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Molecular Characterization of Stress Response in Western Honey Bee (Apis mellifera)

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MOLECULAR CHARACTERIZATION OF STRESS RESPONSE IN WESTERN HONEY BEE (APIS MELLIFERA)

by

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ABSTRACT

Honey bees are incredibly important for the reproduction of flowering plants and the sustainability of agricultural ecosystems. However, they face various stressors such as pesticides, pathogens, habitat loss, and climate change. Extensive research has been conducted to understand how bees respond to these stressors. Scientists have discovered that honey bees exhibit complex physiological and behavioral responses to stress at individual and colony levels. Stress can have a significant impact on their immune function, foraging behavior, and reproductive success (Decourtye et al., 2010). Understanding bee responses to stress is crucial for several reasons. Firstly, honey bees are vital for the pollination of numerous crops, contributing significantly to global food production (Aizen et al., 2008). Declines in honey bee populations due to stress can have far-reaching consequences for food security and ecosystem stability (Vanbergen 2013). Secondly, honey bee declines also have broader implications for biodiversity, as they play a key role in maintaining plant diversity and ecosystem resilience (Winfree et al., 2009). Moreover, honey bees serve as valuable model organisms for studying stress biology and resilience mechanisms in social insects (Winston 1937). Insights gained from honey bee research can inform conservation efforts of sustainable pest management strategies (Johnson and Ellis, 2010). In conclusion, this research on honey bee responses to stress underscores the critical need to protect pollinator populations and mitigate anthropogenic stressors. By integrating knowledge from biochemical, physiological, and ecological perspectives, scientists can inform conservation efforts and promote sustainable beekeeping practices to safeguard honey bee populations and the ecosystems they support.

Keywords: Biotic, Abiotic, Stress, Honey bee, Pathogens, Pesticides, Oxidative, Endoplasmic Reticulum Stress

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CHAPTER I – Background Literature and Significance

The Western honey bee, scientifically known as Apis mellifera, holds a prominent position as the most prevalent species of honey bee worldwide (Mortensen et al., 2013). This eusocial insect undoubtedly plays a crucial role in our lives, offering invaluable contributions through the production of various substances such as honey, propolis, royal jelly, bee wax, and bee venom (Engel 1999). Notably, honey bees, along with other non-Apis bees, are responsible for pollinating an astonishing 80% of the world's plants, including over ninety different food crops (Klein et al., 2007). In the United States alone, the total estimated value of these crop pollination services amounts to a staggering $15 billion annually (Suzuki, 2014). Beyond their agricultural significance and pollination services, honey bees also play a vital role in maintaining ecological balance (Klein et al., 2007; Potts et al., 2010). They contribute to the overall health and diversity of ecosystems, making their conservation of paramount importance. Surprisingly, the conservation status of honey bees has been listed as "data deficient" on the red list of threatened species by the International Union for Conservation of Nature (IUCN) since 2019. This classification highlights the urgent need for further research and monitoring to better understand and protect these vital creatures. Honey bee colonies face a myriad of challenges, including both biotic and abiotic stressors, which can act individually or interact synergistically. Chemical pesticides, mites, and viral pathogens are among the key stressors that have resulted in significant losses over the past two decades (Castelli et al., 2020; Eierman, 2021; G. Li et al., 2018). These stressors have posed significant threats to honey bee populations, necessitating proactive measures to mitigate their impact and safeguard the well-being of these essential pollinators. In conclusion, the Western honey bee, *Apis mellifera*, has earned

its place as a vital species in the natural world. From its role in pollination and agricultural productivity to the production of valuable substances, honey bees have proven indispensable to human life. However, their conservation status and the challenges they face should not be overlooked. It is imperative that we prioritize research, awareness, and sustainable practices to support honey bee populations and ensure the continued well-being of our ecosystems and food systems.

1.1 *Apis mellifera* Physiology

To effectively understand these beneficial insects, humans have designed man-made hives called Langstroth hives that efficiently provide a place for bees to settle in. It is perfectly sheltered from the sun and rain and has a defensible entrance that can prevent most predators from getting into the main chamber where the bees store their brood (immature life stages) and honey. Once you have put a suitable hive in an area of your choice, scout bees can come through to check the area out. If they think the area is suitable for them, they will release a pheromone called Nasonov that lets the swarm, or traveling colony, know of the location. As a characteristic of eusocial insects, this new hive will have three different castes present: the drones, which are male bees, and the two females, the queen, who will singularly populate the hive, and the worker, which is what most of the hive contains. The first three development stages are egg, larvae, and pupa (Free and Spencer-Booth, 1959) and is collectively termed the brood. The last development stage is the adult. The drone, born from an unfertilized egg (24 days to develop from egg to adult) and lives for about a month, is few in number and their only function is to leave the hive and mate with a virgin queen, although very few succeed and once they have done that, they die. When it gets to be colder weather and the hive needs lots of food to survive, the

workers will literally push all these drones out to starve and die since they do not benefit the hive at all in labor and they require the most food, judging by their size since they are much larger than the workers. This division of labor is what makes the hive successful. Each worker bee is born from a fertilized egg and are all female (21 days to develop from egg to adult), and each have a certain task to perform. This could be housekeeping, being undertakers, nurses, queen attendants, nectar collection, fanning the hive, beeswax production, guarding the hive, and probably the most dangerous, foraging. Like the queen, they are female but lack the full development of their reproductive organs and live on average around 27 days (Yang et al., 2017). Anatomically, they are smaller in size and possess pollen baskets on their hind legs, wax glands on their abdomen and mandibular glands that are unable to produce the queen pheromones. The queen, which is a fully developed female that can live for two or more years (16 days to develop from egg to adult), has only two functions which are to lay eggs and to produce chemicals that help maintain colony organization and regulates colony reproduction. She's busy, as she lays around 1500 eggs a day (Merrill 1924; Nolan 1925), so she relies on the workers to feed and groom her body to keep her clean. If the queen is getting weak or dies, the workers will make a new queen by feeding a larvae royal jelly, exclusively for that purpose. As such, at one time, there are usually five or six queens born, and they all must fight to survive. The strongest queen is left standing while the others are either killed or they fly away, and once she's claimed her queen title, she leaves the hive to mate with several drones (Woyke 1960).

1.2 Honey Bee Products

Bees produce a variety of products that are not only important for their own survival but also have economic and practical value for other species like humans. The most well-known is of course their namesake, which is honey. They collect nectar from flowers and transform it into honey through a process of regurgitation and evaporation which they store in the comb. This is a natural sweetener with antibacterial properties that has been used by humans for thousands of years in cooking and baking. It is also used as a food source for adult bees to feed their growing pupae and larvae. Beeswax is another product produced by bees to build the comb in which honey and pollen can be stored, and where the brood is raised. Humans can use this wax to make candles, cosmetics, and skincare products. Royal jelly is produced by worker bees to feed the queen and young larvae as it is a highly nutritious substance that's rich in proteins, vitamins, and minerals. It can also be used as a dietary supplement by humans as it is believed to have health benefits. Resin collected from trees and plants can be used to make propolis, another product produced by bees to seal cracks and gaps formed in their hives, since hives are usually kept outside under various weather conditions. They also produce venom for defense purposes that could be used by humans for health reasons such as venom therapy known as apitherapy. These bees play a crucial role in pollinating plants and crops that humans rely on for food, so their products and services have a significant impact on agriculture and the environment. Thus, beekeeping is an important industry that revolves around the management and production of these bee-derived products.

1.3 Honey Bee Stressors

To better understand the biological processes the bee undergoes, introducing various stressors through their diet is an excellent way. To achieve that, this experiment was designed to understand the molecular and chemical processes when the bee is fed stress-inducing molecules mixed in with their diet. For this, the following six stressors were chosen: tunicamycin, thapsigargin, and metformin for ER stress, and paraquat, hydrogen peroxide, and imidacloprid for oxidative stress. So why were these two stress conditions chosen? For the first one, the endoplasmic reticulum (ER) is a large membrane-enclosed cellular organelle that is found in all eukaryotes. It is at this site that membranes are folded, proteins are secreted, lipids and sterols are synthesized, and free calcium is stored. The problem comes when physiologic stresses like increased secretory load, or pathological stresses like the presence of mutated proteins cannot properly fold in the ER. This can lead to an imbalance between the demand for protein folding and the capacity of the ER for protein folding, which leads to causing ER stress. Thus, as a response to this, eukaryotic cells have evolved a group of signal transduction pathways termed the unfolded protein response, or UPR. The second one is oxidative stress. This is achieved when there is a disturbance in the balance between the production of reactive oxygen species, or free radicals, and antioxidant defenses that can be problematic as it can lead to tissue damage or injury. Damage can occur in a variety of tissues in the organism when there is a deficiency in antioxidant defenses. Hence, causing these various stress conditions could tell us how the honey bee is able to deal with it, or in some cases, how they are not able to deal with it (through death or a decrease in weight, etc.). Additionally, they can have biotic stressors such as numerous pathogens like positive-sense single-stranded RNA viruses, bee

genetics, and queen longevity, or also abiotic stressors such as agrochemical exposure, weather, temperature, light intensity, air humidity, and wind speed. Ectoparasites are perhaps the most stress-causing agents for the bee. Examples of that are the small hive beetle, the greater wax moth, the bee louse, the tracheal mite, and the Varroa destructor mite. All of these can cause an excess number of diseases and viruses such as American foulbrood, chalkbrood, or deformed wing virus. Nevertheless, understanding how the bee deals with these stressors will open the avenue for new research to better protect them.

1.4 *Varroa* Physiology

One of the main pests that threaten the honey bee is the Varroa destructor mite. It is an ectoparasitic mite that has only two stages in its lifecycle. The first stage is known as the adult traveling stage (or phoretic) and the second is known as the reproductive stage. In the adult traveling stage, the mite feeds on an adult honey bee and from there can get into the capped brood cells to begin its reproductive stage, which is where the four distinct life stages are expressed: egg, protonymph, deutonymph, and finally, the adult (Rosenkranz et al., 2010). These mites are interesting since feeding on honey bee brood is the only place that they can reproduce. As soon as the bee caps the cell for the brood to grow in, the mite which was hiding in the food given to the brood (Boecking et al., 2008), has successfully infiltrated the cell. This starts the reproductive stage. In around 70 hours, the bee larva enters the prepupa stage (Boecking et al., 2008) and the mite begins to lay eggs when the bee larva releases a host signal. The first unfertilized egg is remarkably always a male (Garrido et al., 2003) and then, approximately 30 hours later, a fertilized female egg is laid (Rehm et al., 1989). Around 20-to-32-hour intervals after this, at least 3 more female eggs are laid (Martin 1994). This reproductive stage takes only ten days and these new mites

feed just enough on the fat body tissue to properly grow without killing the brood, which then develops into a young bee. When the young bee can exit the cell, the new mites, of which there can now be about 6 to 7 females (Donze et al., 1994; Infantidis 1983), can then spread to other bees to start their lifecycles, which begins again with the phoretic stage. Morphologically, it is very simple to tell the difference between the male and female mite, simply by looking at the color and size. A fully grown male is whiteish with approximate body widths and lengths of 0.700 and 0.715 mm, respectively, while the female is reddish brown in color and can easily be seen with the naked eye since they are significantly larger than the male, generally around 1.1mm wide. (De Jong et al., 1982). Their body shape is akin to a button and their flat form is perfect in being able to feed on honey bees by getting under their abdominal scales. Small bristling setae cover their bodies which aids in them being able to attach to their host (Kirrane et al., 2012). This is a ubiquitous parasite that can be found anywhere honey bees are found. As of 2014, Australia was the only country that did not have any mites but now as of July 2022, they have been detected, making them truly worldwide.

