This assay is more rapid, more linear, and more reproducible compared with the standard Coomassie-dye based Bradford Assay<sup>202</sup>. This assay is based on the binding of proteins to the dye-metal-complex, which causes a shift in the absorbance of the solution, from 450 nm to 660 nm. Before binding, the dye-metal-complex solution appears reddish brown; after proteins bind, the solution turns blueish green. These absorbances are then compared to a bovine serum albumin based standard curve, so as to quantify the total protein content of each sample<sup>202</sup>. The assay was read using a BioTek™ Epoch® spectrophotometer (Fisher Scientific (Waltham, MA)), at 660 nm, per the kit instructions.

#### 2.2.5. Survivorship Assessment with K<sub>ATP</sub> Modulators after Paraquat-Induced ROS

Newly emerged bees (0-24 hours post emergence) were placed into hoarding cages, with 20 bees per cage, with three cages per treatment group per replicate, with three replicates in the study. Treatment groups were fed with 2mM Pinacidil in 1:1 sucrose, while untreated and positive control groups were fed 1:1 sucrose with vehicle only (DMSO). After 24 hours of feeding, paraquat were added to the diet of the experimental and positive control groups at a concentration of 0.5 ug/uL, while the untreated control continued to receive 1:1 sucrose plus vehicle. After 24 hours of exposure to paraquat, and at 24 hour intervals following this point, mortality was assessed for each cage. Mortality was defined as any individual which showed no visible motion within five seconds of observation. All individuals which were found to be dead were removed at the time of observation. Feeding solutions were then made fresh at the time of mortality assessments (every 24 hours) and replaced before returning bees to humidified incubator. Observations continued for a period of 120 hours.

#### 2.2.6. Test the Hypothesis that ROS-Induction Alters Mortality Rates of Bees Heavily Infested with *Varroa* Mites

Newly emerged worker bees were collected from colonies with low (less than 3 mites per 100 bees) and high infestations (>8 mites per 100 bees) of *Varroa destructor* mites, as *Varroa* are the vector for many of the most important honey bee viruses. As before, bees from both colonies were allowed to emerge in a humidified incubator. Bees from both low-mite and high-mite colonies were placed into distinct hoarding cages and fed for 24 hours with untreated sucrose. After 24 hours, a discriminating range of doses of paraquat was added to sucrose solution to cages of both groups to induce ROS. The bees were further allowed to feed until all were dead, with survivorship assessments taken every 24 hours. To enable a statistical comparison of toxicity, we constructed sigmoidal curves using GraphPad Prism 6 software and the time required to kill 50% of the experimental population (ET<sub>50</sub>) was calculated by nonlinear regression (variable slope) using a Hill equation in GraphPad Prism (GraphPad Software, San Diego, CA, USA).

### 2.3. Results

#### 2.3.1. K<sub>ATP</sub> Modulators are Non-Toxic to Adult Honey Bees

The pharmacology of K<sub>ATP</sub> channels is the most developed of all K<sub>IR</sub> channels and consists of both, activators and inhibitors. Molecular structures of pinacidil and diazoxide (activators) as well as glyburide and tolbutamide (inhibitors) are shown in Figure 2.2A. Prior to studying the physiological function of honey bee K<sub>ATP</sub> channels using these pharmacological probes, we tested their impact to honey bee acute toxicity by providing access of 50 caged bees to the compounds through sugar water for 1 week. Compounds were tested at concentrations approaching solubility limits (2-4 mM) and data shown in Figure 2.2B clearly show that that

chronic exposure to these molecules was non-toxic. This was also found to be true for classical  $K_{IR}$  channel inhibitor VU041<sup>123</sup>. The lack of acute toxicity suggests that using these modulators to probe the physiological function of  $K_{ATP}$  channels in bees is a viable approach.

