Biomarkers of Oxidative Stress in Atrazine-treated Honey Bees: A Laboratory and In-hive Study

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ABSTRACT

The decline of honey bee (*Apis mellifera)* colony numbers in recent years presents an economic and ecological threat to agriculture. One outstanding threat to honey bees is the unintended exposure to agricultural pesticides. Previous studies report that acute exposures to the common-use herbicide atrazine elicit oxidative stress in non-target insects; however, little information is currently available on the exposure risk of atrazine to honey bees. This project examined biochemical and molecular oxidative stress response markers of honey bees following laboratory and field treatments of atrazine. Laboratory experiments were conducted with honey bees exposed to increasing concentrations of atrazine for 24 h whereas hive experiments were conducted with bees exposed to one sub-lethal concentration of atrazine for 28 d. The overall antioxidant enzyme activities of atrazine-treated honey bees were decreased compared to the untreated honey bees in both the laboratory and hive experiments. After exposure to atrazine in the laboratory and field, semi-quantitative RT-PCR analysis of antioxidant-encoding genes revealed the differential expression of genes in atrazine-treated bees that are important for oxidative stress tolerance in the laboratory and field experiments. Here, we provide evidence that the laboratory and hive exposure of honey bees to the common-use herbicide atrazine results in oxidative stress responses that can compromise the health

of bee colonies. The data will be discussed with regard to the protection of these pollinators against the untended exposure of agricultural pesticides.

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GENERAL AUDIENCE ABSTRACT

The pollination service provided by insects, primarily honey bees, is estimated to contribute approximately one-third of the diet consumed by the average American. Honey bees are vitally important pollinators due to their broad range of foraging activities and ease of husbandry within a managed colony. In recent decades, colony numbers have decreased in the developed areas of the planet and pesticide usage has been implicated in these losses. Atrazine is the second most commonly used agricultural herbicide in the country and has been linked to oxidative stress in beneficial insects in the past. Oxidative stress is the result of an uncontrolled build-up of reactive oxygen species in an aerobic organism. These reactive oxygen species are dangerous because they are capable of damaging proteins, DNA, and cell walls. Every aerobic organism also possesses antioxidant function which serves to prevent or counteract damage caused by reactive oxygen species. This study examined antioxidant enzyme activities and antioxidant-encoding gene expression levels, which were used as indicators of oxidative stress biomarkers, in honey bees exposed to atrazine in the laboratory and in the hive environment. Honey bees were exposed to atrazine at increasing concentrations in the laboratory for 24 h and at one environmentally relevant dose for 28 d in the hive. After exposure to atrazine in the laboratory and the hive, four out of five antioxidant enzyme levels of honey bees decreased which implied an increase in oxidative stress and a

decrease in antioxidant defenses. Activity of one enzyme, lipid peroxidase, increased in honey bees after exposure to atrazine. Lipid peroxidase is the most common measure of cellular injury during oxidative stress, once again signifying an increase in reactive oxygen species production and oxidative stress. Expression levels of seven antioxidant-encoding genes were examined in honey bees after atrazine exposure and expression levels of some genes changed compared to the untreated control and expression levels in some genes remained the same compared to the untreated control. These changes in antioxidant-encoding gene expression levels may imply an increase in oxidative stress due to exposure of the honey bees to atrazine. This study aimed to examine biomarkers of oxidative stress in honey bees exposed to the commonly used herbicide atrazine with the hope of raising awareness of harmful effects caused by atrazine and protecting these important pollinators from unintended exposure to agricultural pesticides.

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LITERATURE REVIEW

1.1 BASIC BIOLOGY OF THE HONEY BEE

Honey bees are within the order Hymenoptera, which includes wasps, bees, ants, and sawflies. Bees evolved from sphecoid wasps approximately 100 million years ago alongside the appearance of flowering angiosperms. The evolution of bees is believed to be linked to the diversification of these angiosperms, which evolved with different colors, shapes, excess nectar and pollen as reward to bees which could transfer their pollen to nearby plants (Winston 1987). There are approximately 20,000 species of bees contained within 7 families and 150 genera. The honey bee is contained in the family Apidae, in the genus *Apis.* There are currently seven species classified within this genus, although historically the number of species has ranged between six and eleven (Caron 1990). Two of the most notable species within this genus are *A. mellifera*, the western or European honey bee, and *A. cerana*, the eastern or Asian honey bee, which are known for their ability to pollinate a number of crops, store sizable amounts of honey, and can be maintained and manipulated in man-made hives. Honey bees, with the exception of *A. mellifera,* originated and are native to South and Southeast Asia. *Apis mellifera* is believed to have originated in eastern Africa and later spread to Europe and Asia, with a native range spanning from Scandinavia to central Asia and Africa. *Apis mellifera* was deliberately introduced to the Americas in 1622, and has since achieved a near-worldwide distribution (Whitfield 2006).

Honey bees are holometabolous, oviparous, and eusocial insects. Each population is comprised of one reproductive queen, around 20,000-40,000 sterile female workers, and a few hundred fertile males (or drones). Honey bees are haplodiploid insects with the female queens and workers developing from fertilized eggs and drones developing from unfertilized eggs laid by the queen. The queen is the only reproductive entity within the hive, and the ovaries of the working females undergo developmental suppression due to the presence of a pheromone excreted by the queen and brood. The sole purpose of the drones is to mate with the queen, they perform no tasks within the hive and are taken care of by worker bees for the warmer part of the year and are cast out of the hive by the workers when colder weather sets in. Three days after the queen lays an egg, the egg hatches as a larva with a total of five instars. These larvae are tended to by young nurse bees, and are fed either worker jelly if they are meant to become worker bees or royal jelly if the larva is meant to be a queen bee. After six days, the cell is capped by worker bees followed by pupation of the larva. The period of pupal development depends on the type of bee in the cell. The queen bee pupa develops in 7 days whereas worker and drone pupa develop in 12 and 14.5 days, respectively, before emerging as an adult.

The behavior of worker honey bees changes with age, a feature known as temporal polyethism. This phenomenon is controlled by juvenile hormone and vitellogenin (Robinson 1991). Newly emerged adult workers clean the cell after emergence and are referred to as nurse bees after their brood glands develop. Nurse bees stay near the center of the nest and tend to brood by feeding and capping pre-pupal cells, and attend to the queen. As the bees age, they move closer to the edges of the frames, working as comb and cell constructors, transporting and packaging pollen and honey into

cells, and guarding the entrance to the hive. Workers finally become foragers in the last stage of their life, approximately 20-40 days old, and continually leave the hive in search of pollen, nectar, propolis, and water. The progression of task maintenance is dependent on the state of the bees' hypopharyngeal, wax, and alarm pheromone glands, which is altered based on age and physiological development (Seely 1985). Although this order generally occurs in an age-dependent manner, bees may change tasks precociously or retain the same task longer than normal due to environmental conditions within the hive (e.g., dearth of resources or abundance of brood) (Robinson 1991).

1.2 HONEY BEES AS POLLINATORS

Pollination of crops by insects is currently valued at $212 billion worldwide, or approximately 9.5% of the value of total agricultural production (Gallai et al. 2009). Although the number of crops responsible for the majority of human caloric input are not dependent on animals for pollination, the value and acreage of animal-pollinated crops vs. non-animal pollinated crops has increased in the past 50 years (Gallai et al. 2009, Aizen and Harder 2009). Approximately 35% of the human diet is reliant on animal pollination, and of that, 80% is provided solely by honey bees (Klein et al. 2007). The European honey bee (*Apis mellifera)* is the most widely managed pollinator in the world due to their amenability to manipulation within a hive and generalist foraging capabilities. The pollination services which honey bees provide are responsible at least in-part for proper fruit and seed set, yield, and quality of 52 of the 115 leading foodstuffs globally. In the United States alone, over 2.4 million bee colonies are rented out annually for pollination services, at a value of approximately $120-150 per hive. There are a

number of native species responsible for pollination, but their numbers are not sufficient for global pollination and they are largely unmanageable (Gallai et al. 2009). Honey bees and native pollinators have seen fluctuations in numbers globally in recent decades, the factors of which seem to be multiple and interacting (Williams and Osbourne 2009).