1.5 Physiology of Honey Bee-Mite Interaction

It should be noted here, that although the mites will feed on any of the bees, their favorite target is the drone brood cells. This is because the drone brood cells take an extra three days to properly develop, and these three days are what is detrimental to the bee but beneficial to the mite. These extra days of feeding are what can cause genetic defects such as deformed wings in the bee. It's also ample time to spread viruses and fungi in the bee (Ramsey et al., 2019). Since the queen mates with several drones in her lifetime, if the

drone is infected with DWV, the virus can be vertically passed on to the worker bee through the queen and infected drone (Amiri et al., 2016).

Previous studies assumed that these ectoparasites feed exclusively on the hemolymph of the bee, but it has recently been proven that this was not the only thing the mite fed on (Ramsey et al., 2019). The bee is severely weakened since the adult mite feeds not on the hemolymph, but primarily on the fat body of adult and bee larvae and this is problematic because the fat body is fundamental for bodily functions such as energy, hormone regulation, immunity, and pesticide detoxification. When the fat body is eaten or damaged, this leaves the bee in a weakened state. Furthermore, when a mite feeds, it leaves an open wound on the bee which can become a site for virus infections or diseases. Mites are known to spread anywhere from five to eighteen different kinds of debilitating bee viruses. At the very least, heavy infestations of mites can cause the whole collapse of the hive so making sure a hive is not infested becomes paramount to the success of the bee. Even if the hive isn't heavily infected, RNA viruses like the deformed wing virus (DWV) can still happen (Ramsey et al., 2019). As a result of their affinity for brood cells, the mites favorite place to occupy is around the "nurse" bees since they spend the most time near the broods which lets them have easy access and also, because of recent advances in understanding, these nursing bees have been shown to contain most of the fat bodies as compared to a regular worker bee (Ramsey et al., 2019). Once a hive has become infected, the mite can damage the bee in two ways. First is through feeding on the fat body, causing the bee to weaken and easily die from any external or internal factors that a bee with an intact fat body would easily survive. This causes them to also have a shorter life span than ordinary worker bees and they tend to be absent from the hive, possibly since their

navigation or energy regulation is severely debilitated. The second way is through the transmission of the many viruses that the mite is a vector of, including sacbrood, deformed wing virus, black queen cell virus, chronic bee paralysis virus, acute bee paralysis, Kashmir bee virus, Israeli acute paralysis virus, and slow bee paralysis virus (Rosenkranz, 2010, and Bee-health, 2019).

1.6 Honey Bee Cell Culture

There is an urgent need to protect honey bees from fatal levels of virus infection and the non-target effects of insecticides used in agricultural settings, as colony loses driven by V. destructor mites and their associated aggravation of viral loads is becoming widespread. To achieve this protection, a continuously replicating cell line derived from the honey bee would provide a valuable tool for the study of molecular mechanisms of virus–host interaction, for the screening of antiviral agents for potential use within the hive, and for the assessment of the risk of current and candidate insecticides to the honey bee. Still, the establishment of a continuously replicating honey bee cell line has proved challenging, especially since no bee cell line is free from the deformed wing virus (Guo et al., 2020). However, even if the cells are infected with DWV, infecting them with another virus may show us the viral effects of immune genes as they pertain to how the cells deal with a high viral load, especially when the cell can be infected with other viruses that could harm the bee. The DWV is a persistent infection so while we can not completely get rid of it, we could either reduce it or increase it to determine the effects of an immune gene function.

1.7 Knowledge Gaps in Honey Bee Literature

The study of honey bees is an ongoing and dynamic field, but there are several gaps and areas where further research is needed to better understand various aspects of honey bee biology, health, behavior, and interactions with the environment. Some of the key gaps in honey bee literature include: pesticide interactions and synergies: while the effects of individual pesticides on honey bees are well-studied, there is a need for more research on how combinations of different pesticides and other stressors interact and synergize to impact honey bee health and behavior; sublethal effects of pesticides: many pesticides have sublethal effects on honey bees that are not immediately fatal but can impact behaviors like foraging, learning, and immune function. More research is needed to understand the long-term consequences of such effects on colony dynamics and survival; long-term impact of climate change: while studies have examined short-term impacts of climate change on honey bees, there is a need for more research to understand the long-term effects of changing climatic conditions on honey bee populations, behaviors, and interactions with plants and other species; microbiome and health: the role of honey bee gut microbiota in health and disease resistance is an emerging area of research. Further studies are needed to explore how the microbiome influences honey bee health and the potential for microbiome-based interventions; quantifying pollination services: while the economic value of pollination services by honey bees is recognized, there's room for improvement in accurately quantifying and valuing their contributions to agricultural productivity and food security; genetic basis of behavioral traits: understanding the genetics underlying honey bee behavioral traits, such as hygienic behavior, foraging behavior, and aggression, is crucial for selective breeding efforts. More research is needed to identify specific genes

associated with these traits; parasite-pathogen interactions: honey bee colonies are often exposed to multiple parasites and pathogens simultaneously. Investigating how these stressors interact within colonies and influence disease dynamics is important for developing effective disease management strategies; urban bee ecology: urban environments provide unique challenges and opportunities for honey bee colonies. Research on how honey bees adapt to urban landscapes, interact with other urban species, and contribute to urban ecosystems is limited; honey bee-pathogen-plant interactions: Investigating the tripartite interactions between honey bees, pathogens, and plants can provide insights into how plant health and immune responses impact honey bee health and vice versa; and ethical considerations in genetic engineering: as genetic engineering techniques advance, there is a need for more research and discussion around the ethical implications of genetically modified honey bees, including potential impacts on wild populations and ecosystems. Addressing these gaps requires collaboration between researchers, beekeepers, policymakers, and various stakeholders. By filling these knowledge voids, the scientific community can contribute to more effective honey bee management, conservation, and the preservation of this vital pollinator species.

Although most of the studies done are for managed honey bee populations, another thing that has been linked to the decline of wild bees is anthropogenic stress (Siviter et al., 2023). More specifically are urban areas that humans use products such as pesticide bug sprays that are lethal to both managed bees and wild bees that unfortunately come through the area. The best way to stop this would be to restrict agrochemical use in urban environments, especially in areas where the economic benefits are limited (Siviter et al., 2023) but educating people on this is an extremely difficult endeavor. Therefore, this study

was designed to understand the molecular and chemical processes when the bee is fed stress-inducing molecules mixed in its diet. The eight treatments used were tunicamycin inhibits N-linked glycosylation which will induce Endoplasmic Reticulum (ER) stress (Hirata et al., 2021); Thapsigargin inhibits SERCA of sarco endoplasmic reticulum Ca2 ATPase. This sets off UPR and if protein misfolding is not resolved, will induce ER stress (Quynh Doan et al., 2015); Metformin helps against redox stress but induces distinct ER stress pathways in cardiomyocytes (Pernicova et al., 2014); Paraquat catalyzes the formation of ROS through accepting electrons from photosystem I and transferring them to molecular oxygen (Kennedy et al., 2021); Hydrogen peroxide works by producing destructive hydroxyl free radicals that could attack membrane lipids, DNA, and other essential cell components (Brudzynski 2020); Imidacloprid acts on several types of post-synaptic nicotinic acetylcholine receptors in the nervous system which are located within the central nervous system of insects; following binding to the nicotinic receptor, nerve impulses are spontaneously discharged at first, followed by failure of the neuron to propagate any signal; Sustained activation of the receptor results from the inability of acetylcholinesterases to break down the pesticide, an irreversible process that induces excessive ROS production (Nicodemo et al., 2014); and finally two controls, one with water and one with PBS (to be used as the control for metformin).

At base value, this would cover two stress conditions that would further our understanding of how bees deal with external stress induced in bioassay arenas in a laboratory setting. The first stress condition is oxidative stress. This is achieved when there is a disturbance in the balance between the production of reactive oxygen species, or free radicals, and antioxidant defenses (di Conza & Ho, 2020). This is problematic as it can lead

to tissue damage or injury. Any deficiency in antioxidant defenses can cause damage which occurs at various tissue levels of the organism. The second stress condition is the endoplasmic reticulum (ER) stress. The ER is a large membrane enclosed cellular organelle that is found in all eukaryotes (Hirata et al., 2021). It is at this site that proteins are synthesized, folded, secreted, lipids and sterols are synthesized, and free calcium is stored (di Conza & Ho, 2020). The problem comes when physiologic stresses like increased secretory load, or pathological stresses like the presence of mutated proteins cannot properly fold in the ER. This can lead to an imbalance between the demand for protein folding and the capacity of the ER for protein folding, which leads to causing ER stress (di Conza & Ho, 2020). Consequently, as a response to this, eukaryotic cells have evolved a group of signal transduction pathways termed the unfolded protein response, or UPR.

As of now, there is not a single cause for the depletion of bees due to bee diseases since many studies have found synergistic effects of pesticides and microbial infection with each other (Alaux et al., 2010; Aufauvre et al., 2012; Boncristiani et al., 2012; Doublet et al., 2015; Pettis et al., 2013; Vidau et al., 2011) so focusing on the impact of interactions between various stressors will give a more holistic view (Johnston et al., 2016). So, for this study, causing these various stress conditions in bees infected with deformed wing virus (DWV) could tell us how the honey bee is able to protect itself, or in some cases, how they are not able to be protected (through mortality or a decrease of weight, etc.). Understanding how the bee deals with these stressors will open the avenue for new research to better protect them on a larger scale.