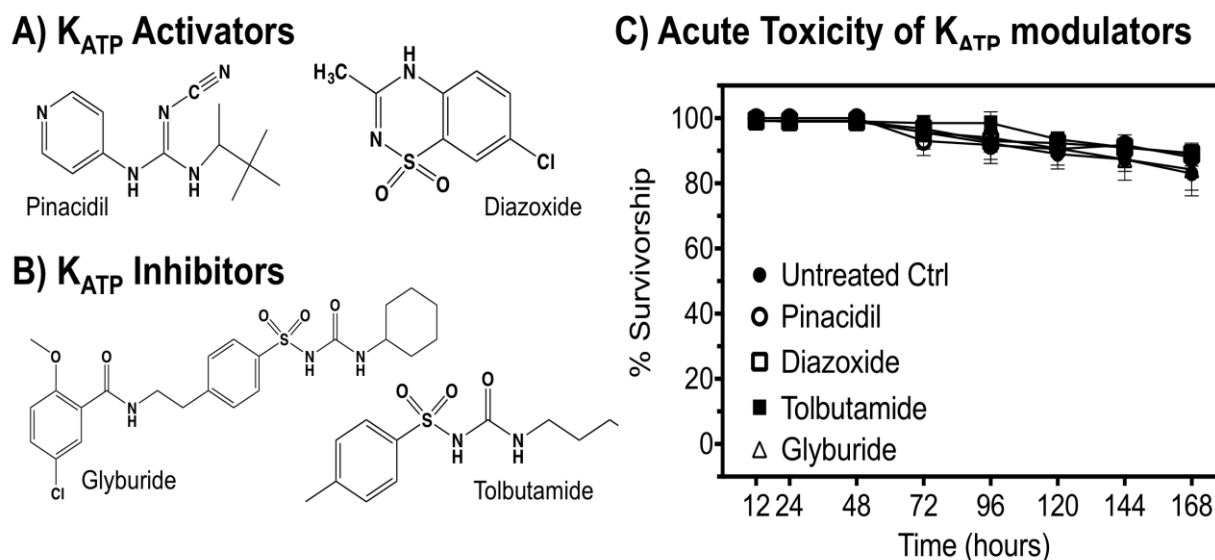


Figure 2.2.  **$K_{ATP}$  modulators and Acute Toxicity.** Molecular structures of  $K_{ATP}$  channel activators (A) and inhibitors (B). Acute toxicity of  $K_{ATP}$  modulators to honey bees. Data points represent mean (n=150) % survivorship with error bars representing SEM.

### 2.3.2. Identification of Paraquat Concentration to Induce ROS

A goal of this study was to measure the ability of  $K_{ATP}$  modulators to regulate the levels of ROS and thus, the induction of ROS is necessary to determine if  $K_{ATP}$  modulators increase or decrease ROS levels. The standard method to induce ROS into individual honey bees is through injection of the herbicide paraquat, as it yields reproducible levels of ROS, whereas flight and other measures are highly variable. Thus, we measured mortality of individual honey bees after exposure to paraquat at concentrations of 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL. Concentrations of paraquat at 1 mg/mL and 2 mg/mL resulted in near 100% mortality at 72-

hours post exposure and approximately 50% mortality at 48 hours. The onset of toxicity was slower with 0.5  $\mu\text{g/mL}$  paraquat where we observed  $20 \pm 7\%$  mortality at 48 hours and  $58 \pm 11\%$  toxicity at 72 hours. Less than 100% toxicity was observed at 96 hours, which was the end of the analysis. Thus, we deemed 0.5 mg/mL paraquat to be the appropriate concentration to use for subsequent studies with  $K_{\text{ATP}}$  modulators due to the slower, yet linear rate of toxicity when compared to other concentrations (Figure 2.3).

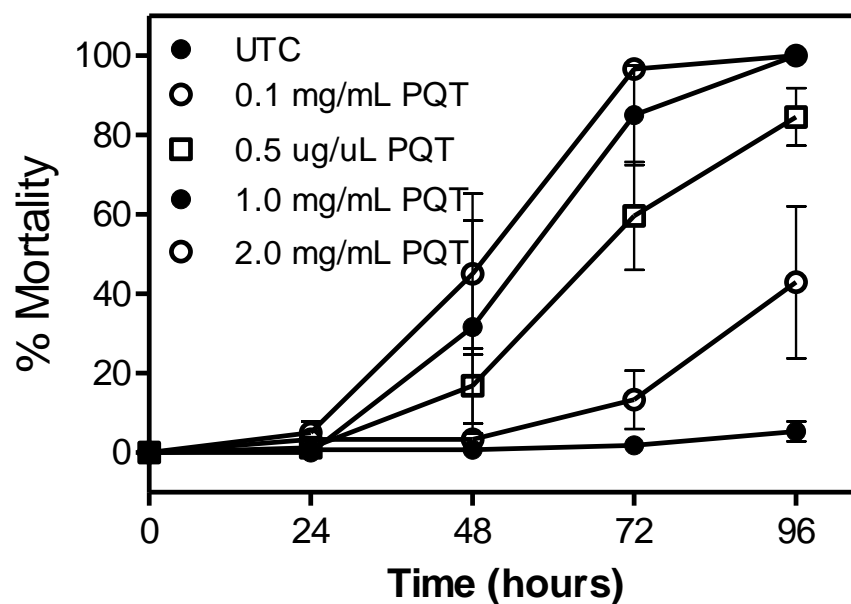


Figure 2.3. **Rate of mortality of honey bees exposed to varying concentrations of paraquat.** Mortality was assessed at 24 hour intervals and data points represent mean ( $n=75$  bees) % mortality. Error bars represent SEM.