1.3 HONEY BEE DECLINE

Global numbers of managed bee colonies have increased since the 1950's mostly in developing areas, but at a rate which is unsustainable with the demand for insect-dependent crop pollination (Aizen and Harder 2009). Colony numbers have declined in developed areas of the world since the 1960's, with the current losses between 1961 and 2007 decreasing in Europe by approximately 26.5% and North America by approximately 49.5% (FAO 2009). The decline of bee colony numbers in recent years presents an economic and ecological threat to agricultural systems and the pollination services provided by bees. Mass colony loss is not a new phenomenon, with alarming rates of decline reported in the United States in 1897, the 1960s and 1970s (Stokstad 2007). In the 1980s, there were ~ 78,000 managed bee colonies in Virginia. Today, there are ~ 40,000 bee colonies. Beekeepers and growers are losing an estimated $1.3 - 1.8 million annually (i.e., $120 - 150 per colony) from reduced honey sales, loss of pollination fees, and colony replacement costs. Nearly 12,000 bee colonies are rented on an annual basis for the pollination of agricultural crops across Virginia. This number exceeds two million bee colonies across the United States. Overwintering has historically been a leading factor in colony loss, with losses averaging 5-10% each year. In 2007–2008 in the United States, the average winter mortality was 35.8% with the total

loss in individual states ranging from 7.3% to 56.2% (vanEngelsdorp et al. 2008). An estimated 30% of colonies are lost every year in Virginia, a rate consistent with the national average. In 2015, the total managed colony loss rate in Virginia was 45.5%, which was slightly higher than the national colony loss rate of 42.1% (Lee et al. 2015)

These recent losses have been associated with a phenomenon referred to as Colony Collapse Disorder, or CCD. CCD was first described in 2006 and is characterized by the loss of adult bees, with no signs of a large die-off inside or outside of the hive. The colonies will generally have a queen present with a small retinue of adult bees, capped brood and undisturbed food stores (Cox-Foster et al. 2007, Oldroyd 2007, Stokstad 2007). Approximately 23% of major beekeeping operations in the United States claimed CCD losses over the winter of 2006–2007, with those responding beekeepers losing an average of 45% of their hives (vanEngelsdorp 2007). No one factor can be singled out as the cause of CCD; however, one hypothesis is that an infectious agent or group of agents is currently unknown to the beekeeping world, a theory supported by anecdotal evidence that reusing equipment from CCD hives can transmit the disorder (Cox-Foster et al. 2007).

1.4 FACTORS IMPLICATED IN HONEY BEE DECLINE

1.4.1 Varroa Mite

The varroa mite, *Varroa destructor*, has been proclaimed the most damaging parasite of honey bees today and has spread to almost every continent in which *A. mellifera* is found (vanEngelsdorp et al. 2007, Le Conte et al. 2010, Rosenkranz et al. 2010). Varroa mites inflict damage on honey bee hives by feeding on the hemolymph of brood and adult bees

and are capable of transmitting up to 21 different viruses. The feeding behavior of the Varroa mite causes physical damage to the bee, reduces the weight and protein content of the bee, and may disrupt certain organ development (Bowen-Walker and Gunn 2001). The viruses vectored by the mite can cause physical deformities and suppress the immune system of the bees, allowing for an increase in viral titers and co-infection with other pathogens (Yang and Cox-Foster, 2005, 2007; Le Conte et al. 2010). One virus, Israeli Acute Paralysis Virus (IAPV) was found to be strongly associated with CCD in a 2007 metagenomic survey of colonies (Cox-Foster et al.) Another virus, Deformed Wing Virus (DWV) is found ubiquitously in bee hives but may also be vectored by and replicate more prodigiously within the Varroa mite. DWV has been recently declared the single most important virus for colony health, and symptomatic adults experience vastly shortened lifespans and reduced vigor (Ryabov et al. 2014). Since the introduction of the Varroa mite in the 1980s, colony numbers in the United States have decreased from 4.5 million colonies in 1980 to 2.3 million in 2008 (vanEngelsdorp 2007, NASS 2009). Before the introduction of *V. destructor,* winter losses were estimated between 5–10% and after the introduction, losses ranged from 15–25% (vanEngelsdorp et al. 2008).

1.4.2 Pathogens

Known pathogens of honey bee colonies include bacteria, fungus, parasitic mites, and viruses. Bacterial pathogens of bees include American Foulbrood (*Paenibacillus larvae*) and European Foulbrood (*Melissococcus plutonius*). American Foulbrood is historically considered to be the most serious ailments affecting honey bees and treatment with antibiotics may be suggested for hives with low-level infections; however, most states

mandate burning of infected hives in order to stop the spread of this pervasive disease. European Foulbrood is a less severe bacterial infection associated with colony stress and easily treated with antibiotics or alleviation of environmental stressors. Fungal pathogens of honey bees include Nosema (*Nosema apis* and *Nosema ceranae*) and Chalkbrood (*Ascosphaera apis*). Chalkbrood affects larvae which are then overgrown by fungal mycelia and the disease is characterized by larval chalk-like "mummies". Chalkbrood is thought to be caused by environmental stressors and may resolve itself through requeening or alleviation of environmental stress (Caron 1990). Nosema is a microsporidian disease complex in bees composed of two species, *Nosema apis* and *Nosema ceranae.* Historically *N. apis* was thought to be the only infective species in honey bees, but in 1994 a new species, *N. ceranae*, was found in the Asian honey bee (*Apis cerana)* (Fries et al. 1996). In 2006 it was discovered that *N. ceranae* had broadened its host range to include *A. mellifera*, and it has quickly spread across the world with some hypothesizing an eventual displacement of *N. apis* (Paxton et al. 2007). Nosema spores are ingested through contaminated food or comb and infect the epithelial cells of the adult honey bee's midgut (Webster 1993). *Nosema apis* infection is typically characterized by dysentery-like symptoms found on the front of the hive, and infection with either species is thought to suppress immune function and causes a reduction in the life span of the bee (Higes et al. 2007). Viral pathogens present another challenge to the immune system of the bee, with the Varroa mite vectoring numerous viruses, the most debilitating of which were previously described; however, other viral pathogens exist within the hive including Sacbrood virus (*Morator aetatulas*). Sacbrood is analogous to

influenza in honey bee brood, with appearance fluctuating according to the season. Treatment for Sacbrood includes requeening and mitigation of environmental stressors.

1.4.3 Other Factors

A host of other factors may contribute to a decline in colony health. Historically, overwintering losses have accounted for the largest decline in colony numbers. Inadequately prepared hives are likely to freeze or starve over winter due to lack of food stores. Queen quality is another factor which is of utmost importance, with "poor queens" being consistently ranked near the top of the list of reasons for colony loss by beekeepers (vanEngelsdorp et al. 2008). Inclement weather, migratory beekeeping, poor forage and subsequent nutritional deficiencies are additional players implicated in colony loss, a list in which the factors increasingly appear to be multiple and interacting.

1.5 PESTICIDES

Pesticide usage is another factor frequently associated with bee losses. Crop protection has developed over centuries for the prevention of crop losses due to pests in the field (pre-harvest losses) and during storage (post-harvest losses). Synthetic pesticides work to diminish pre-harvest losses, and while farmers can never expect to reach a 100% potential yield, they can minimize losses to an acceptable level using pesticides in an integrated approach. Pesticides are therefore an integral part of the agricultural sector, and ensure a measure of protection against weeds, arthropod pests, viruses, and fungi to maintain high crop yields. The United States uses more pesticide annually than any other country in the world, with a reported $12.5 billion spent on pesticides in 2007,

accounting for approximately 32% of the world's pesticide expenditure that year. That survey by the US EPA determined that the US used approximately 1.1 billion pounds of pesticide active ingredient, with a usage breakdown of: herbicides (47%), insecticides (8%), fungicides (6%), and other (including nematicides, fumigants, other conventional pesticides, and miscellaneous chemicals used as pesticides, 39%).

Historically, the effects of pesticides on beneficial arthropods such as natural enemies and pollinators has relied on the establishment of a median lethal dose or concentration (LD_{50} or LC_{50}), but more recent studies highlight the importance of identifying sub-lethal effects of pesticides on non-target species. Sub-lethal effects include any effect of pesticide exposure that does not kill the insect outright and is generally broken into two broad groups: behavioral and physiological effects. While sub-lethal effects in honey bees are much more likely to be encountered than a direct poisoning by pesticides, they can often be more difficult to observe and research. One particular class of insecticide, the neonicitinoids, has been targeted for honey bee colony losses by the media and environmental groups who champion studies linking the use of this class to sub-lethal effects including reduced lifespan, changes in learning behavior, and decreases in foraging success (Decourtye et al. 2003, 2004, Ramirez-Romero et al. 2005, Henry et al. 2012). One study examined the interaction between neonicitinoid exposure and Nosema infection, and observed a decrease in life span and glucose oxidase (an established marker of social immunity) activity in bees (Alaux et al 2010). Although these and other studies may point to pesticides as the main driver of bee decline, the majority of scientists studying the issue agree that pesticides may be just one of many

factors interacting to weaken the immune system of the bees and make them more susceptible to pathogen attack and eventual collapse.

1.6 HERBICIDES

In many cropping systems, weeds are the most important and devastating pest group. Due to mechanical means of control and the use of synthetic herbicides, farmers are capable of controlling weeds more effectively than crop losses resulting from diseases or animal pests. The efficacy of combined control measures of pathogens and animal pests reaches 32 and 39%, respectively, whereas efficacy of control for weeds is approximately 75% (Oerke 2006). Broad-spectrum herbicides account for *ca*. 50% of the total pesticides used in the US each year (Kellogg et al. 2000, US EPA 2007). An estimated 531 million pounds of herbicidal active ingredient was used in the US in 2007, as opposed to 93, 70, 163, and 276 millions of pounds of active ingredient of insecticides, fungicides, other conventional pesticides (including nematicides, fumigants, and other miscellaneous pesticides), and "other" (including sulfur, petroleum, sulfuric acid, and insect repellants), respectively. The majority of herbicides used in the US each year are consumed by the agricultural market, which relies on herbicides to protect crop systems from resource competition introduced by weeds. Herbicides and their metabolites were detected in *ca.* 50% of all samples collected from honey bee hives in a 2010 survey, with detection hits found in all sampled matrices including bees, pollen, and wax (Mullin et al. 2010).