1.8 Study Limitations

Honey bee research, like any scientific investigation, comes with its own set of limitations. Identifying and acknowledging these limitations is essential for understanding the scope of the research and the validity of its findings. Since most of the bee studies are conducted on specific bee colonies or in specific geographic regions, extrapolating findings from these limited samples to a broader population of bees can be challenging. Bee behavior, physiology, and colony dynamics can also vary significantly throughout the year so studies conducted during one season may not accurately represent bee behavior and responses during other seasons so potential impact of seasonal variation should be considered. Bees are also highly sensitive to environmental conditions like climate, habitat, and availability of food sources. Changes in environmental factors can influence their behavior and health, regardless of virus infection. Thus, laboratory studies may offer controlled environments but may not fully replicate natural conditions for bees, so field studies would provide a more realistic setting but could be subject to uncontrolled variables that impact research outcomes. Many honey bee phenomena like colony health and population dynamics play out over extended periods so short-term studies may not capture these long-term trends. Despite all these limitations, ongoing research on bees is critical for understanding their biology, behavior, and health as well as for addressing the various challenges they may face like habitat loss, pesticides, and diseases. By acknowledging these limitations and conducting well thought-out studies, researchers can contribute to our knowledge of bees and develop strategies for their conservation and sustainable management.

CHAPTER II – Specific Aim and Rationale of the Project

The economic contribution of insect pollination to global food production is $215 billion (Gallai et al., 2009). Seventy-five percent of our crop species benefit from insect pollinators (Klein et al., 2003), and managed honey bees provide about half of this ecosystem service to all insect-pollinated crops on earth (Kleijin et al., 2015). Honey bee colonies face many biotic and abiotic stressors that can act individually or synergistically. These stressors include chemical pesticides, mites, and viral pathogens, and have led to significant losses during the last two decades (Castelli et al., 2020; Eirman 2021; Li et al., 2018). Honey bee's biological process and response to stressors have been investigated in cage experiments, which provide a more controlled environment and limit the implication of other variables on the outcomes (Alburaki et al., 2017; 2019). Bee colonies can maintain and return to homeostasis despite stressors such as parasite infestations or exposure to pesticides; this is known as social resilience (Ulgezen et al., 2021), which helps the whole bee population. There is not a single cause for the depletion of bees due to bee diseases since many studies have found synergistic effects of pesticides and viral infection. Hence, the impact of various stressors on the bee response is needed to develop new tools to safeguard the bee populations. Understanding how bees deal with multiple stressors will open the avenue for new research to better protect them on a larger scale.

The vital significance of honey bees (*Apis mellifera*) to world food production and the crucial role of *Varroa destructor* (hereafter; Varroa) in honey bee colony losses make it one of the most severe ectoparasites. Varroosis, the mite infestation, is a significant threat to apiculture (Traynor et al., 2022). In the United States, it has been reported that bee colonies would never survive unless beekeepers use chemical treatments (Webster and

Delaplane, 2001). A Deformed Wing Virus (hereafter: DWV) is a positive-strand RNA virus transmitted to immature honey bees by Varroa foundresses, which causes significant damage to honey bee hives. The control and prevention of Varroa remain primarily based on chemical acaricides. Even more worrying observations include increasing resistance in Varroa to available acaricides, and its vectored viruses are becoming more virulent (Traynor et al., 2020). It is now widespread and growing resistance to tau-fluvalinate, flumethrin, coumaphos, and amitraz (Rodriguez-Dehaibes et al., 2011), leaving many areas with no effective control measures against Varroa during much of the beekeeping season. The current lack of alternative control methods may undermine the future of sustainable apiculture globally (Dietemann et al., 2012) and, hence, general food security. Clearly, current control strategies are insufficient; more effective and novel approaches must be adopted to tackle this global problem. We hypothesize that bees differentially regulate the antioxidants and Unfolded Protein Response (UPR) genes to buffer bees against the harmful effects of stressors, including pesticides and ectoparasites. Specifically, the hypothesis will be tested by pursuing the following aim using a combination of molecular approaches.

2.1 Aim 1: determine the impact of abiotic stressors on the expression of oxidative and ER stress genes in bees.

We routinely maintain Deformed Wing virus-B -infected mites and bees at the University of Southern Mississippi. We will use sub-lethal doses of six stressors, including tunicamycin, thapsigargin, metformin, paraquat, hydrogen peroxide, and imidacloprid, to induce oxidative and ER stress in bees naturally infected with DWV. A transcriptional gene expression assay will be performed to determine the responses of oxidative and ER stress

responses in bees. DWV-B load will also be determined using qPCR assay. These experiments fill the critical gaps in fundamental knowledge of bee's stress biology to develop new tools to protect honey bees.

Approach: A sizable cohort will have 11 days to feed on a predetermined dosage of a stressor. On days 0, 3, 5, 7, 9, and 11, a sample of 9 bees will be taken to check their gene expression of 12 preselected stress genes by using qRT-PCR. These genes are antioxidant genes, and genes involved in activating anti-ER stress pathways (antioxidants, unfolded protein response, ERAD pathway). Differential expression of above-mentioned genes would also provide a measure of robustness of honey bees regarding antioxidants and anti-ER genes which might be useful for further studies. We will look at the infection level of each bee prior to looking at the stress genes; that way we can use the bees with low infection levels as a control to look at bees with a high DWV load.

Expected results: Preliminary results show that these bees are heavily infected with deformed wing virus variant B. Depending on which days will have the most infection verses the day they have the least will tell us the functional role the gene in question plays in the virus infection.

CHAPTER III – Honey Bee Responses to Oxidative Stress Induced by Pharmacological and Pesticide Compounds

**[This chapter contains previously published work, "Tahir F, Goblirsch M, Adamczyk J, Karim S and Alburaki M (2023). Honey bee *Apis mellifera* L. responses to oxidative stress induced by pharmacological and pesticidal compounds. Front. Bee Sci. 1:1275862. doi: 10.3389/frbee.2023.1275862".]

3.1 Introduction

The vital role of the western honeybee, *Apis mellifera* L., as a pollinator cannot be overstated. Not only does it contribute to the pollination of 80% of the world's flowering plants and over ninety different food crops (Rader et al., 2016), but it also produces valuable hive products. The value of honey bee pollination alone is estimated at $17 billion annually in the United States (Calderone 2012). However, honey bee colonies face numerous challenges that have led to significant losses over the past two decades (Vanengelsdorp et al., 2011; Steinhauer et al., 2014; Kulhanek et al., 2017; Li et al., 2018). Biotic and abiotic stressors such as diseases, parasites, pathogens, and chemical pesticides all contribute to these losses. Understanding and mitigating these stressors is crucial to the survival and health of honey bee populations (Alaux et al., 2010; Vidau etl., 2011; Aufauvre et al., 2012; Boncritiani et al., 2012; Doublet et al., 2015). To study the biological processes and responses of honey bees to stressors, researchers have conducted laboratory cage experiments (Evans et al., 2009; Gregorc et al., 2018; Alburaki et al., 2019a; Alburaki et al., 2022). These experiments provide a controlled environment that allows for a more precise examination of honey bee behavior and physiological changes. By limiting the impact of other variables, these studies have shed light on the impact of stressors such as

nutritional deficiencies and pathogenic infections, like Deformed Wing Virus and Nosema ceranae, on honey bee colony strength. One of the first physiological responses to stress in honey bees is the manifestation of cellular oxidative stress. This occurs when there is an imbalance between the production of reactive oxygen species (ROS) or free radicals, and the antioxidant defenses of the bees (Di Conza and Ho 2020). Insufficient antioxidant defenses can lead to damage at various tissue levels of the honey bee. Another physiological response to stress occurs at the endoplasmic reticulum (ER) level. The ER is a crucial cellular organelle involved in folding membranes, protein secretion, lipid synthesis, and calcium storage (Di Conza and Ho 2020; Hirata et al., 2021). When stress persists and is not adequately addressed at the cellular level, an imbalance can occur between the demand for protein folding and the ER's capacity, resulting in protein damage. Understanding these physiological responses to stress in honey bees is essential for developing strategies to mitigate colony losses.

By identifying and addressing the root causes of stress, researchers and beekeepers can work together to protect honey bee populations and promote the health of our ecosystems. Maintaining homeostasis in the face of stressors is a challenge for all living organisms, including honey bee colonies. These superorganisms have the remarkable ability to return to a state of balance, known as social resilience, even when faced with challenges such as parasite infestations or exposure to pesticides (Ulgezen et al., 2021). This resilience is particularly evident in feral honey bee colonies, where the survival of the entire population depends on their ability to adapt and overcome stressors. Managed honey bee populations, on the other hand, have been experiencing constant losses, partly due to anthropogenic stressors (Siviter et al., 2023). Pesticides used for pest management in urban

landscapes and home backyards pose a threat to both managed and feral honey bees, contributing to the decline of feral populations. Understanding the genetic mechanisms that allow honey bees to cope with stress is crucial for implementing effective beekeeping management strategies. Gene regulation plays a vital role in honey bee resilience. Some genes have been identified as key players in specific stress responses, while others are still being investigated. For example, major royal jelly protein 1 (mrjp1) is involved in honey bee nutrition and larvae development (Srisuparbh et al., 2003; Li et al., 2021). Transcription factors Xbp1 and IRE1 regulate the expression of genes crucial for immune system functioning and cellular stress responses (Johnston et al., 206; Adames et al., 2020). Sodesque (Sodq) controls the levels of reactive oxygen species, essential for maintaining cellular balance (Wang et al., 2018). Two selenoproteins, SelT and SelK, are associated with both endoplasmic reticulum (ER) stress and redox stress, contributing to cellular protection and calcium regulation (Alburaki et al., 2019b; Pothion et al., 2020; Xia et al., 2022). Apismin and apideacin, known as Anti-Microbial Peptides (AMP) genes, are believed to play roles in nutrition and pathogen infections (Casteels et al., 1989; Shen et al., 2007). Lastly, cytochrome P450 (Cp450) codes for enzymes that detoxify xenobiotics, maintaining cellular metabolism and homeostasis (Zhang et al., 2018). Understanding the intricate web of gene regulation in honey bees provides valuable insights into their ability to cope with stress. By harnessing this knowledge, beekeepers can improve management techniques and limit losses in managed colonies. Additionally, preserving natural habitats and reducing the use of pesticides in urban areas can help protect feral honey bee populations and promote their resilience. Ultimately, by prioritizing the well-being of honey bees and their genetic resilience, we can ensure the survival of these vital pollinators

for generations to come. Title: Investigating the Impact of Oxidative and ER Stresses on Honey Bees. Introduction: The delicate balance of honey bee populations is crucial for the environment and agriculture. However, various stressors, including pesticides and pharmacological inducers, can disrupt their well-being. In this study, we sought to explore the toxicological effects of sublethal doses of these stressors on honey bees.