### 2.3.3. Determine if Pretreatment with $K_{\text{ATP}}$ Modulators Affects Survivorship during Paraquat-Induced ROS

We aimed to determine the influence of  $K_{\text{ATP}}$  channel activators and inhibitors to mortality that resulted from generation of ROS. To do this, we pretreated groups of 20 newly emerged bees with either  $K_{\text{ATP}}$  modulators in 1:1 sucrose, or sucrose with vehicle (DMSO) only.

After 24 hours, 0.5 ug/uL of paraquat was added to the sucrose diet, and survivorship was then assessed in 24 hour intervals after exposure to paraquat. First, we analyzed the impact of  $K_{ATP}$  inhibitors to paraquat-induced mortality and found that glibenclamide, 5-hydroxydecanoic acid (data not shown), or tolbutamide (Figure 2.4A) did not alter paraquat-induced toxicity over the 120 hour feeding period. Next, we studied an array of structurally distinct  $K_{ATP}$  activators (Table 1.3) and found that VU063, diazoxide, or minoxidil did not alter paraquat induced mortality (data not shown). However, pinacidil was found to significantly increase the rate of toxicity with an  $ET_{50}$  of approximately 40 hours whereas 0.5 mg/mL paraquat alone resulted in an  $ET_{50}$  of approximately 68 hours (Figure 2.4A), a statistically significant difference ( $P < 0.05$ ).

Next, we microinjected pinacidil in honey bees that were fed paraquat to ensure the increased rate of mortality was not a byproduct of feeding the bees pinacidil plus paraquat. The pattern of toxicity remained the same with pinacidil increasing the rate of toxicity and tolbutamide not altering paraquat induced mortality. As expected, the rate of mortality was much faster when compared to oral exposure with the pinacidil/paraquat combination, resulting in an  $ET_{50}$  of approximately 20 hours whereas 0.5 mg/mL paraquat alone resulted in an  $ET_{50}$  of approximately 75 hours (Figure 2.4B).