1.7 ATRAZINE

Atrazine (6-chloro-*N*-ethyl-*N´*-(1-methylethyl)-1,3,5-triazine-2,4-diamine), a triazine-class herbicide, is the most commonly used herbicide in the US to treat pre- and post-emergence broadleaf and grassy weeds, and the second most commonly used pesticide in 2007 at 73-78 million pounds of active ingredient applied (US EPA 2012). Atrazine is applied in crops such as corn, sugar cane, sorghum, pine, canola, and on turf such as residential lawns and golf courses. It works as a photosynthesis inhibitor, binding to the D-1 plastoquinone-binding protein in photosystem II of chloroplasts, causing a disruption in electron transport through inhibition of the Hill reaction (Shimabukuro and Swanson 1969). With no way to convert light energy to chemical energy for sustenance, the plant dies from starvation and the oxidative damage caused by the inhibition of the electron transport chain. Injury symptoms of plants exposed to atrazine include yellowing of leaf tissue followed by death of the tissue. Triazines such as atrazine are taken up into the weed via the roots or foliage and move in the xylem to plant leaves (Shimabukuro et al. 1970, Pfister and Arntzen 1979). The crops which atrazine protect possess one or more detoxification mechanisms, such as hydroxylation of a functional group which converts the phytotoxic atrazine into a completely harmless derivative or a dealkylation pathway which detoxifies by way of breakdown into more stable intermediates (Shimabukuro 1967). Atrazine was ranked among the top ten most commonly detected pesticides within hives and was detected in up to *ca.* 20%, of the bee, pollen, or wax samples analyzed in a North American survey of pesticide residues in hives (Mullin et al. 2010). While still efficacious and used heavily in the US (primarily on corn in the Midwest) and Australia, atrazine has been steeped in controversy since the late 1980's and its use is banned in several nations.

1.7.1 Problems with Atrazine

Atrazine degrades relatively quickly in soil (typically 14-109 days, US EPA 2003), but can easily migrate from soil to groundwater where it is more persistent and slow to degrade. Atrazine has been shown to persist in water for years and as of 2001 it was the most commonly detected pesticide in groundwater, at concentrations ranging from 1-25 µg/L (Gilliom et al. 2006) and at particularly high concentrations at bodies of waters adjacent to agricultural fields the first rainfall after application. Atrazine is reported by some to affect humans and other vertebrates as an endocrine disruptor. In the 1980's, there were several reports documenting the deleterious effects of atrazine to fish, including a decrease in fecundity and gonad abnormalities in multiple species (Kettle et al. 1987, Tillitt et al. 2010). Endocrine related effects, such as altered steroid hormones, have been observed in fish after exposure to atrazine at environmentally relevant concentrations (Moore and Lower 2001) and high exposures to atrazine (Spanò et al. 2004). Numerous other studies link atrazine exposure with hormone disruption in aquatic animals including fish (Solomon et al. 2008, Hayes et al. 2011, Paulino et al. 2012, Xing et al. 2015), and controversial studies link atrazine to changes in reproductive organs and the feminizing of male frogs (Hayes 2002, Hayes et al. 2002, Withgott 2002). Due to its various effects on aquatic vertebrates and detection in surface and groundwater around the country, environmental and human advocacy groups were quick to demand studies investigating effects of atrazine exposure on humans. Concerns regarding possible links to hormonal disruption, teratogenic effects, or cancer in humans has been researched in recent years, the conclusions of which are controversial. Some studies (Munger et al. 1992, Winchester et al. 2009, Waller et al. 2010, Agopian et al. 2014) report teratogenic

effects due to atrazine such as prematurity, gastroschisis, genital deformations, low birth weights, and intrauterine growth retardation after prenatal exposure through the mother. No definitive links have been found between atrazine exposure and cancer in humans, even among pesticide applicators (Freeman et al. 2011). These studies rely on birth records and self-reporting in the majority of cases, so the EPA conducted a meta-analysis of sorts for atrazine risk assessment.

In 2003, the US EPA conducted an Interim Reregistration Eligibility Decision (IRED) and concluded that "the Agency has found that there is a reasonable certainty that no harm will result to the general US population, infants, children, or other major identifiable subgroups of consumers from aggregate exposure (from food, drinking water, and non-occupational sources) to cumulative residues of atrazine and the other chlorinated triazine pesticides," and in 2007, the EPA stated that atrazine does not adversely affect amphibian sexual development and that no additional testing was warranted. Although still available for use by licensed and registered pesticide applicators in the US, use of atrazine was banned in the European Union (EU) in 2004, when the EU found groundwater levels exceeding the limits set by regulators.

1.8 REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

The activation of molecular oxygen (O_2) is required in aerobic organisms for many cellular processes. The reduction of O_2 to water (H_2O) is up to eighteen times more energetically efficient than the analogous reaction in anaerobic organisms. This benefit is counteracted by the fact that the reaction gives rise to oxidation states of oxygen which are highly reactive with other molecules, creating complexes such as superoxide anion

radical, hydrogen peroxide, and hydroxyl radical, and are known as reactive oxygen species, or ROS (Pardini 1995). These reactive oxygen species can accumulate in the system and inflict detrimental intracellular reactions including cellular membrane damage and the oxidation of proteins and DNA, a phenomenon referred to as oxidative stress. Endogenous oxidative stress is accumulated in all aerobic organisms due to the inherent toxicity of activated oxygen. This stress is compounded by the influx of dietary or environmental sources of pro-oxidant substances, referred to as exogenous oxidative stress. The oxidative stress theory states that the accumulation of reactive oxygen species elicits oxidative stress and physiological damage that contributes to aging and compromises the health and lifespan of an organism, including insects (Williams et al. 2008).

Honey bees undergo a great deal of oxidative stress due to their age-dependent foraging activity. The wing muscles found within the thorax of bees comprises *ca.* 35% of the total body mass and contract at approximately 240 wing beats per second (Harrison et al. 1996). The flight of bees increases metabolic rates up to 100-fold as compared to resting behavior, thus inducing a great amount of ROS within the mitochondria of these flight muscle cells (Suarez et al. 1999). In addition, all bees within the hive are exposed to known pro-oxidants through feeding and interactions with the external environment. Alaux et al. (2011) reported that increased oxidative stress in queen and worker bees cause physiological changes that impact colony health and bee longevity. This suggests that bees, along with all aerobic organisms, must possess some sort of mechanism to counteract the effects of this oxidative stress. The innate capacity to mitigate the effects of oxidative stress is achieved through the antioxidant system.

1.9 ANTIOXIDANTS

The health of bees is affected by their capacity to minimize oxidative stress. The antioxidant activity of an insect can mitigate the damage caused by oxidative stress, but at a physiological cost (Williams et al. 2008). Bees have an advanced antioxidant system analogous to higher vertebrates, which works to either deter the formation of free radicals or neutralize them before they can inflict cellular damage. This system is composed of numerous enzymes and metabolites found within the hemolymph, tissue, or exogenously derived from food sources. Enzymatic antioxidants include three major classes: the superoxide dismutases (SOD), catalases (CAT) and glutathione (GSH) peroxidases (Sies, 1997). Additional enzymes include glutathione-S-transferase (GST), and glutathione reductase (GR). SODs work to convert O_2 to H_2O_2 which is then eliminated by CATs and glutathione peroxidases. GSTs work to neutralize toxic compounds by way of conjugating them to glutathione for future removal from the organism. Some GSTs also have glutathione peroxidase activity, which provides an additional oxygen-detoxifying function which is vital to insects because they are deficient in the other (vertebrate-type, selenium-dependent) glutathione peroxidases (Pardini 1995, Weirich 2002).

1.10 ATRAZINE AND OXIDATIVE STRESS

Atrazine is a known inducer of oxidative stress in numerous vertebrate and invertebrate species. There is no test available to quantify oxidative stress as a definitive endpoint with a universal unit of measure; however, studies frequently measure concentrations of known antioxidants in the system along with concentrations of harmful byproducts of oxidative damage after exposure to a known ROS inducer. As stated previously, all

aerobic organisms experience some degree of oxidative stress due to the natural formation of free radicals (ROS) during oxygen metabolism, and possess mechanisms to prevent or neutralize this damage known as antioxidant function. Some commonly found components of this antioxidant system include glutathione, glutathione peroxidase, superoxide dismutase, catalase, glutathione *S*-transferase, and glutathione reductase, among others. Recent studies have shown that atrazine has the potential to induce oxidative stress in fish (Jin et al. 2010, Xing et al. 2012, Blahová et al. 2013), rats (Pogrmic-Majkic et al. 2012, Singh et al. 2008), and endocrine disruption (Oka et al. 2008, Salaberria et al. 2009, Jin et al. 2013) in different organisms. Atrazine was shown to be genotoxic by causing single and double strand breaks in DNA through the formation of ROS (Song et al. 2009). In a 2008 study, Singh et al. demonstrated that atrazine induced oxidative stress in terms of increased malondialdehyde (MDA) production in rats, which is the byproduct of lipid peroxidation and the most common measure of cellular damage caused by oxidative stress.