Additionally, we investigated the transcriptional response and protein damage caused by oxidative and endoplasmic reticulum (ER) stresses. Understanding the Stressors: To induce oxidative stress in caged honey bees, we employed six different abiotic stressors. Two pesticides, imidacloprid and paraquat, were used. Imidacloprid, a neonicotinoid insecticide, is highly toxic to bees and commonly used in agriculture for pest management. This pesticide targets various post-synaptic nicotinic acetylcholine receptors in the insect nervous system, leading to the failure of neuronal signal propagation. Acetylcholinesterase, an enzyme primarily found at postsynaptic neuromuscular junctions (Dvir et al., 2010), plays a crucial role in terminating neuronal transmission and signaling between synapses (Smulders et al.2004; Dvir et al., 2010), . However, prolonged exposure to imidacloprid prevents this enzyme from breaking down the pesticide, resulting in excessive production of reactive oxygen species (ROS) (Dvir et al., 2010; Nicodemo et al., 2014; Alburaki et al., 2019a). Paraquat, on the other hand, catalyzes the formation of ROS by accepting electrons from photosystem I and transferring them to molecular oxygen (Kennedy et al., 2021). These ROS can cause damage to cellular components, including membrane lipids and DNA. We also employed four pharmacological compounds to induce ER stress: tunicamycin, thapsigargin, metformin, and hydrogen peroxide. Tunicamycin inhibits N-linked glycosylation, triggering ER stress (Hirata et al., 2021). Thapsigargin inhibits

SERCA, leading to unfolded protein response (UPR) and ER stress when protein misfolding is not resolved (Quynh Doan et al., 2015). Metformin, known for its ability to reduce redox stress, has been reported to induce distinct ER stress pathways in cardiomyocytes (Pernicova et al., 2014). Hydrogen peroxide, a powerful oxidizing agent, generates destructive hydroxyl free radicals that can attack essential cell components (Brudzynski 2020). Analyzing the effects: In our study, we aimed to assess the toxicological impact of sublethal doses of these stressors on honey bees. We examined their transcriptional response, looking for changes in gene expression patterns that may indicate cellular stress or damage. Additionally, we assessed protein damage caused by oxidative and ER stresses. Through our research, we hope to gain a deeper understanding of how these stressors affect honey bee health and well-being. By exploring the molecular responses and damage caused by oxidative and ER stresses, we can work towards mitigating the negative impacts on honey bee populations. In conclusion, protecting honey bees and their vital role in the ecosystem requires a comprehensive understanding of the stressors they face. Our study investigates the toxicological effects of sublethal doses of pesticides and pharmacological compounds on honey bees. By examining the transcriptional response and protein damage caused by oxidative and ER stresses, we contribute to the growing body of knowledge surrounding honey bee health. Ultimately, this research may inform efforts to promote the well-being and sustainability of honey bee populations.

3.2 Materials and Methods

3.2.1 Laboratory Bioassay Design

In order to understand the impact of different substances on honeybees, a carefully designed experiment was carried out at the cage level. The study aimed to investigate the effects of tunicamycin, thapsigargin, paraquat, hydrogen peroxide, imidacloprid, and metformin on honey bees' survival and molecular makeup. Two control groups were also included for comparison purposes. The experiment involved 800 one-day-old sister bees, which were obtained from a genetically characterized *Apis mellifera* ligustica colony with the mtDNA haplotype C1. These bees were incubated under controlled conditions (35°C, RH 55%) and randomly divided into eight different cages. The cages used in this study were specifically designed for feeding experiments, as described in detail by Gregorc et al. 2017. Sublethal concentrations of the substances were carefully chosen based on available toxicological data from the ECOTOX database (US Environmental Protection Agency EPA) and previous investigations. Tunicamycin was administered at 19,600 ppb, thapsigargin at 195 ppb, metformin at 129,000 ppb, paraquat at 1,000 ppb, hydrogen peroxide at 4,000 ppb, and imidacloprid at 20 ppb.

The experiment lasted for 13 days and consisted of two phases. The first phase was a two-day acclimation period, during which the bees were allowed to adapt to the cage conditions. No data was reported during this period. The second phase, known as the treatment period, spanned 11 days and involved investigating the effects of substance exposure on both survival and molecular levels. During the acclimation period, the bees in the cages were provided with 1:1 sugar syrup without any additional treatments. At the beginning of the treatment period (day 0), the cages were randomly assigned to the eight

different treatments. The substances were administered ad libitum through the sugar syrup using 20 mL syringes. To monitor sugar syrup consumption, the syringes were weighed daily using a sensitive scale with an accuracy of ± 0.01 g. Throughout the experiment, dead bees were collected on a daily basis and counted for each cage. Additionally, nine bees from each treatment were sampled at two time points, specifically day 5 and day 9, for further molecular analyses. These bees were immediately frozen on dry ice and stored at -80°C until further analysis. This comprehensive study provides valuable insights into the effects of various substances on honeybees. By carefully controlling the experimental conditions and utilizing state-of-the-art techniques, researchers were able to shed light on the survival rates and molecular changes in honeybees exposed to tunicamycin, thapsigargin, paraquat, hydrogen peroxide, imidacloprid, and metformin. These findings contribute to our understanding of the potential risks associated with these substances and their impact on honeybee populations.

3.2.2 RNA Extraction

The extraction of RNA from individual bees was conducted using the whole-body tissue method. To ensure accuracy and consistency, three bees were selected for RNA extraction for each treatment and date of collection. The Trizol extraction method, with some modifications to the original protocol by Chomczynski (1993), was employed. In this process, the individual whole bee bodies were placed in 1 mL of TRIzol and crushed using sterile pestles. The resulting lysate was then homogenized by pipetting it up and down several times. To further facilitate the breakdown of the bee tissues, the lysate was sonicated for a total of 10 cycles, with each cycle consisting of a 30-second pulse followed by a 30-second rest at 4ºC. After the homogenization and sonication steps, the samples

were transferred to a rocker and incubated for 10 minutes at room temperature. Following this, centrifugation was carried out at approximately 15,000 g for 10 minutes at 4ºC. This step allowed for the separation of the supernatant from the rest of the sample. The resulting supernatants were carefully transferred to fresh RNase free tubes and incubated for an additional 5 minutes at room temperature to ensure complete dissociation of the nucleoproteins. To further purify the RNA, 200 µL of chloroform was added to each tube, which was then vigorously vortexed. The samples were then incubated on a rocker at 4ºC for 10 minutes before being centrifuged at 15,000 g for 20 minutes at 4ºC. This centrifugation step facilitated the separation of the aqueous phase from the rest of the sample. The aqueous phase, containing the desired RNA, was carefully transferred to a new tube. To precipitate the RNA, 600 µL of isopropanol was added to each sample, followed by thorough vortexing. The samples were then incubated at -20ºC for 10 minutes. Subsequently, centrifugation at 15,000 g for 15 minutes at 4ºC was performed to separate the RNA pellet from the supernatant. The supernatant was discarded, and the RNA pellet was washed with 600 µL of 70% ethanol. The pellet was pipetted up and down three times and then centrifuged at 15,000 g for 5 minutes at 4ºC. After the ethanol wash, the supernatant was discarded, and the RNA pellet was air dried for 5 minutes. Finally, the RNA pellet was resuspended in 50 µL of molecular-grade RNAse-free water. The quality and quantity of the extracted RNA were assessed using a NanoDrop ND 1000 spectrophotometer (Thermo Scientific). To ensure preservation, the RNA extracts were stored at -80°C. This method of RNA extraction from individual bees using whole-body tissue, with the Trizol extraction method and the modifications made to the protocol, allowed for the successful isolation and collection of RNA for further analysis. The

rigorous steps employed in this process ensured the purity and integrity of the extracted RNA, making it suitable for future molecular studies and investigations.

3.2.3 Transcriptional Analysis

Gene expression of twelve major antioxidant and developmental genes were evaluated from three whole honey bee samples taken from timepoint day 5 and day 9 (Acetylcholinesterase 2, Apisimin, Apidaecin, Major royal jelly protein 1, Sodesque, Cytochrome P450, Selenoprotein T, Selenoprotein K, Inositol-requiring enzyme 1, X-box binding protein, Derlin 1 and Heat shock 70-kDa protein cognate 3). cDNA was produced from total RNA using BioRad iScript Kit following the manufacturer's protocol. Previously published primers were confirmed on their respective targets and used in this study, found in Table 1. The twelve target genes were normalized against two housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein S5) known for their stability in honey bee tissues (Scharlaken et al., 2008; Alburaki et al., 2017). All RT-qPCR runs consisted of 3 biological replicates per treatment and time combination and each biological sample was run with three technical replicates per gene using the following cycling protocol: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and a final melting step of 95°C for 10 s and a 0.5°C increment for 5 s from 65°C to 95°C. All qPCR plates were normalized using an inter-plate calibrator ran on each plate and the two housekeeping genes (Table 1). Gene analysis was conducted using relative normalized expression calculated via the ΔΔCt values through the BioRad Maestro Software. Datasets were subsequently transferred to the R environment (Team 2011) for statistical analysis and figure generation.

Table 1. List of the target genes analyzed in this study. Primer sequence for each gene and amplicon size is given as well as the NCBI accession number. Two housekeeping genes known for their stability across honey bee tissues were used to standardize the qPCR.

Gene	Name	F	R	Size bp	NCBI Accession
Target Genes					
AChE-2	Acetylcholinesterase 2	GACGCGAAGACCATATCCGT	TCTGTGTCCTTGAAGTCCGC	140	NM_001040230.1
Apis	Apisimin	TAGCTGCCTTCTGCGTAGCC	CACGTTCGATTCGCCTTTGACAC	80	NM_001011582.1
Apid	Apidaecin	GGAATACCAACCTAGATCCGCCTA	TATTGCCGGGTTCAGCTTT	162	NM_001011613.1
mrjp1	Major royal jelly protein 1	TGACCAATGGCATGATAAGATTTT	GACCACCATCACCGACCT	98	NM_001011579.1
Sodq	Sodesque	ATTTACCGCTTAGATGTTATGTAGGAG	AGCACACGCAAATTTATTACTTCCA	179	XM_006558333.2
cp450	Cytochrome P450 305	CAGGTGATATATTGGCAAAAGCTACGA	TGTGGCCCTTTACCAGGAAC	94	XM_623618.6
SelT	Selenoprotein T	GACAGCCACCAGCATCATTG	TGGACCACACAGGAACATCAT	150	XM_623426.6
SelK	Selenoprotein K	TGGAAGCGTTTTATGTGGTACT	ATCTACGAGTTGGTGGACGTG	182	NM_001278332.1
Ire1	Inositol-requiring enzyme 1	GGGCAAAAATTGGTTCAGTCAA	CAGCTACGGATCGTCCATCA	320	XM_006565606.3
Xbp1	X-box binding protein 1	GGAACAACACAGCTGGCATC	TTTTGATGTCTGCCCCACCA	275	XM_392383.7
Derl-1	Derlin 1	TCCATGGGTATTGTTTGCGT	CGTCCCCAATTATGACCACCA	267	XM_001122972.5
Hsc70	Heat shock 70-kDa protein cognate 3	TCGAAGTCGTGCTGAGAGTA	CCATCAGCAGTAAATGCCACA	350	NM_001160052.1
Housekeeping Genes					
RpS5	Ribosomal protein S5	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA	115	XM_006570237.3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	TACCGCTTTCTGCCCTTCAA	GCACCGAACTCAATGGAAGC	142	XM_393605.7

3.2.4 Quantification of Protein Damage

A thorough assessment was conducted to evaluate the potential post-transcriptional damage caused by the various treatments administered. To achieve this, a protein carbonyl content assay was carried out on the last sampling date (Day 9). The aim was to quantify the extent of protein damage induced by the treatments. In order to conduct the assay, protein was extracted individually from each bee in triplicate. A protein extraction buffer, composed of 20mM Tris-HCl pH 8.0, 30mM NaCl, and 10% glycerol, was used for this purpose. The tissues were carefully crushed using a pestle and subsequently sonicated utilizing a Bioruptor Pico (Diagenode) sonication device. The sonication process involved 10 cycles, with each cycle comprising a 30-second pulse and a 30-second rest, all performed at a temperature of 4°C.