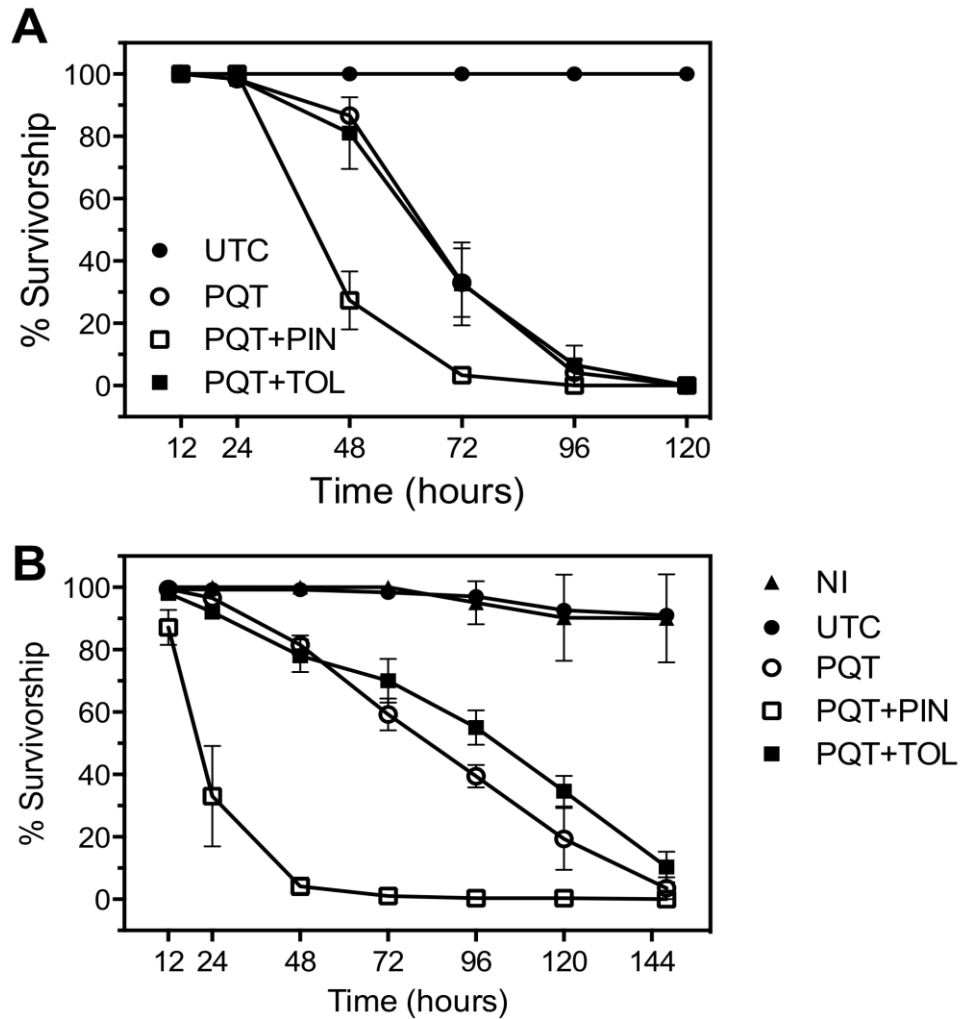


Figure 2.4. **Influence of pinacidil and tolbutamide to paraquat induced mortality.** Toxicity was assessed with routes of pinacidil exposure being oral (A) and injection (B). Mortality was assessed at 24 hour intervals and data points represent mean (n=75 bees) % mortality. Error bars represent SEM. Abbreviations used: NI (no injection), UTC (untreated control), PQT (paraquat only), PQT+PIN (paraquat plus pinacidil), PQT+TOL (paraquat plus tolbutamide).

#### 2.3.4. Effect $K_{ATP}$ Modulator Pinacidil on Paraquat-Induced ROS in Honey Bees

To measure the ability of  $K_{ATP}$  channel activator pinacidil to affect levels of ROS in individual honey bees, we pretreated groups of 10 newly emerged bees with 2 mM pinacidil by oral exposure. Other  $K_{ATP}$  modulators were not studied due to the lack of impact to paraquat induced mortality (Figure 2.4.). Paraquat was incorporated into the sugar diet to chemically

induce ROS 24 hours after pinacidil exposure and the total antioxidants in individual bees were quantified as an indirect measure for ROS. Pinacidil alone did not reduce antioxidant levels when compared to control at 24 hours post treatment. Both paraquat alone and paraquat/pinacidil combination reduced the total levels of antioxidants with a 1.3- and 1.6-fold reductions, respectively when compared to control (1.25  $\mu$ M A.O. /  $\mu$ g/mg protein), which were statistically significant reductions ( $<0.05$ ) from control but not from each other. At 48 hours, the antioxidant level after exposure to pinacidil alone was found to be approximately 15% increased over control, which was not statistically significant (P-value: 0.4) when compared to control. However, paraquat was shown to statistically reduce antioxidant levels when compared to control levels by 1.4-fold (Figure 2.5). Additionally, a dramatic reduction of antioxidants was observed at 48 hours for pinacidil/paraquat combination when compared to untreated control or paraquat alone with a 3.0- and 1.7-fold reduction, respectively, which are both statistically significant reductions ( $P<0.05$ , Figure 2.5). Contrary to 24- and 48-hours, pinacidil alone was found to reduce antioxidant levels by 1.5-fold at 72-hours post exposure, which was a statistically significant reduction ( $P<0.05$ ), and was not statistically different from paraquat alone at 72 hour. No data for paraquat/pinacidil combination was collected at 72-hours post exposure because greater than 95% of bees were dead. Interestingly, the total antioxidant levels for the paraquat/pinacidil combination continued to be reduced between the 24 and 48 hour time points (and presumably, were reduced to the point of lethality between the 48 and 72 hour time points), whereas bees exposed to paraquat alone did not have significantly different antioxidant levels between 24-, 48-, and 72-hours post exposure (Figure 2.5).