While numerous studies link atrazine to increased oxidative stress and deleterious effects in mammals and invertebrates, including insects (Anderson et al. 2008, Thorton et al. 2010, Vogel 2015), relatively little information exists regarding atrazine exposure to honey bees and any resulting changes in antioxidant composition or function. Helmer et al. (2015) investigated environmentally relevant doses of herbicides, such as atrazine, on lipid peroxidase and exogenous antioxidant levels in caged honey bees, but made no mention of effects caused by exposure of bees to atrazine in a field setting. The research described herein will assess any induction of reactive oxygen species and oxidative stress in honey bees exposed to the commonly-used agricultural herbicide atrazine. This study

examines the levels of enzymatic antioxidant components in field and laboratory bees post-exposure to environmentally relevant concentrations of atrazine, and determine gene expression levels of antioxidant-encoding genes exposed to this herbicide.

CHAPTER 2

LABORATORY AND IN-HIVE EFFECTS OF ATRAZINE ON ANTIOXIDANT ENZYMES AND ANTIOXIDANT-ENCODING GENES IN HONEY BEES

2.1 INTRODUCTION

Honey bees are the most widely managed pollinator in the world due to their amenability to manipulation within a hive and generalist foraging capabilities. Insect pollination contributes 35% of total global food production, and 80% of these pollination services are attributed solely to honey bees (Klein et al. 2007). The pollination services that honey bees provide equates to approximately $14.6 billion in the United States (US) and $220 billion worldwide (Gallai et al. 2009). In the US alone, over 2.4 million honey bee colonies are rented out every year for pollination services, at a value of approximately $145 per hive for a total of $350 million annually (USDA 2009). While global numbers of managed honey bee colonies have actually risen since the 1950's, these increases are observed primarily in developing areas of the globe and regional losses in Europe and the US are still a cause of concern (Aizen et al 2009). Honey bees and native pollinators have fluctuated in their global numbers in recent decades; however, there are multiple interacting factors implicated in these losses (Williams and Osbourne 2009).

Pesticide usage has become a factor associated with the managed colony losses. Over 75% of pesticide applications are performed by the agricultural sector to ensure protection of crops against weeds, arthropods, and microbial pests to maintain higher crop yields. The US uses more pesticide annually than any other country in the world,

with a reported $12.5 billion spent on pesticides in 2007, accounting for approximately 32% of the world's expenditure that year (US EPA 2008). This survey by the EPA determined that the US used approximately 1.1 billion pounds of pesticide active ingredient, with herbicides accounting for approximately 50% of all pesticides used. Herbicides are used in agriculture to stem the resource competition imposed by weeds on cash crops. An estimated 531 million pounds of herbicidal active ingredient was used in the US in 2007 (US EPA), and the second most commonly used herbicide active ingredient was atrazine.

Atrazine is a triazine-class herbicide and is most commonly used herbicide to treat broadleaf and grassy weeds (US EPA 2012). Atrazine is a known surface and groundwater contaminant, and a commonly detected pesticide in groundwater. It is also an inducer of oxidative stress in numerous vertebrate and invertebrate species. Recent studies have shown that atrazine has the potential to induce oxidative stress in fish (Jin et al. 2010, Xing et al., 2012, Blahová et al., 2013), rats (Pogrmic-Majkic et al. 2012, Singh et al. 2009), and cause endocrine disruption in different organisms (Oka et al., 2008; Salaberria et al., 2009; Jin et al., 2013). Atrazine was shown to be genotoxic by causing single and double strand breaks in DNA through the formation of reactive oxygen species (ROS) (Song et al. 2009). There is evidence for induction of oxidative stress in insects exposed to atrazine (Barata et al. 2005, Anderson et al. 2008, Thorton et al. 2010, Vogel 2013), but relatively little information exists regarding atrazine-induced oxidative stress in honey bees. The health of honey bees is affected by their capacity to minimize this oxidative stress. Oxidative stress is a byproduct of oxygen metabolism in aerobic organisms. The antioxidant activity of an insect can mitigate the damage caused by

oxidative stress, but at a physiological cost (Williams et al. 2008). Honey bees have an advanced antioxidant system analogous to higher vertebrates, which works to either deter the formation of free radicals or neutralize these free radicals before they can inflict cellular damage.

This study examined the antioxidant enzyme activities and antioxidant-encoding gene expression levels of honey bees exposed to atrazine in the laboratory and in the hive. The hypothesis of this study is that the exposure of honey bees to atrazine will elicit production of reactive oxygen species, and in turn, oxidative stress. The data gathered from this study provide new information regarding changes in antioxidant function both enzymatically and biochemically in honey bees exposed to the common-use herbicide atrazine in both controlled laboratory experiments and natural in-hive settings. This study aims to elucidate a link between herbicide exposure and honey bee health and is intended to promote the protection of these pollinators against the unintended exposure of agricultural pesticides.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Technical grade atrazine was purchased from ChemService Inc. (West Chester, PA). Total antioxidant, total glutathione, glutathione peroxidase, and lipid peroxidase assay kits were purchased from Cayman Chemical (Ann Arbor, MI). Bicinchoninic acid, Triton X-100, bovine serum albumin, 1-chloro-2, 4-dinitrobenzene (CDNB), triethanolamine, radioimmunoprecipitation assay (RIPA) buffer, and copper sulfate were purchased from Sigma Aldrich (St. Louis, MO, USA). Chloroform, ethanol, isopropanol,

metaphosphoric acid, sodium phosphate monobasic and sodium phosphate dibasic were purchased from Fisher Scientific (Fair Lawn, NJ). Trizol reagent was purchased from Invitrogen (Carlsbad, CA).

2.2.2 Laboratory Exposure of Honey Bees to Atrazine

Honey bees were collected from untreated hives maintained at the Virginia Tech Price's Fork Research Center (Blacksburg, VA). Approximately 200 honey bees were sampled from the center of brood nest frames and brought back to the laboratory for the analysis of antioxidant enzyme activities. Honey bees were placed into experimental cages in an environmental chamber maintained at 32 °C with 60% relative humidity. The honey bees were fed *ad libitum* with a 50% sucrose solution overnight. If higher than 10% mortality in these untreated honey bees was observed overnight the collection and caging of honey bees was repeated. If lower than 10% mortality was observed in the caged honey bees, treatment with atrazine was applied. The honey bees were exposed to nominal concentrations of atrazine at 0.1, 1, or 10 μg/L (parts per billion or ppb) in 50% sucrose solution (v/v) for 24 h, and the control group was fed a 50% sucrose-only treatment. Following the exposure period, the honey bee were collected and stored at -80 °C for antioxidant enzyme activity and antioxidant-encoding gene expression analysis.

2.2.3 Field Exposure of Honey Bees to Atrazine

Ten standard Langstroth hives were used in the field exposure portion of this experiment. Six new medium-depth hive bodies and frames were constructed in March of 2013 and new packages of honey bees were installed in April at Moore Farm in Blacksburg, VA.

The remaining four hives were full-depth hive bodies and frames previously constructed and new packages of honey bees installed in April at Kentland Farm in Blacksburg, VA. All experimental hives contained sister queens in order to reduce genetic variation. The hives were given approximately four months to construct comb and build up resources and honey bee numbers before sampling began the last week of July. Five of the hives were designated control and the remaining five were treated with atrazine at 10 µg/L (parts per billion or ppb). The treatments were contained within a 40% sucrose solution in quart mason jars, two of which were inverted over the inner cover of each experimental hive and surrounded by an empty hive box. Lids of the jars were poked with small holes and allowed the honey bees to move up in the brood box and feed *ad libitum*. Jars were refilled every week. The jars were usually empty at the end of each week and it was assumed that the treated or untreated sucrose solution was distributed to honey bees throughout the hive through trophallaxis. Trophallaxis is the well described act of mouth to mouth food-sharing within a honey bee colony (Wheeler 1923). The exposure period was 28 days and included two sampling periods: day 0 (pre-exposure) and day 28 (post-exposure). During a sampling period, four samples were collected from each hive in 50 mL conical tubes (approximately 100 honey bees each). These honey bees were collected from different locations in the hive, two collections on periphery frames and two collections from central brood-nest frames to ensure collection of adult honey bees of multiple life stages. The samples were immediately placed on ice, brought back to the laboratory and placed in the -80 °C freezer for analysis.