Following sonication, the homogenates were subjected to centrifugation at 5,000 g for 10 minutes at 4°C. This step ensured the separation of the supernatants from the remaining debris. The supernatants, containing the protein samples of interest, were then collected for further analysis. To estimate the protein carbonyl contents in the samples under investigation, a Sigma-Aldrich Kit (MO, USA) was utilized. The estimation process was carried out in accordance with the manufacturer's protocol, ensuring accuracy and reliability in the results obtained.

By conducting this protein carbonyl content assay, valuable insights were gained into the potential post-transcriptional damage caused by the various treatments administered. The results obtained shed light on the extent of protein damage and provide a basis for further understanding the impact of these treatments on the overall health and well-being of the bees. This information is crucial in formulating effective strategies to mitigate any negative effects and ensure the preservation of bee populations and their important contributions to ecosystems.

3.2.5 Statistical Analysis

The experiment involving cages was conducted at an individual bee level, with a total of eight treatments. Each treatment had three biological replicates, and each gene had three technical replicates. All statistical analyses for this study were performed using the R environment, specifically RStudio version 2022.12.0+353. To begin, the Shapiro test was used to check the normality of each dataset. Syrup consumption, which was recorded daily, was then calculated at the cage level for each of the eight groups under study. An ANOVA was conducted at a 95% confidence interval on normally distributed data with three levels of significance ($P < 0.05$*, < 0.001**, < 0.0001**). For data that did not pass the normality

test, the Kruskal-Wallis rank test, a nonparametric test, was employed. Multiple comparisons and p-values were adjusted using the Benjamini-Hochberg method with the "FSA" Library. To assess the survival probability and cumulative hazard of each treatment group, the Kaplan-Meier survival probability model was utilized in R. This analysis relied on three packages: "dplyr," "survival," and "survminer." Figures illustrating the results were generated in the same environment, making use of four main libraries: "ggplot2," "doby," and "plyr." For gene regulations, relative normalized expression was displayed per date and overall averages. Heatmaps were created using the "pheatmap" library, illustrating gene expression either by date (Day 5 and 9) or overall. To examine the correlation between gene regulations in different treatments, the R libraries "PerformanceAnalytics" and "corrplot" were employed, with an intermediate level of significance ($p < 0.01$). Principal coordinate analysis (PCA) was conducted using the "factoextra" library to estimate the expression of each variable on a three-dimensional scale and determine the similarity between treatment groups. Additionally, box and whisker plots were constructed to visualize the data, displaying the median, first and third quartiles, as well as the maximum and minimum values for each condition.

3.3 Results

3.3.1 Toxicity of Stressors

The Kaplan-Meier model showed significant differences ($p < 0.001$) in survival rates among administered stressors, Figure 1. The lowest level of mortality during the 11-day

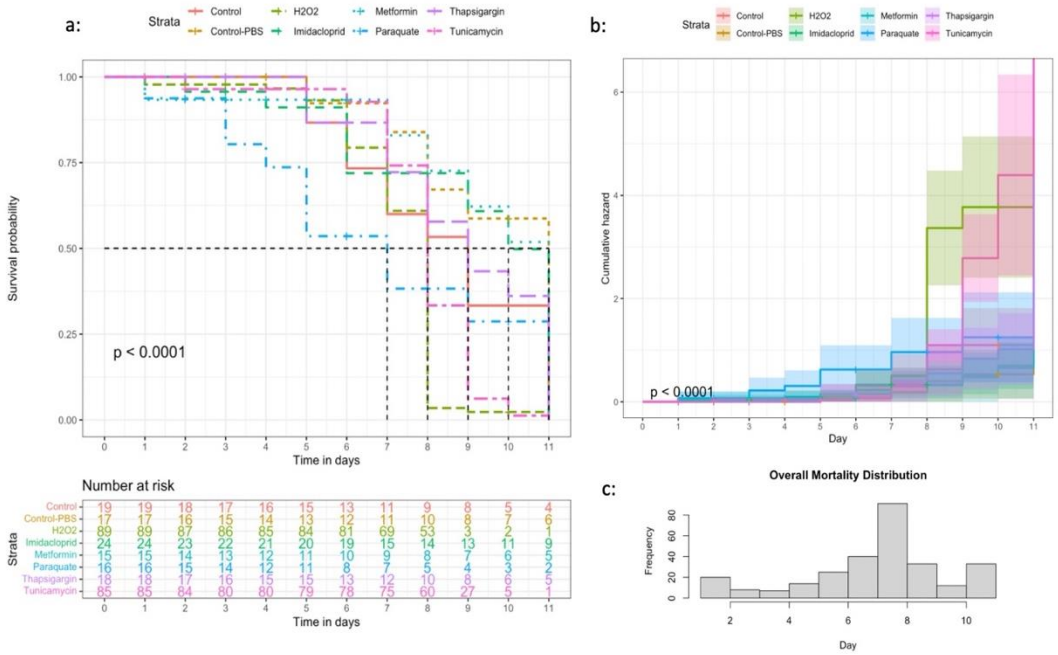

*Figure 1. Kaplan-Meier survival probability (a) and cumulative hazard (b) models conducted on the honey bee groups subjected to six different treatments and two controls. The number of honey bees at risk is estimated through the same model for each treatment. Distribution of overall mortality throughout the experiment in day (c). Significant mortality differences among treatments were identified at $p < 0.001$***.*

experiment was recorded in cages exposed to the Control-PBS treatment while the lowest survival rate was observed in bees exposed to paraquat. Paraquat induced an early mortality starting at day 3 post-administration followed by exposure to hydrogen peroxide (H2O2), which led to 100% mortality at day 9. Metformin and imidacloprid had a significantly greater survival rate than the control, while tunicamycin led to lower survival rate compared to the control. Caged bees exposed to both thapsigargin, and control had similar

survival rates. The cumulative hazard of paraquat was chronic, started early at day 3 but did not lead to total mortality. However, hydrogen peroxide's cumulative hazard sharply increased at day 8 causing complete mortality of caged bees. For syrup consumption, caged

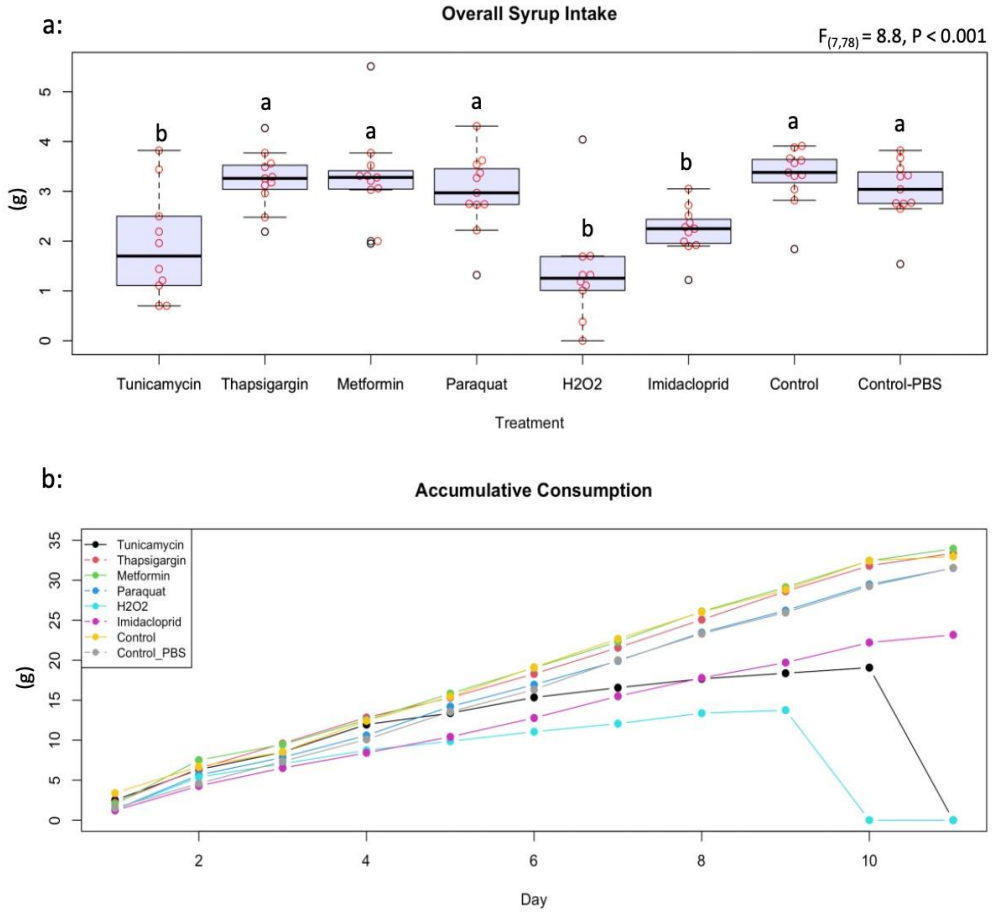

Figure 2. Average at libitum syrup intake (a) and accumulative syrup intake (b) displayed by treatment. Accumulative consumptions of the eight administered treatments and controls (tunicamycin, thapsigargin, metformin, paraquat, hydrogen peroxide, imidacloprid, control and control in PBS buffer) are shown in a longitudinal manner. ANOVA was conducted at three levels of significance(p<0.05, <0.001**, <0.0001***). Boxplots with different alphabetical letters are statistically significant.*

bees consumed significantly ($p < 0.001$) lower amounts of tunicamycin, H_2O_2, and imidacloprid compared to all other treatment compounds including both controls, Figure

1.2a. No significant differences were found in the amount of syrup consumed among thapsigargin, metformin, paraquat, and both controls. The accumulative consumption graph confirms this finding over the 11-day experiment, Figure 1.2b.