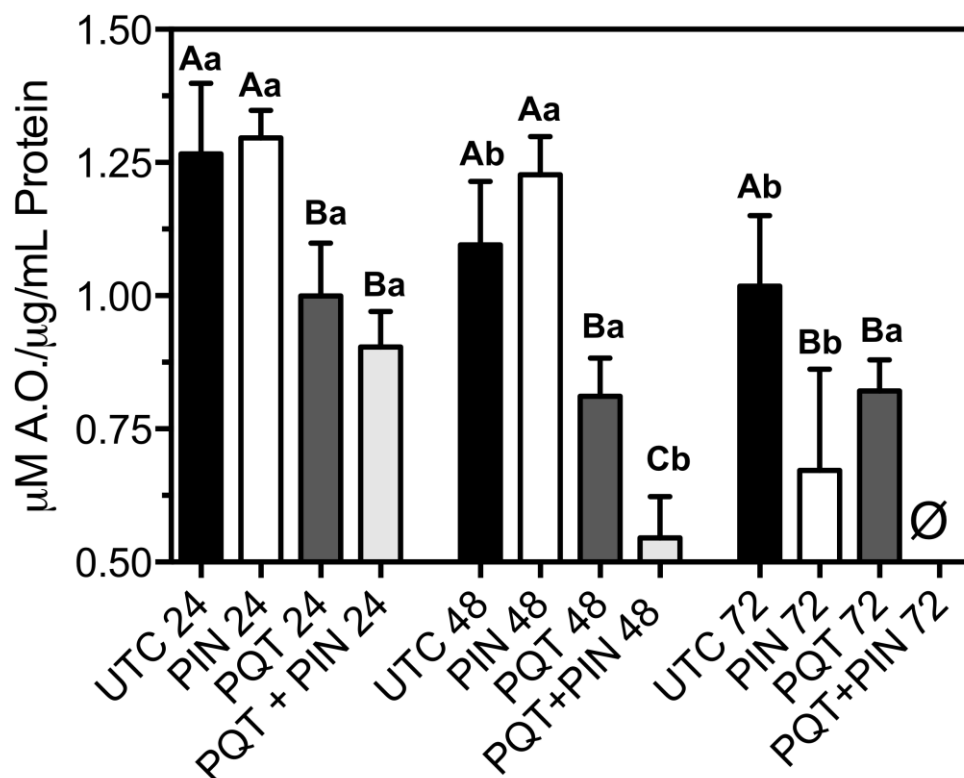


Figure 2.5. **Change in total antioxidant capacity over the course of 72 hours after exposure to paraquat alone or paraquat/pinacidil combination.** Bars represent mean (n=50-100 bees) antioxidant levels and error bars represent SEM. Uppercase letters represent statistical significance ( $P<0.05$ ) within the same time point and lowercase levels represent statistical significance ( $P<0.05$ ) of the same chemical treatment group across the time points. Significance was determined by one-way ANOVA with Tukey's posttest.

### 2.3.5. ROS Induction Reduces Rate of Mortality in Colonies Heavily Infested with *Varroa*

ROS is known to cause significant cellular damage through enhanced lipid peroxidation of cell membranes, and is considered a significant detriment to honey bee health<sup>110,203</sup>. Yet, ROS have also been shown to serve as a secondary messenger for the activation of immune responses in mammals<sup>152,184,204</sup>, leading us to develop the hypothesis that honey bees use ROS as a stimulator for immune function and the biological response is dependent upon concentration and pathogen titers within the bee. To test this hypothesis, we extracted honey bees from colonies that had a heavy- (>8 mites/100 bees) or low- (<3 mites/100 bees) mite infestation and



fed them varying concentrations of paraquat, which is known to exert injurious effects through ROS<sup>205-207</sup>. Presumably, honey bees from high mite colonies would benefit from an increased immune response through ROS-mediated activation since bees from high mite colonies are likely to have higher virus titers when compared to low mite colonies. Indeed, data show that exposure of bees from high mite colonies to 0.1 mg/mL paraquat resulted in a significantly reduced mortality at all time points when compared to low mite colonies (Figure 2.6A). As expected, the ability to rescue mortality was reduced when bees were exposed to a greater concentration of paraquat (0.5 mg/mL) (Figure 2.6B) and no significant difference in mortality was observed at 1 mg/mL and 2 mg/mL paraquat (Figure 2.6C-D).