2.2.4 Antioxidant Enzyme Activities of Honey Bees Exposed to Atrazine in the Laboratory and Hive

The total antioxidant activities of honey bees untreated and treated with atrazine in the laboratory and the hive were measured using a Cayman Chemical Co. Total Antioxidant Kit (Ann Arbor, MI) following the manufacturer's protocol. The head and thorax were dissected from the atrazine-untreated and -treated honey bees and homogenized in 1 mL of ice-cold 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% Triton X-100 (v/v). The homogenates were centrifuged at 12,000 x g at 4 °C for 15 min. The supernatant was collected from the centrifuged homogenate and used as the source for measuring total antioxidant activity. The total antioxidant activity assay allows for the colorimetric measurement of available metmyoglobin in each honey bee sample to inhibit the oxidation of ABTS. The absorbance of ABTS oxidation was measured at 750 nm with a multimode spectrophotomer (Molecular Devices, Sunnyvale, CA) and standardized using Trolox reagent.

The total glutathione (GSH) content of honey bees untreated and treated with atrazine were measured using a Cayman Chemical Co. Total GSH Kit (Ann Arbor, MI) following the manufacturer's protocol. The head and thorax were dissected from the atrazine-untreated and -treated honey bees and homogenized in 1 mL of ice-cold 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% Triton X-100 (v/v). The homogenates were centrifuged at 12,000 x g at 4 °C for 15 min. The supernatant was collected from the centrifuged homogenate and used as the source for measuring total GSH concentrations. The supernatant was deproteinated using metaphosphoric acid, triethanolamine, and water to reduce interference with protein-associated sulfyhyrdryl

groups. The assay measures the glutathione reductase-mediated reduction of a 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) substrate to a 5-thio-2-nitrobenzoic acid (TNB) product. The absorbance of TNB product was measured at 405 nm with a multimode spectrophotomer (Molecular Devices, Sunnyvale, CA).

The total glutathione *S*-transferase (GST) activities of honey bees untreated and treated with atrazine were measured according to Zhu et al. (2000), with slight modifications, using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The head and thorax were dissected from the atrazine-untreated and -treated honey bees and homogenized in 1 mL of ice-cold 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% Triton X-100 (v/v). The homogenates were centrifuged at 12,000 x g at 4 °C for 15 min. The supernatant was collected from the centrifuged homogenate and used as the source for measuring GST activities. The conjugation of glutathione towards CDNB was determined by recording the change in absorbance at 340 nm for CDNB for 1 min at 10-sec intervals using a multimode microplate reader (Molecular Devices, Sunnyvale, CA). Non-enzymatic controls were performed in parallel to correct for non-enzymatic conjugation.

The glutathione peroxidase activities of honey bees untreated and treated with atrazine were measured using a Cayman Chemical Co. Glutathione Peroxidase Kit (Ann Arbor, MI) following the manufacturer's protocol. The head and thorax were dissected from the atrazine-untreated and -treated honey bees and homogenized in 1 mL of ice-cold 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% Triton X-100 (v/v). The homogenates were centrifuged at 12,000 x g at 4 °C for 15 min. The supernatant was collected from the centrifuged homogenate and used as the source for measuring

glutathione peroxidase activity. The assay measures the glutathione peroxidase-mediated reduction of glutathione to oxidized glutathione. The absorbance of oxidized glutathione was measured at 340 nm with a multimode microplate reader (Molecular Devices, Sunnyvale, CA).

The lipid peroxidation activities of honey bees untreated and treated with atrazine in the field and the hive were measured using a Cayman Chemical Co. TBARS Kit (Ann Arbor, MI) following the manufacturer's protocol. The head and thorax were dissected from the atrazine-untreated and -treated honey bees and homogenized in 1 mL of ice-cold RIPA buffer. The homogenates were centrifuged at 1,000 x g at 4 °C for 10 min. The supernatant was collected from the centrifuged homogenate and used as the source for measuring lipid peroxidation activity. The assay measures the total amount of malondialdehyde (MDA), a natural product of lipid peroxidation, following the conjugation of MDA with the substrate thiobarbituric acid (TBAR). The absorbance of the MDA-TBAR conjugate was measured at 530 nm with a multimode spectrophotomer (Molecular Devices, Sunnyvale, CA).

The total protein contents of honey bees untreated and treated with atrazine in the laboratory and the hive were measured using the method of Smith et al. (1985) using bovine serum albumin as a standard. The total protein content was measured at 560 nm with a multimode spectrophotometer (Molecular Devices, Sunnyvale, CA). The antioxidant enzyme activity assays were standardized against the total protein content of the atrazine untreated and treated honey bees.

2.2.5 Semi-quantitative RT-PCR of Antioxidant-Encoding Genes in Honey Bees Exposed to Atrazine in the Laboratory and Hive

The total RNA of honey bees untreated and treated with atrazine in either the laboratory or the hive was extracted using the following procedures. The head and thorax of each honey bee was placed into a sterilized 1.7 mL microcentrifuge tube and homogenized in 1 mL of Trizol reagent using a sterile plastic pestle. The homogenates were centrifuged at 12,000 x g for 15 min. at 4 °C. The supernatants were transferred to a clean 1.7 ml microcentrifuge tube containing 200 µL chloroform and incubated at room temperature for 3 min. followed by centrifugation 12,000 x g for 15 min. at 4 °C. The aqueous phase was collected, added to 500 µL isopropanol, incubated at room temperature for 10 min., and centrifuged at 12,000 x g for 10 min. at 4 °C. The remaining pellet was washed with 1 ml 75% ethanol and centrifuged at 7,500 x for 5 min. at 4 °C. The ethanol was discarded from the samples and the samples were incubated at room temperature for 5 min. to evaporate the remaining ethanol. The RNA pellet was then resuspended in 50 µL of molecular-grade water followed by a dry heat bath incubation for 15 min at 60 °C. The total RNA concentration and purity of each sample was estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The semi-quantitative reverse-transcription (RT)-PCR of antioxidant-encoding genes of honey bees untreated and treated with atrazine in the laboratory and the hive was conducted using the following procedures. The semi-quantitative RT-PCR was conducted with antioxidant-encoding gene specific primers as described by Corona et al. (2005). These genes included: *CuZn Sod-1* (cytoplasmic Cu-Zn superoxide dismutase 1), *Mn Sod-2* (mitochondrial Mn superoxide dismutase 2), *Catalase*, *Trxr-1* (thioredoxin

reductase 1), *Gtpx-1* (glutathione peroxidase 1), *Tpx-3* (mitochondrial thioredoxin peroxidase 3), *Gst-1* (glutathione *S*-transferase), and *Msr A* (methionine sulfoxide reductase A), all of which are found in the mitochondrial antioxidant system. The primers for *β-actin* derived from *A. mellifera* were used as the housekeeping gene for each sample. All samples were diluted in deionized water to a concentration of 2,000 ng/µL prior to reverse transcription. A reverse transcription (RT) master mix was then prepared using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). The master mix included 10 x RT buffer, 25x dNTP mix, 10x RT random primers, *Multiscribe* reverse transcriptase, RNAse inhibitor, and nuclease-free H_20. Ten microliters of RT master mix was mixed with 10 µL of diluted RNA sample in a 0.2 mL centrifuge tube and held on ice until RT. RT was performed on a thermal cycler (Bio-Rad, Hercules, CA, USA) using a standard RT protocol of 10 min at 25 °C , 120 min at 37 °C, and 5 min at 85 °C. The cDNA was quantified and checked for purity using the NanoDrop 2000 spectrophotometer. The cDNA samples were used for semi-quantitative RT-PCR. The semi-quantitative RT-PCR mixture of Apex Blue 2x mastermix (New England Biolabs, Ipswich, MA, USA), forward and reverse primers at 10 µmol, cDNA, and deionized water to a final volume of 25 µL. The PCRs were cycled as follows: 15 min at 95 °C (1 cycle); 30 s at 95 °C (denaturation step), 30 s at the appropriate annealing temperature for the primer set (between 54-59 °C) and 1 min at 72 °C (extension step) for 35 cycles, finally samples were then incubated for 5 min at 72 °C (1 cycle). The PCR products were electrophoresed alongside 100 bp and 1 kb ladders (New England Biolabs, Ipswich, MA, USA) on a 1.5% agarose gel containing ethidium bromide. The agarose gels were run in 1X TBE buffer at 90 V for 90 min. at room temperature. The PCR

products were photographed on a benchtop transilluminator (UVP, Upland, CA, USA). Each gel examined a single antioxidant-encoding gene of interest on D0 (pre-exposure) and D28 (post-exposure) for eight (8) bees from a given hive in the treatment group. The relative levels of antioxidant-encoding and *β-actin* mRNA transcripts were indirectly quantified by calculating the ratio of intensity of the antioxidant genes to that of a *β-actin* gene using Image J (National Institutes of Health, https://imagej.nih.gov/ij/). The PCR products were sequenced and identified at the Virginia Bioinformatics Institute (Blacksburg, VA).