3.3.2 Transcriptional Analysis and ER Stress

Honey bee response to stresses induced by pharmacological molecules and agricultural pesticides was evaluated by identifying the transcript level of genes involved in the mitigation of oxidative and ER stress. AChE-2 was significantly ($p < 0.001$) upregulated in day 9 compared to day 5 irrespective of the treatments, while both apisimin and apidaecin showed no effect of time or treatment on their regulation, except higher ($p < 0.05$) regulation of *apisimin* at day 5 for metformin compared to imidacloprid, Figure 3.

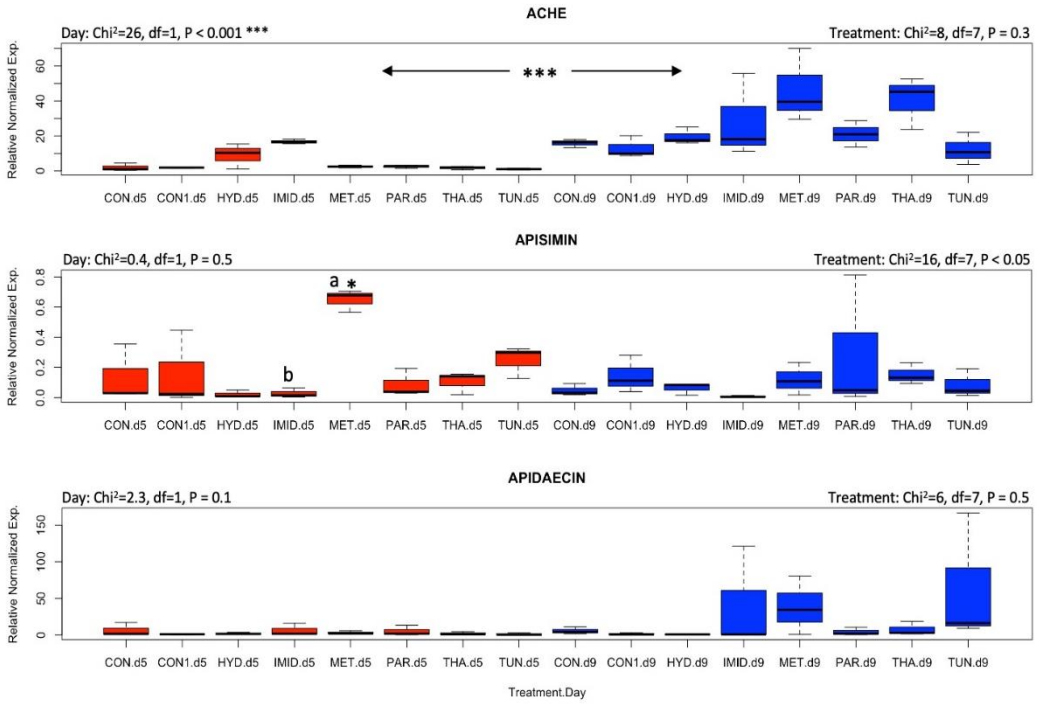

Figure 1. Overall gene expression of AChE, apisimin and apidaecin across eight treatments and days of exposure (Day 5: red boxplots and day 9: blue boxplots). Non-parametric Kruskal-Wallis test was conducted at a 95% confidential interval with three levels of significance (p < 0.05, < 0.001**, < 0.0001***) to determine statistical differences among treatments and dates. Boxplots with different alphabetical letters are statistically significant.*

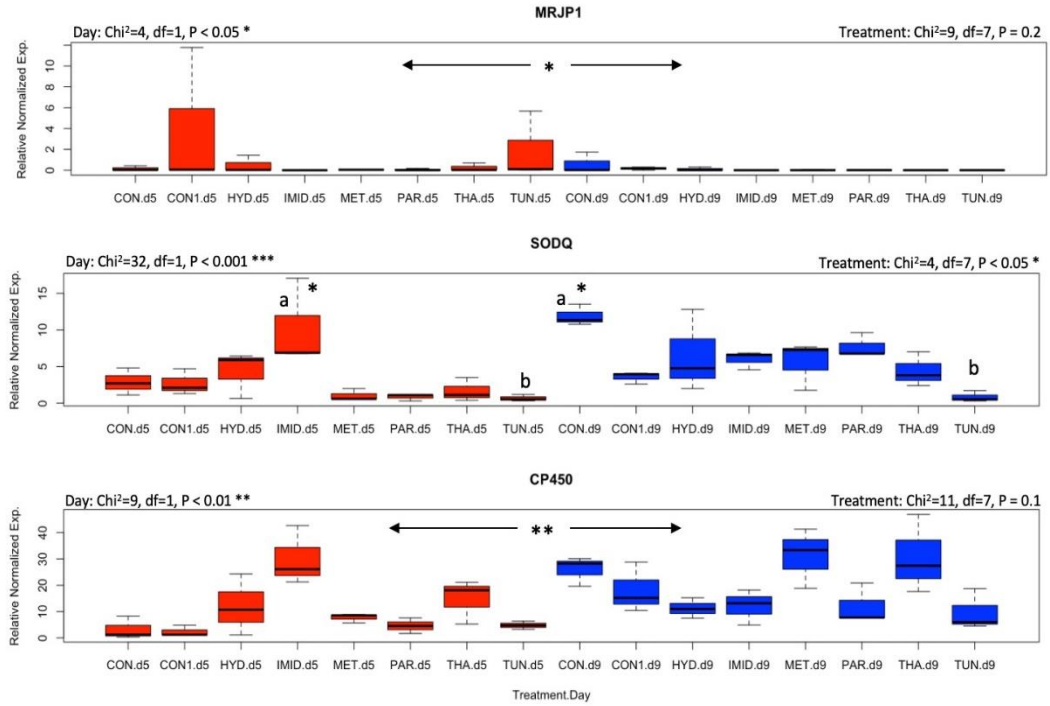

Figure 2. Overall gene expression of mrjp1, Sodq and cp450 across eight treatments and studied dates (Day 5: red boxplots and day 9: blue boxplots). Non-parametric Kruskal-Wallis test was conducted at a 95% confidential interval with three levels of significance (p < 0.05, < 0.001**, < 0.0001***) to determine statistical differences among treatments and dates. Boxplots with different alphabetical letters are statistically significant.*

Treatments did not affect the regulation of *mrjp1*, however, an overall significant ($p < 0.05$) drop in its regulation was observed at day 9, Figure 4. A similar finding was recorded for the regulation of *P450* with significant ($p < 0.05$) upregulation at day 9. Regulation of *Sodq* gene however, was not affected by time, and was significantly higher ($p < 0.05$) for imidacloprid at day 5 compared to tunicamycin, and significantly upregulated for the control compared to tunicamycin at day 9, Figure 4.

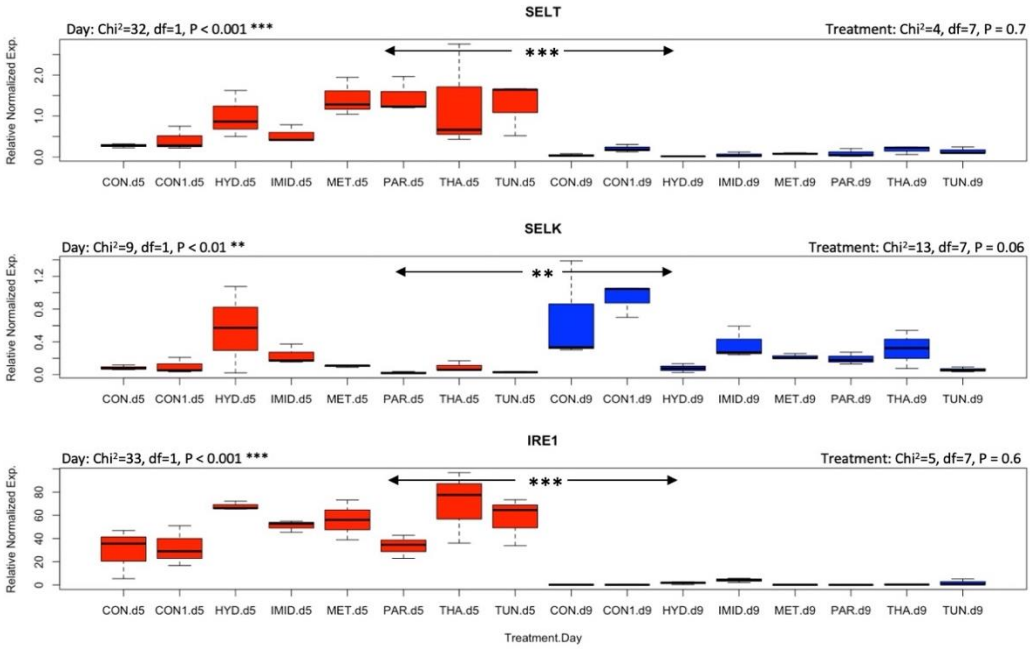

Figure 5. Overall gene expression of SelT, SelK and Ire1 across eight treatments and studied dates (Day 5: red boxplots and day 9: blue boxplots). Non-parametric Kruskal-Wallis test was conducted at a 95% confidential interval with three levels of significance ($p < 0.05$, < 0.001**, < 0.0001***) to assess statistical differences among treatments and dates.*

Both studied selenoprotein genes (*SelT* and *Selk*) were not affected by the treatments similar to *Ire1,* Figure 5. Nonetheless, each of these three genes changed regulations vis-à-vis time. *SelT* and *Ire1* were downregulated in day 9, while *SelK* was upregulated in day 9, Figure 5. *Xbp1* was significantly ($p < 0.05$) upregulated for metformin and thapsigargin compared to imidacloprid at day 5 with a significant ($p < 0.001$) overall downregulation at day 9 irrespective of the treatments, Figure 6. Regulations of both *Derl-1* and *Hsc70* were not affected by the treatments, Figure 6.