To enable statistical comparison of low- and high-mite data, we determined the time required to achieve 50% mortality (ET<sub>50</sub>) for each concentration of paraquat. The largest difference in ET<sub>50</sub> values was found with 0.1 mg/mL paraquat where a 2.1-fold difference between low- and high mite colonies was observed, which was a statistically significant difference ( $P < 0.05$ ). Less of a difference, albeit still statistically significant ( $P < 0.05$ ), was observed at 0.5 mg/mL with bees with high mite infestations dying 1.5-fold slower when compared to bees from low-mite colonies. No significant difference in the rate of mortality between low and high-mite colonies was observed at paraquat concentrations of 1 mg/mL or higher (Table 2.1).

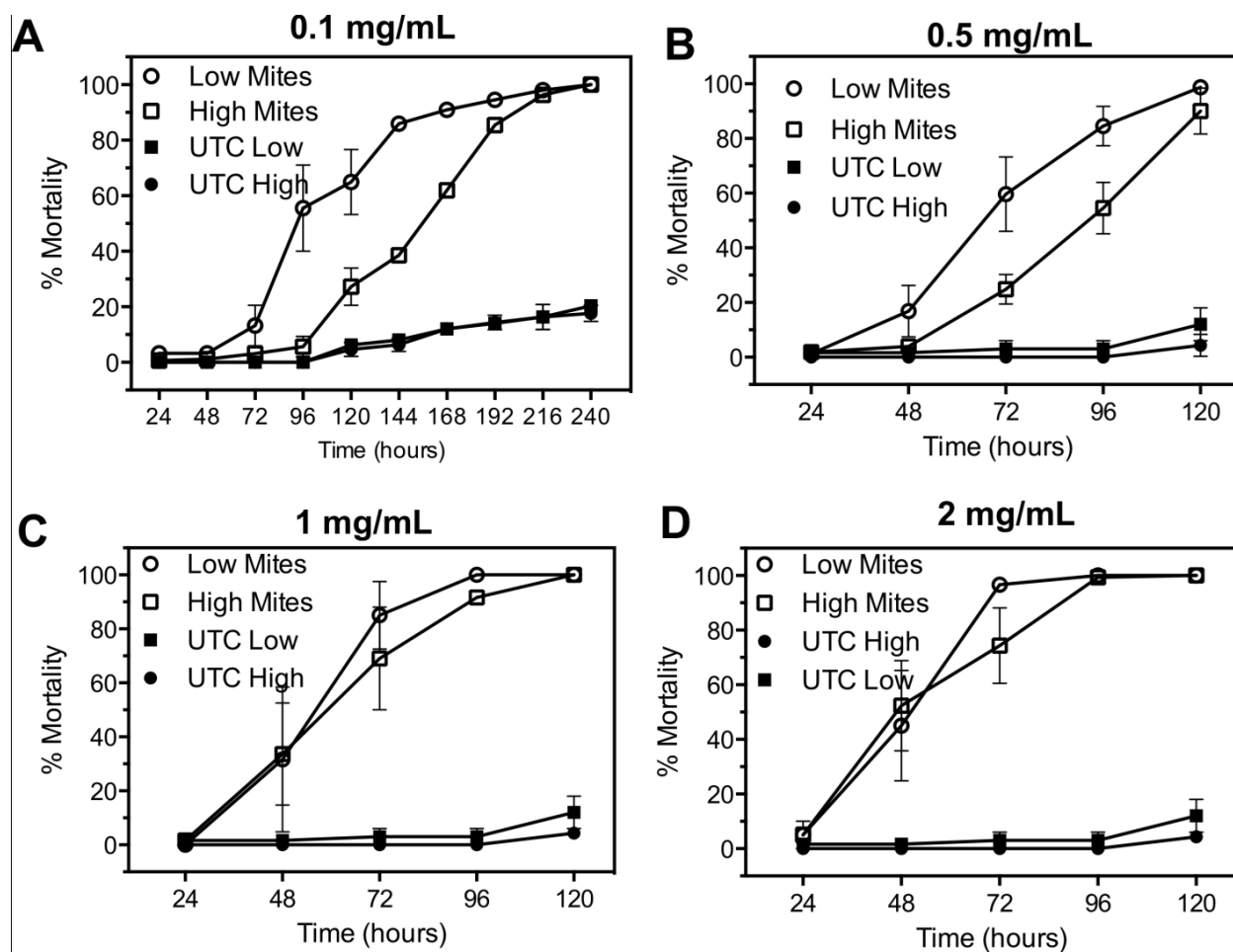


Figure 2.6. **Mortality of honey bees from high- and low-mite colonies after ROS induction.** Honey bees from low ( $\circ$ ) or high ( $\square$ ) mite colonies were exposed to paraquat at 0.1 mg/mL (A), 0.5 mg/mL (B), 1 mg/mL (C), or 2 mg/mL (D) and mortality was assessed at 24 hour intervals. Data points represent mean ( $n=75$  bees) % mortality. Error bars represent SEM.