2.2.6 Statistical Analysis

The antioxidant enzyme activities of bees untreated and treated with atrazine in the laboratory were statistically analyzed using a one-way analysis of variance with a Tukey's multiple comparison test (GraphPad Software, La Jolla, CA). The antioxidant enzyme activities of honey bees untreated and treated with atrazine in the hive were statistically analyzed using a mixed-model analysis of covariance with enzymatic activity of day 0 honey bees as the covariate (JMP 12.1, Cary, NC). This mixed-model analysis of covariance allowed for an investigation of significant interaction between treatment (atrazine untreated and treated honey bees) or the covariate as well as any interaction in a treatment*covariate factorial on the day 28 enzyme activity of honey bees. The natural variation in honey bee samples was accounted for using a nested design of honey bees [Group, Hive]. *Post-hoc* tests were performed using a least squares means student's t-test and significance was selected as $\alpha = 0.05$. Similarly, the antioxidant-encoding gene expression levels of bees untreated and treated with atrazine in the hive after 28 days

were statistically analyzed using a mixed-model analysis of covariance using gene expression levels of day 0 honey bees as the covariate (JMP 12.1) The expression level *β-actin*, a common housekeeping gene, was estimated in each sample of atrazine untreated and treated honey bees as a method to standardize the expression levels of antioxidant-encoding genes in atrazine untreated and treated honey bees following 0- and 28-day treatments in the hive. Prior to the mixed-model analysis of covariance, the data were examined for normality and transformed using a Box-Cox transformation if necessary. The statistical analysis of the antioxidant-encoding gene expression levels in atrazine untreated and treated honey bees was completed using a mixed model analysis of covariance with a *post-hoc* student's t-test. The means of expression intensity were measured using ImageJ, while blanking between hive replicates with *β-actin* intensity. Once again, the significant effects of treatment, covariate, and a treatment*covariate factorial were examined and the variation between the means of both day 0 treatments (atrazine untreated and treated) were accounted for using the mixed-model analysis of covariance.

2.3 RESULTS

2.3.1 Antioxidant Enzyme Activities of Honey Bees Exposed to Atrazine in the Laboratory and Hive

The antioxidant enzyme activities were measured in honey bees untreated and treated with atrazine in the laboratory at 0.1, 1, and 10 µg/L for 24 h. The antioxidant enzymes include total antioxidants, total glutathione, glutathione peroxidase, glutathione *S*-transferase, and lipid peroxidase. The total antioxidant activities of honey bees exposed

to atrazine at 0.1, 1, and 10 µg/L were significantly decreased *ca*. 28%, 33%, and 38%, respectively, compared to the untreated honey bees (Fig. 2.1). The total glutathione contents of honey bees exposed to atrazine at 0.1, 1, and 10 µg/L were significantly decreased *ca*. 21%, 25%, and 27%, respectively, compared to the untreated honey bees (Fig. 2.1). The glutathione peroxidase activities of honey bees exposed to atrazine up to 1 and10 µg/L was significantly decreased *ca*. 30% and 58%, respectively, compared to the untreated honey bees (Fig. 2.1). The glutathione *S*-transferase activities of honey bees exposed to atrazine up to 1 and 10 µg/L was significantly decreased *ca*. 21 and 24%, respectively, compared to the untreated honey bees (Fig. 2.1). The lipid peroxidase activities of honey bees exposed to atrazine up to 0.1, 1, and 10 µg/L was significantly increased *ca*. 52%, 55%, and 36%, respectively, compared to the untreated honey bees (Fig. 2.1).

The effects of treatment and time on D28 (post-exposure) field-treated honey bees' total antioxidants are shown in Fig. 2.2. There is a significant effect of both treatment and time (treatment: DF = 1, DF den = 325.8, F-ratio = 279.8612, Prob>F = <0.0001*; time: DF = 1, DF den = 350.5, F-ratio = 4.7854, Prob>F = 0.0294*). The significant effects of atrazine treatment is denoted by an asterisk and time by a plus sign. These interactions were determined by a mixed model analysis of covariance with a least square means student t-test. After accounting for variation between antioxidant levels in D0 honey bees, post-exposure control and atrazine-treated field honey bees had a total antioxidant level of 0.0422 mM/mg protein and 0.0226 mM/mg protein, respectively. Atrazine-treated field honey bees experienced a 46.6% decrease in total antioxidant levels compared to untreated honey bees.

The treatment- and time-dependent effects of lipid peroxidase levels in post-exposure field-treated honey bees are shown in Fig. 2.3. There is a significant effect of treatment (DF = 1, DF den = 54.98, F-ratio = 10.3266, Prob>F = 0.0022*) on lipid peroxidase levels in post-exposure honey bees. The significant effect of atrazine treatment on honey bees is denoted by an asterisk and determined by a mixed model analysis of covariance with a least square means student t-test. After 28 days of exposure, control and atrazine-treated field honey bees had a lipid peroxidation level of 8.7392 µM MDA/mg protein and 12.0831 µM MDA/mg protein, respectively. Field bees treated with atrazine experienced a 38.3% increase in lipid peroxidase activity compared to untreated honey bees.

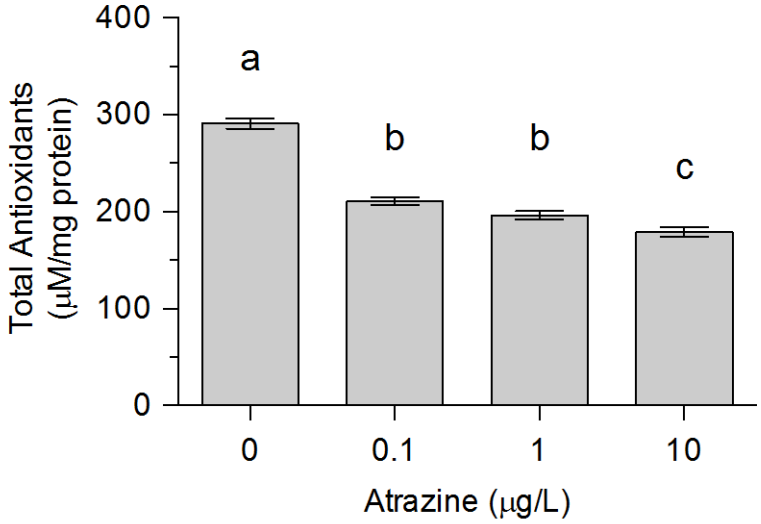
2.3.2 Semi-quantitative RT-PCR of Antioxidant-Encoding Genes in Honey Bees Exposed to Atrazine in the Laboratory and Hive

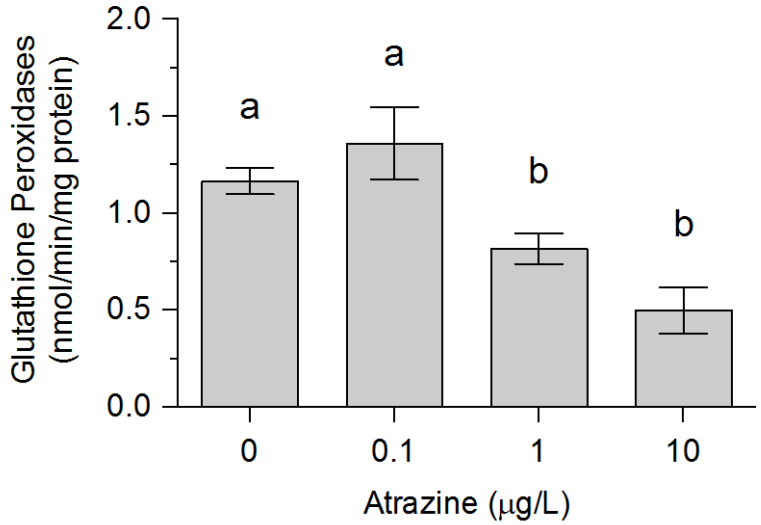
The semi-quantitative gene expression levels of seven antioxidant-encoding genes were measured in honey bees were treated with 0, 0.1, 1, 10, 100, and 1,000 µg/L (ppb) atrazine for 24 h in the laboratory. Relative RT-PCR transcript levels are presented as the mean ± standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$). *β-actin* was used to indirectly calculate the ratio of intensity of the antioxidant genes for each atrazine treatment. The expression level of *CuZnSod-1* increased *ca.* 33%, 35%, and 37% in honey bees treated with atrazine at 10, 100, and 1,000 µg/L, respectively, compared to untreated honey bees (Fig. 2.4). Expression of *MnSod-2* in honey bee treated with atrazine at 0.1, 1, and 10 µg/L