3.3.3 Gene Interaction and Correlation

The study of the overall gene regulation displayed as heatmaps revealed three different sets of genes: 1- Genes upregulated (Ire1, AChE-2 and Cp450) and 2- Gene downregulated (SelK, SelT, Apisimin, mrjp1) and 3- Genes with mix regulations

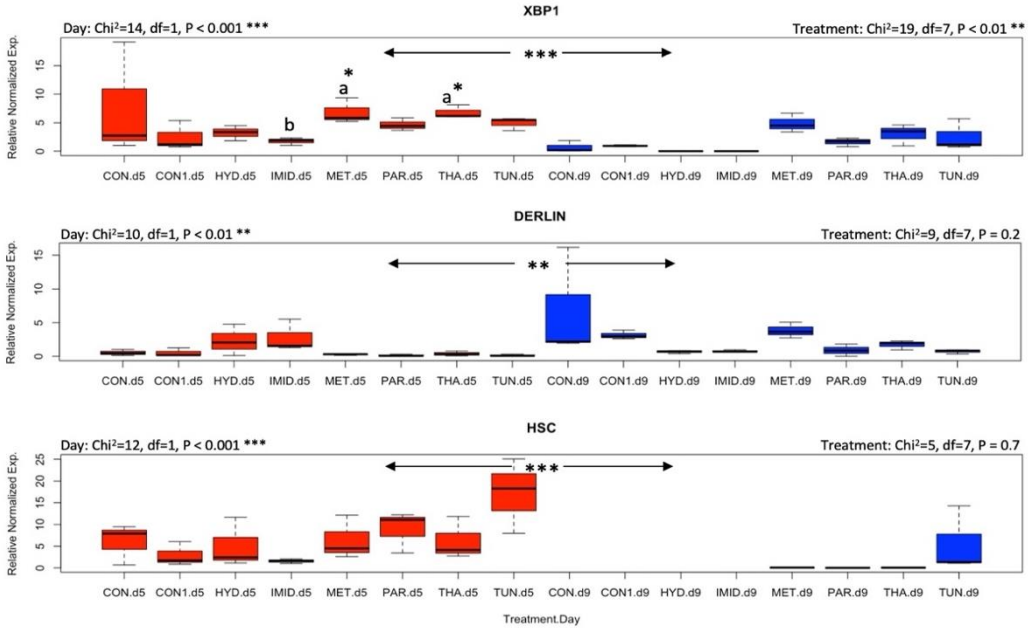

Figure 6. Overall gene expression of Xbp1, Derl-1 and Hsc70 across eight treatments and studied dates (Day 5: red boxplots and day 9: blue boxplots). Non-parametric Kruskal-Wallis test was conducted at a 95% confidential interval with three levels of significance (p < 0.05, < 0.001**, < 0.0001***) to assess statistical differences among treatments and dates. Boxplots with different alphabetical letters are statistically significant.*

(Apideacin, Sodq, Hsc70, Xbp1, Derl-1), Figure 7a. The heatmap dendrogram distinguished four genes which exhibited the closest similarity in their overall regulations across treatments (CONT, PAR, MET, THA), Figure 7a. However, this pattern of regulations was not constant and differed by dates. For instance, at day 5, the highest expressed gene among all treatments and genes was Ire1, while at day 9 upregulations were observed for Apid, Sodq, AChE-2 and Cp450 genes, Figure 7b. The correlation analysis conducted on the overall and date-by-date gene regulations and treatments reveals significant ($p < 0.01$) positive correlations within each date only, Figure 8. No significant negative correlations among genes were found at any time point.

3.3.4 Principal Component Analysis

The PCA conducted on the regulation of all studied genes revealed that 40.7% of the variables were expressed on Dim1, 27.8% on Dim2 and 12.4% on Dim3, Figure 9. According to the estimated number of clusters (K=3-4) and the visual distribution of the individual variables (Treatments), the PCA discriminates on Dim1 (40.7%) and Dim 2 (27.8%), four major groups which are: 1-(HYD and IMID), 2- (CONT and CONT1), 3- (MET and THA) and 4- (PAR and TUN), Figure 9a. However, on Dim1 (40.7%) and Dim 3 (12.4%), treatments grouped into three clusters only: 1- (HYD, CONT1, IMID), 2- (CONT) and 3- (TUN, THA, PAR, MET), Figure 9a.

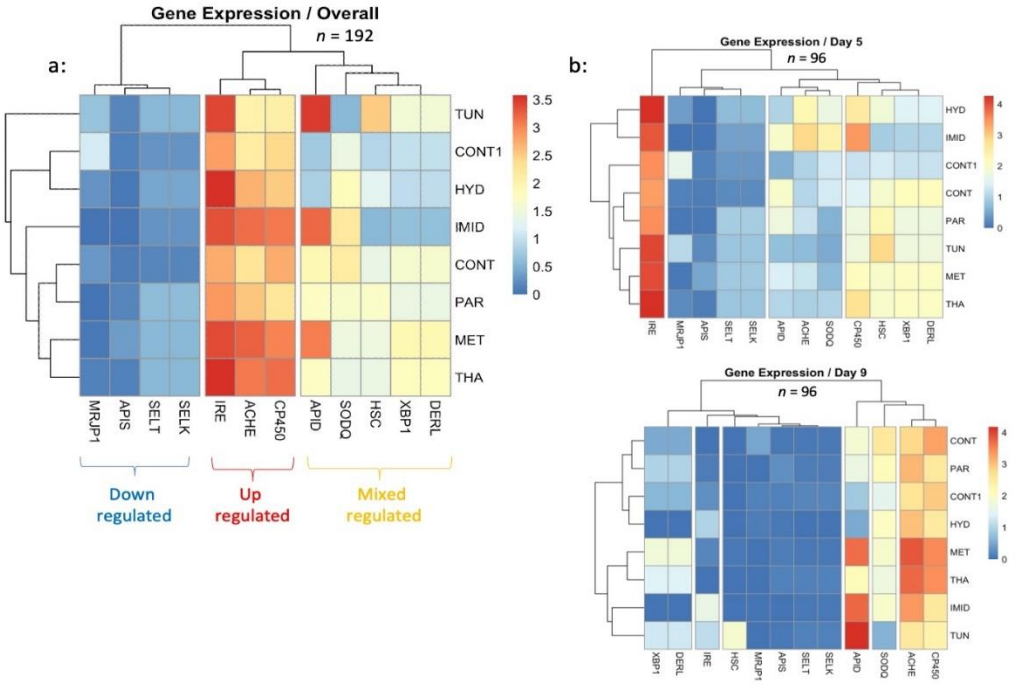

Figure 7. Heatmaps conducted on the overall regulation of the twelve studied genes in eight different treatments (a) as well as their regulations per date (b), (Day 5 and 9). Analysis of the overall gene regulation distinguished three major gene clusters showing upregulated (Ire1, AChE-2, Cp450), downregulated (SelK, SelT, Apis, mrjp1), and mixed regulated genes (Apis, Sodq, Hsc70, Xbp1, Derl)

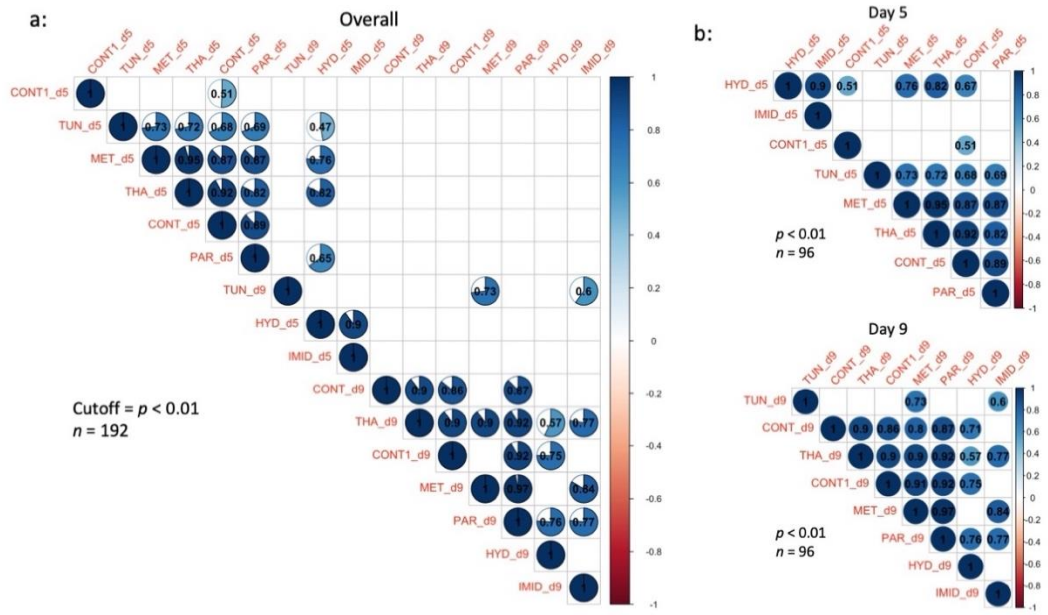

Figure 8. Correlation matrices of the gene regulation conducted on the eight studied treatments and displayed by overall (a) and date-by-date expressions (b). Correlation analysis was conducted at an intermediary level of significance ($p < 0.01$). Correlation R-values are given in each circle and blank squares represent non-significant correlations at the cutoff level of $p < 0.01$.

Concerning the variable behavior (Regulation of the genes) regarding the treatments, the PCA displayed by "variables" showed two sets of genes with opposing function and regulation vis-à-vis the treatments: 1-(*cp450, Sodq)*, and 2- (*Ire1, Apis, SelT, SelK, Apid, Xbp1, Derl-1, Hsc70)*, while *mrjp1 and AChE-2* exhibited neutral regulation, Figure 9b.

3.3.5 Oxidative Stress and Protein Damage

The protein carbonyl contents assay was conducted on caged bees sampled at day 9 of the treatment. The highest carbonyl contents were identified in bees fed paraquat and

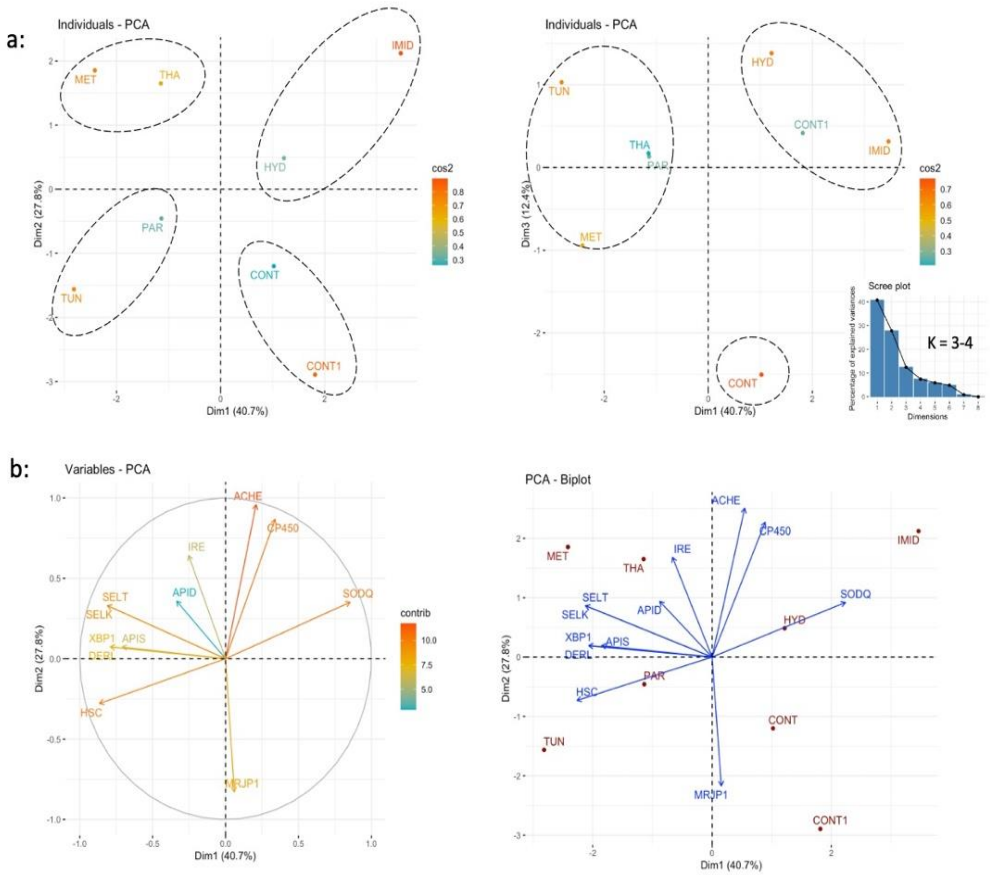

Figure 9. Principal Component Analysis (PCA) conducted on the overall regulation of the twelve studied genes in eight treatments. Percentages of individual variables expressed on components 1 and 2, 1 and 3 (a) graphically visualized in a 3-dimensional space. Expression and direction of each variable (genes) on Dim1 and 2 are given along with the PCA biplot of both expressed variables and treatments on Dim1 and 2 (b). The scree plot shows the mathematically calculated number of estimated groups (k).

imidacloprid, which contained significantly ($p < 0.001$) higher protein damage than all other treatments. The control_H2O, metformin, thapsigargin, and tunicamycin did not statistically differ in their carbonyl contents, Figure 10. The significantly lowest protein damage was identified in the control syrup containing PBS buffer (Control_PBS), Figure 10.