Table 2.1. Rate of mortality after paraquat exposure to bees from low- and high-mite colonies.

Paraquat Concentration	<b>ET<sub>50</sub> (Hours; 95% CI)</b>	
	Low mites	High Mites
<b>0.1 mg/mL</b>	82 (51-99) Aa	169 (142-177) Ba
<b>0.5 mg/mL</b>	66 (52-80) Ab	99 (71-136) Bb
<b>1 mg/mL</b>	54 (38-70) Ab	52 (22-84) Ac
<b>2 mg/mL</b>	49 (42-56) Ab	47 (38-63) Ac

Table 2.1. Low mite colonies were defined as <3 mites/100 bees and high mite colonies were defined as >8 mites/100 bees. The uppercase letters represent statistical significance within the row (low vs high mite) and lowercase letters represent statistical significance within the column (paraquat concentrations). Rows or columns not labeled by the same letter represents statistical significance at P<0.05 as determined by a one-way analysis of variance with multiple comparisons posttest.

## 2.4. Discussion

Considering the economic and agricultural importance of domesticated honey bees and the increasing evidence that bee decline is largely driven by viral pathogens, the mechanisms involved in the antiviral immune response of bees remain poorly characterized. This gap in knowledge pertaining to the complex relationship between host and pathogen limits our understanding of how viruses and immune deficiencies affect bee health and is restricting our ability to develop more effective strategies to mitigate colony loss. Bees are known to utilize an antiviral RNAi response<sup>160,161</sup>, along with other immune pathways (*e.g.* Toll, Jak), for protection from virus infection, but there is little information regarding the mechanisms honey bees use to initiate the RNAi response available. Previous work has demonstrated a correlation between ATP-sensitive inwardly rectifying potassium (K<sub>ATP</sub>) channels, virus mRNA replication, and

honey bee survivorship<sup>127,128,162</sup>. these data showed that reduced expression or function of K<sub>ATP</sub> channels leads to a strong increase of virus that results in an accelerated death of Flock House Virus-infected flies<sup>128,163</sup> and bees<sup>127</sup>. Similarly, viral-mediated mortality is alleviated through activation of K<sub>ATP</sub> channels with pinacidil. This previous work has revealed an evolutionarily conserved relationship between K<sub>ATP</sub> channels and the antiviral immune response of honey bees, yet the mechanism for this putative linkage remains unknown. Thus, the goal of this thesis work was to begin to understand the relationship between K<sub>ATP</sub> channels, ROS generation, and survivorship in honey bees.

Biologically “normal” levels of ROS are critical to animal survival, as they function as signal messengers for the immune system after virus infection, and thus a moderate increase in ROS benefits animal health by enhancing immune function<sup>152</sup>. Unfortunately, the mechanisms driving ROS production in honey bees are significantly understudied. The coupling of K<sub>ATP</sub> channels to the metabolic state of the cell suggests that these channels may be involved in the regulation of ROS for cellular protection<sup>164</sup>. Indeed, studies on mammalian K<sub>ATP</sub> channels have shown that pharmacological activation of K<sub>ATP</sub> channels with diazoxide results in functional uncoupling of the mitochondria (loss of chemiosmotic potential that drives ATP production), yielding an increase in ROS production<sup>165,166</sup>. On the contrary, pharmacological inhibition of K<sub>ATP</sub> channels results in a decrease in ROS concentration within the animal<sup>167,168</sup>. Importantly, the role of K<sub>ATP</sub> channels and ROS modulation is conserved between mammals and insects as it has been shown that K<sub>ATP</sub> channels provide a protective role against hypoxic stress in *Drosophila*<sup>162</sup>. Considering the role of K<sub>ATP</sub> channels and ROS in mammals, it was surprising that exposure to pinacidil only, a K<sub>ATP</sub> channel activator, did not statistically alter antioxidant levels at 24- or 48-hours post exposure when compared to control groups. However, we observed a 15%