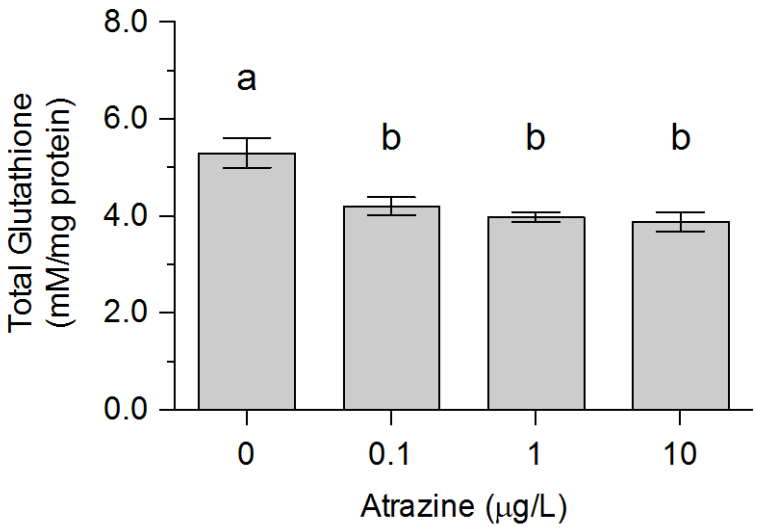
significantly increased *ca*. 15%, 20%, and 13%, respectively, compared to untreated honey bees. However, *MnSod-2* expression was significantly decreased *ca*. 17% and 11% in honey bees exposed to atrazine at 100, and 1,000 µg/L, respectively, compared to the untreated honey bees (Fig. 2.4). *Catalase* expression levels increased *ca.* 15%, 16%, 18%, 24%, and 25% in honey bees treated with atrazine at 0.1, 1, 10, 100, and 1,000 µg/L, respectively, compared to the untreated control (Fig. 2.4). Expression levels of *Tpx-3* did not change after exposure to any atrazine treatment (Fig. 2.4). *Gtpx-1* expression in honey bees treated with 10 µg/L atrazine was increased *ca*. 15% whereas 100 and 1,000 µg/L atrazine decreased *Gtpx-1* expression *ca.* 25% and 32%, respectively, in honey bees compared to the untreated control (Fig. 2.4). *Trxr-1* expression levels in honey bees did not change after exposure to any concentration of atrazine (Fig. 2.4). Expression levels of *MsrA* decreased *ca*. 12% in honey bees treated with 0.1 µg/L atrazine compared to the untreated control (Fig. 2.4).

The expression levels of seven antioxidant-encoding genes on day 28 (post-exposure) in honey bees treated with atrazine in the hive are shown in Fig. 2.5. The significant effects of atrazine treatment or time is denoted by an asterisk or a plus sign, respectively. These interactions were determined by a mixed-model analysis of covariance with a least square means student t-test. Neither time nor treatment had any significant effect on the expression levels of *Catalase*, *Trxr-1*, *MsrA* and *Gst-1*. Time did have a significant effect on the expression of *CuZnSod-1* (DF = 1, DFDen = 60.48, F Ratio = 5.3315, Prob>F = 0.0244*). Both time and treatment had an effect on the expression levels of *Tpx-3* (time: DF = 1, DFDen = 74.41, F-ratio = 4.8388, Prob>F = 0.0309*; treatment: DF = 1, DFDen = 7.007, F-ratio = 28.2207, Prob>F = 0.0011*) and

Gtpx-1 (time: DF = 1, DFDen = 73.04, F-ratio = 8.3911, Prob>F = 0.0050; treatment: DF = 1, DFDen = 6.982, F-ratio = 54.0010, Prob>F = 0.0002*) Expression of *Tpx-3* in atrazine treated honey bees on day 28 increased 186.5% compared to the untreated control. Expression of *Gtpx-1* in atrazine treated honey bees on day 28 increased 93.2% compared to the untreated control.



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(mM/mg protein)
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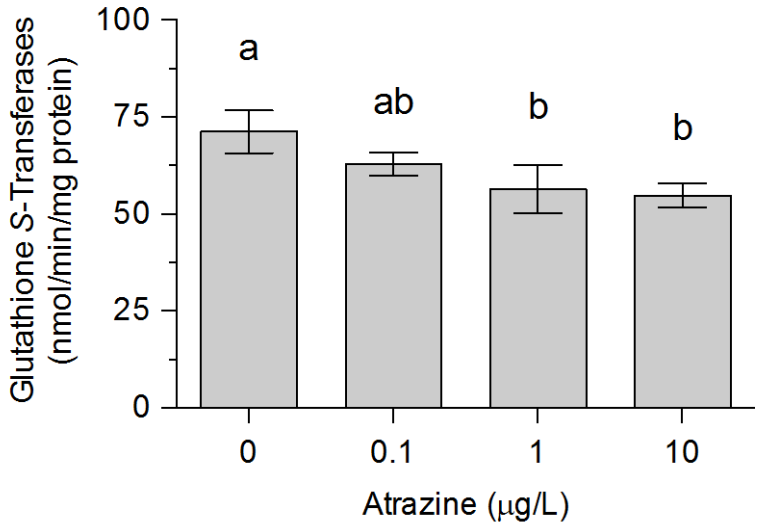
4.0
2.0
0.0
a
b
b
b
0
0.1
1
10
Atrazine (μg/L)


Glutathione S-Transferases
(nmol/min/mg protein)
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75
50
25
0
a
ab
b
b
0
0.1
1
10
Atrazine (μg/L)

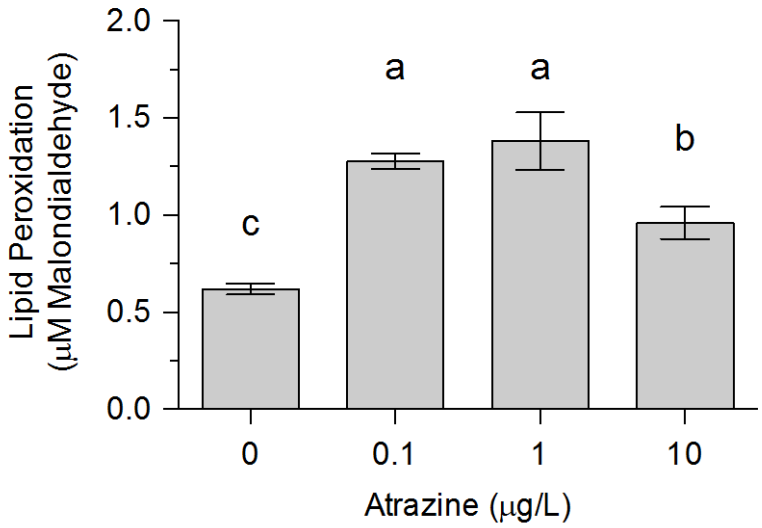


Fig. 2.1. Antioxidant activities of laboratory honey bees treated with the broad-spectrum herbicide atrazine. Bees were treated with 0, 0.1, 1, and 10 µg/L atrazine for 24 h. Antioxidant activities are presented as the mean ± standard error (n =5 for total antioxidants, n = 3 for glutathione peroxidase, total glutathione, glutathione-*S*-transferase, and lipid peroxidase). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

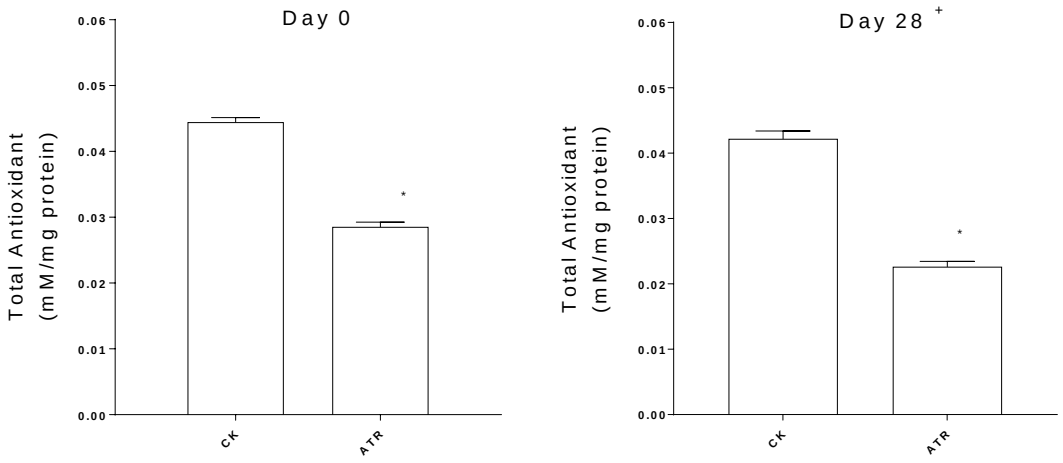


Figure 2.2. Effect of atrazine on the total antioxidant activities of bees before and following a 28 day exposure period. Vertical bars indicate the standard error of the mean. A mixed model analysis of covariance compared the treatment and time effects at day 28 while accounting for treatment variation at day 0. Asterisks on the bars indicate the means are significantly different due to treatment at day 28. Positive signs on the bars indicate a significant difference due to time using a least squares means student's t-test ($\alpha = 0.05$). CK = control, or untreated bees; ATR = atrazine treated bees at 10 μg/L (parts per billion or ppb)