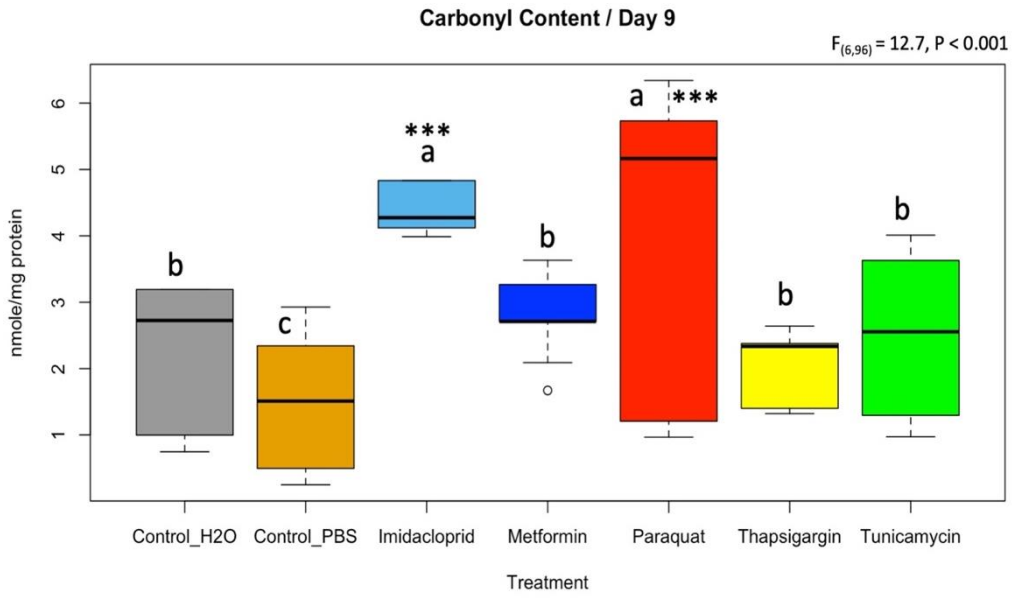

Figure 10. Level of protein damage quantified by Protein Carbonyl Content Assay on honey bee samples of Day 9 subjected to different treatments. ANOVA was conducted at a 95% confidential interval with three levels of significance ($p < 0.05$, < 0.001**, < 0.0001***) to assess statistical differences among treatments. Boxplots with different alphabetical letters are statistically significant.*

3.4 Discussion

The relationship between sublethal doses of pharmacological inducers and agricultural pesticides on honey bee gene regulation and oxidative stress has been the subject of extensive research. This study aimed to shed light on the effects of various oxidative stress inducers, including paraquat (herbicide), imidacloprid (neonicotinoid insecticide), and hydrogen peroxide (cellular byproduct of oxidative stress), as well as newly tested pharmacological compounds and antibiotics. Oxidative stress, which refers to the imbalance between reactive oxygen species (ROS) and a biological system's ability to neutralize them, can cause significant damage to living organisms (Pizzino et al., 2017). This imbalance can lead to endoplasmic reticulum (ER) stress, a condition where proteins are improperly folded or conformed (Yamamoto and Ichikawa, 2019). Honey bees, with

their short-lived worker bees and complex social structure, provide an excellent model for studying oxidative stress and its association with senescence (Kramer et al., 2021). ER stress is also used to investigate diabetes by examining insulin-producing cells in drosophila (Katsube et al., 2019). The toxicological analysis conducted in this study revealed that paraquat exhibited the most chronic toxicity among the tested molecules, even at sublethal concentrations (Figure 1). Paraquat triggers the production of ROS by accepting electrons from photosystem I and transferring them to molecular oxygen (Kennedy et al., 2021). Interestingly, the transcriptional results did not indicate a specific gene response to counteract the effects of paraquat on honey bees. However, the highest levels of protein damage were observed in honey bees exposed to this treatment (Figure 10). Furthermore, the honey bees exposed to tunicamycin showed the closest overall gene regulation similarity to those exposed to paraquat, as evidenced by the PCA analysis (Figure 9a). Previous studies have shown that tunicamycin and oxidative stress work synergistically in C. elegans studies involving ER stress (Taylor et al., 2021). Similar results were also observed in drosophila experiments, where genes involved in oxidative stress resistance were transcriptionally affected by paraquat, hydrogen peroxide, and tunicamycin (Girardot et al., 2004). Tunicamycin, an antibiotic that inhibits N-linked glycosylation, induces ER stress (Yamamoto and Ichikawa, 2019). In summary, this study highlights the potential impact of pharmacological inducers and agricultural pesticides on honey bee health. It emphasizes the significance of oxidative stress and ER stress in understanding the physiological responses of honey bees and other organisms. Further research is warranted to explore the intricate mechanisms underlying these responses and to develop strategies for mitigating the detrimental effects of oxidative stress on honey bee

populations. By safeguarding the well-being of honey bees, we can protect the vital role they play in pollination and the overall ecosystem.

Chemical exposure can have detrimental effects on honey bees, impacting their health and overall well-being. In a recent study, researchers investigated the toxicological effects of various substances on honey bees, including tunicamycin, paraquat, neonicotinoids, and metformin. One of the key findings of the study was that tunicamycin, an antibiotic, exhibited acute toxicity at day 8 of exposure. This was evidenced by an induced transcriptional response for Hsc70, a heat shock 70-kDa protein that plays important roles in cellular function and homeostasis. The higher regulation of Hsc70 at day 5 seemed to alleviate the rate of protein damage observed at day 9 for tunicamycin, which was one of the most toxic molecules tested. Furthermore, the study confirmed previous findings on the exposure to neonicotinoids, a group of insecticides commonly used in agriculture. It was found that exposure to imidacloprid, a specific neonicotinoid, led to elevated expressions of AChE-2, an enzyme associated with neurological function. This finding aligns with previous investigations reporting avoidance of sugar syrup tainted with imidacloprid by honey bees. Additionally, significantly higher protein damage was observed in caged bees exposed to this insecticide. Interestingly, the study also revealed that exposure to metformin, a medication commonly used to treat diabetes, resulted in the upregulation of Apisimin, a gene associated with honey bee nutrition and antimicrobial defense. This finding suggests a potential impact of metformin on honey bee health, although further research is needed to fully understand its implications. Overall, this study highlights the importance of understanding the impact of chemical exposure on honey bees. The findings emphasize the need for responsible and informed chemical usage to ensure

the preservation of honey bee populations and their crucial role in pollination and ecosystem health. Continued research in this field is essential to develop sustainable agricultural practices that minimize the negative impact on honey bees and other pollinators.

Honey bees play a crucial role in our ecosystem as pollinators, ensuring the reproduction of countless plant species. However, their populations have been facing numerous challenges in recent years, including exposure to various environmental stressors and pesticides. Understanding the genetic responses of honey bees to these factors is essential for developing effective conservation strategies. In this article, we will delve into the latest research findings that shed light on the intricate genetics underlying honey bee health. A recent study conducted by Lv et al. (2023) examined the impact of different treatments, including tunicamycin, hydrogen peroxide, and imidacloprid, on honey bees. While tunicamycin is associated with detoxification properties, the study revealed that the treatments may not have reached lethal levels, thus inhibiting the detoxification process. The authors also observed downregulation of mrjp1, a gene involved in behavioral development, and the absence of regulation in Hsp70, a gene associated with stress responses (Oliveira et al., 2022). These findings suggest that the stress experienced by bees, such as confinement, can disrupt their normal physiological processes. The study also explored the impact of hydrogen peroxide, which produces destructive hydroxyl free radicals capable of damaging essential cellular components (Brudzynski, 2020). Interestingly, the bees actively avoided hydrogen peroxide, indicating their ability to recognize and mitigate potential threats. Additionally, the gene response to hydrogen peroxide exhibited similarities to that of imidacloprid, a widely used insecticide (Figure

9a). These results highlight the intricate interplay between environmental stressors and the genetic response of honey bees. Furthermore, the study uncovered crucial data related to genes potentially linked to the behavioral caste development in honey bees (Figures 4, 5, 6). The regulation of genes such as mrjp1, Cp450, SelT, SelK, Derl-1, Ire1, and Hsc70 was found to be dependent on the age of the honey bees, irrespective of the treatments administered. Mrjp1, in particular, has been previously identified as a physiological marker for behavioral development (Corona et al., 2023). However, further investigations are required to fully understand the age-related regulations of the other genes and their implications for honey bee development. In conclusion, the study observed signs of disinclination in post-ingestion and enhanced survivorship in bees treated with tunicamycin, hydrogen peroxide, and imidacloprid compared to those in the control group fed sugar syrup. Although a few antioxidant genes showed significant regulation under different treatments, the research shed light on age-related regulation of other major genes, revealing positive correlations between them. Notably, the treatments of paraquat and imidacloprid resulted in significant protein damage in honey bees when administered over an extended period (11 days). These findings underscore the importance of considering the genetic responses of honey bees to various stressors in efforts to safeguard their health. Understanding the intricate genetics underlying honey bee health is crucial for mitigating the threats they face and ensuring their vital role as pollinators. The research discussed in this article provides valuable insights into the genetic responses of honey bees to environmental stressors and highlights the age-related regulation of essential genes. By unraveling these mechanisms, scientists and conservationists can develop targeted

interventions to protect honey bee populations and safeguard the biodiversity and productivity of our ecosystems.

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