increase in antioxidant levels with pinacidil alone at 48-hours post exposure, and although this increase was not statistically significant, it is likely to be biological relevant as it has been shown small increases in ROS cause a genetic upregulation of genes encoding antioxidants<sup>83</sup>. Further, we observed a significantly greater reduction of antioxidant levels after co-exposure to paraquat + pinacidil when compared to paraquat alone, indicating a synergistic relationship between the two molecules. This reduction of antioxidant levels after co-exposure to paraquat + pinacidil was mirrored by a significantly increased rate of mortality. We anticipate that pinacidil alone is indeed inducing small amounts of ROS due to 1) the 15% increase in antioxidant levels at 48-hours when compared to control, 2) the significant reduction in antioxidants at 72-hours after pinacidil only exposure, and 3) the synergistic relationship of pinacidil + paraquat. Thus, we speculate that  $K_{ATP}$  channels are associated with honey bee redox biology and activation of these channels causes a small increase in ROS, but the amounts of induced ROS at 24-48 hours is below the limits of detection with the utilized antioxidant assay and prevents quantification. These data provide the first indication that pinacidil is likely capable of stimulating the production of ROS within individual honey bees and importantly, the quantity of ROS generated is small and does not induce acute mortality. Additional studies are needed that will provide conclusive evidence for this functional linkage and to shed more light on the physiological mechanism driving the generation of ROS after activation of  $K_{ATP}$  channels.

Previous work has clearly shown that pinacidil is capable of augmenting the innate antiviral immune pathways in honey bees<sup>127</sup>, and, considering our data showing pinacidil driving ROS generation (Figure 2.5), we tested the hypothesis that honey bees use ROS as a stimulator for immune function. Our data indicate that chemically induced ROS is indeed capable of reducing mortality stemming from high mite infestations, which was used as a proxy

for heavy viral loads, and these data support previous studies showing a reduction in Flock House Virus mortality after treatment with pinacidil. As expected, these data show that the biological response to ROS is dependent upon concentration as we observed a reduction in rescued mortality with increasing concentrations of paraquat. The reduced mortality in bees from high mite colonies when compared to bees from colonies with low mite infestation levels at low, but not higher concentrations of paraquat supports the notion that ROS represents a type of chemical hormesis in bees. For example, a bee experiencing infection with an already low level of ROS is likely to benefit from a slight increase of ROS because this would lead to an enhanced immune response. If the ROS levels in the infected bee were to increase dramatically, however, they may promote a pathological response, such as autoimmunity, and lead to cell death. Thus, a moderate of quantity of ROS is beneficial to the health of the organism, while an excess of ROS leads to cell damage and death. The data presented in Table 2.1 challenge the current paradigm that claims ROS only impacts bee survivorship negatively, by providing evidence that ROS is beneficial at low to moderate concentrations when the bee is infested with the virus-vectoring mite, *Varroa destructor*. Thus, we speculate that the reduced bee mortality observed in high-mite infested colonies is resultant from ROS stimulating immune function, leading to increased antiviral immunity. Additional work is required to validate this hypothesis.

## **2.5. Conclusion**

In summary, we have revealed a possible role for ion channel regulation of the honey bee antiviral immune response by providing a putative linkage between  $K_{ATP}$  channels, ROS, and antiviral immunity. We have shown that treatment with the  $K_{ATP}$  channel agonist pinacidil increases ROS of individual honey bees; this is likely the mechanism causing a decreased viral replication following infection with flock house virus that has been shown in previous studies.

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. Admittedly, the data presented in this thesis are not conclusive evidence validating a functional linkage between  $K_{ATP}$  channels and ROS production, and additional work is necessary to confirm this speculated role of  $K_{ATP}$  channels and ROS to innate antiviral immunity in bees. However, this work does represent a promising area of future research that will aim to increase our understanding of honey bee immune responses and may provide tractable biochemical targets that can be exploited through product development campaigns.

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## **Vita**

Christopher Joel Fellows was born in Jasper, Georgia, and lived there until moving north to the mountains, to attend Young Harris College. It was at Young Harris that Christopher found his passion for honey bees, and began to dream of a career in honey bee research. After completing a Bachelor of Science in Biology, Christopher accepted a position at Eurofins Agrosience Services, and moved to Mebane, North Carolina. Here, Christopher worked on honey bee ecotoxicology studies for three years before leaving to pursue a Master of Science in Entomology at Louisiana State University in Baton Rouge, Louisiana. Once Christopher has completed his master's degree, he will begin pursuit of his doctorate, under the direction of Dr. Daniel Swale.