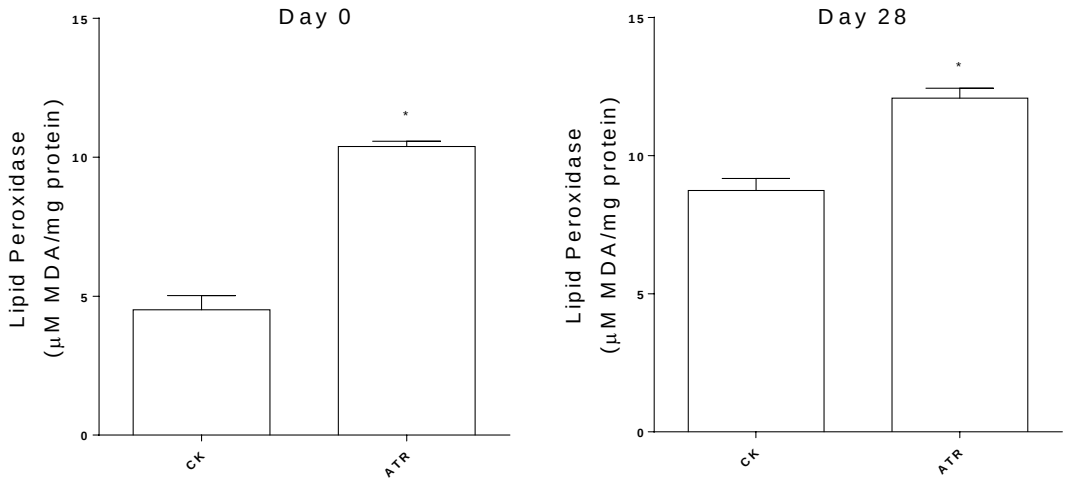
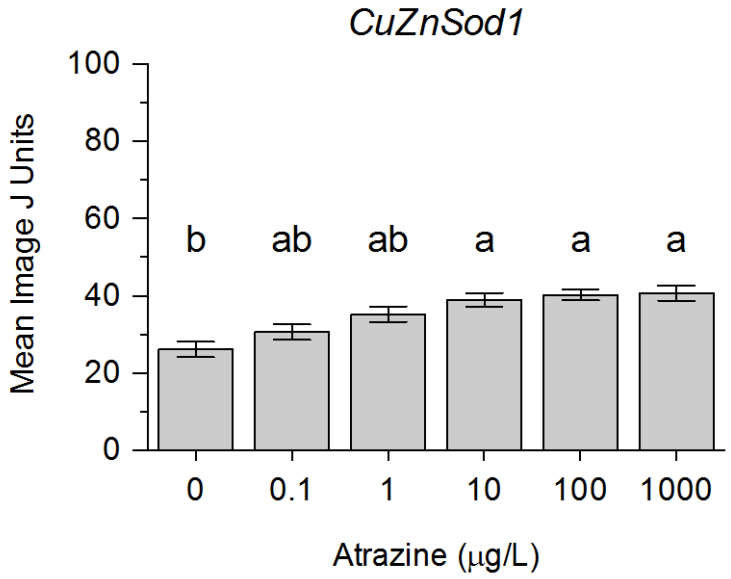


Figure 2.3. Effect of atrazine on the lipid peroxidase activities of bees before and following a 28 day exposure period. Vertical bars indicate the standard error of the mean. A mixed model analysis of covariance compared the treatment and time effects at day 28 while accounting for treatment variation at day 0. Asterisks on the bars indicate the means are significantly different due to treatment at day 28. CK = control, or untreated bees; ATR = atrazine treated bees at 10 μg/L (parts per billion or ppb).


CuZnSod1
Mean Image J Units
100
80
60
40
20
0
b
ab
ab
a
a
a
0
0.1
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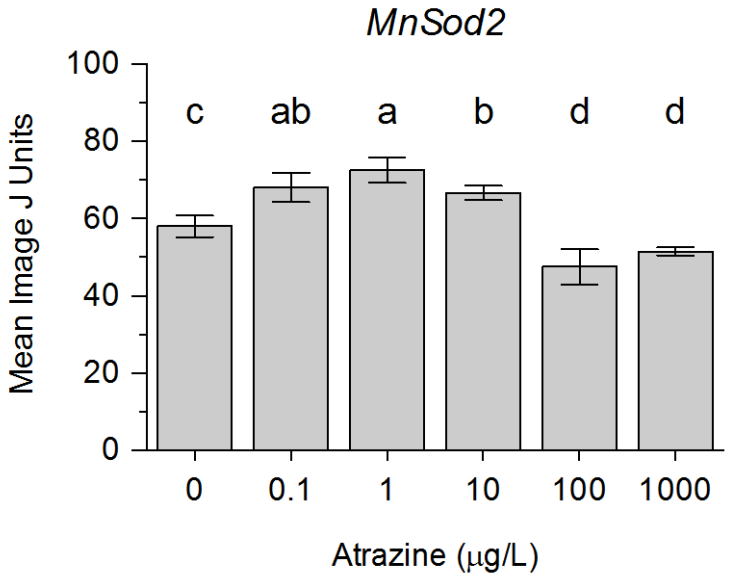
10
100
1000
Atrazine (μg/L)


MnSod2
Mean Image J Units
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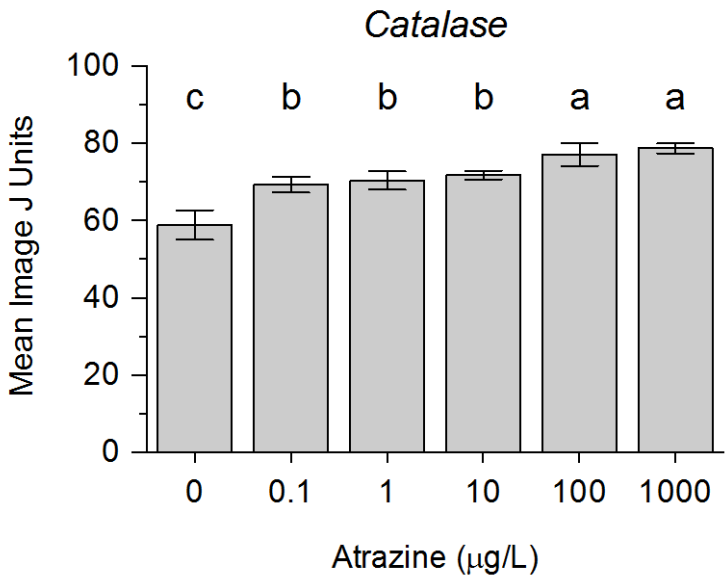
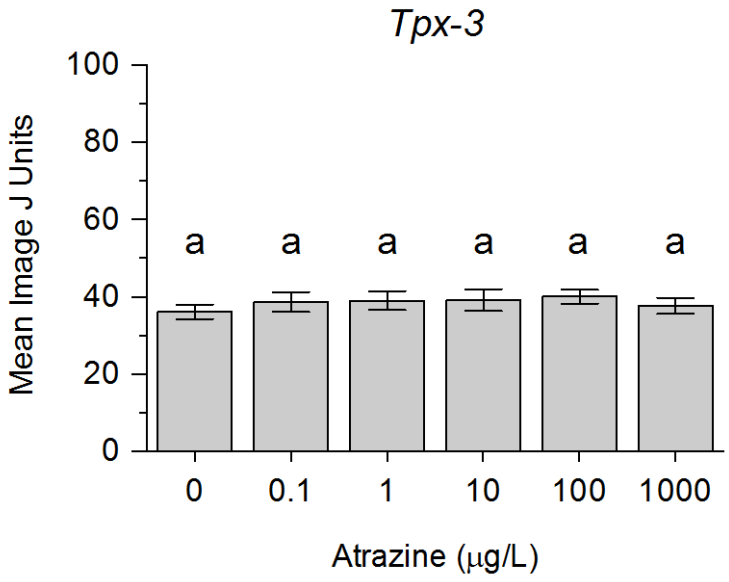
80
60
40
20
0
c
ab
a
b
d
d
0
0.1
1
10
100
1000
Atrazine (μg/L)


Catalase
Mean Image J Units
100
80
60
40
20
0
c
b
b
b
a
a
0
0.1
1
10
100
1000
Atrazine (μg/L)


Tpx-3
Mean Image J Units
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60
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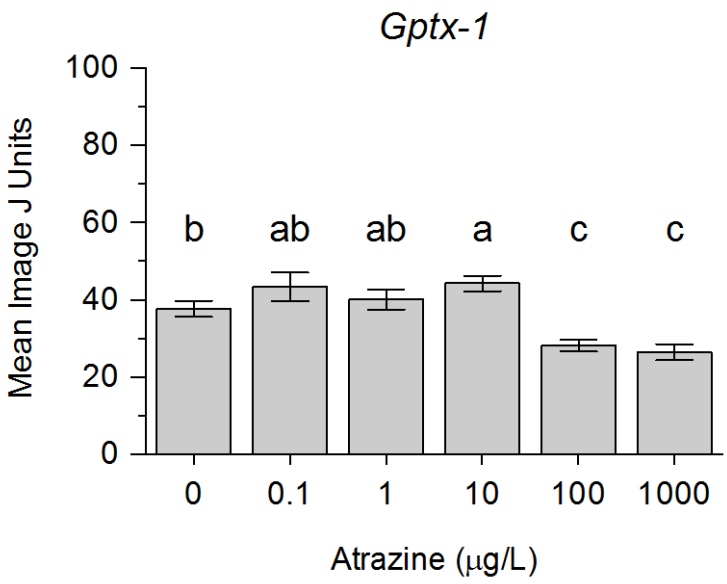
100
1000
Atrazine (μg/L)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Mean Image J Units
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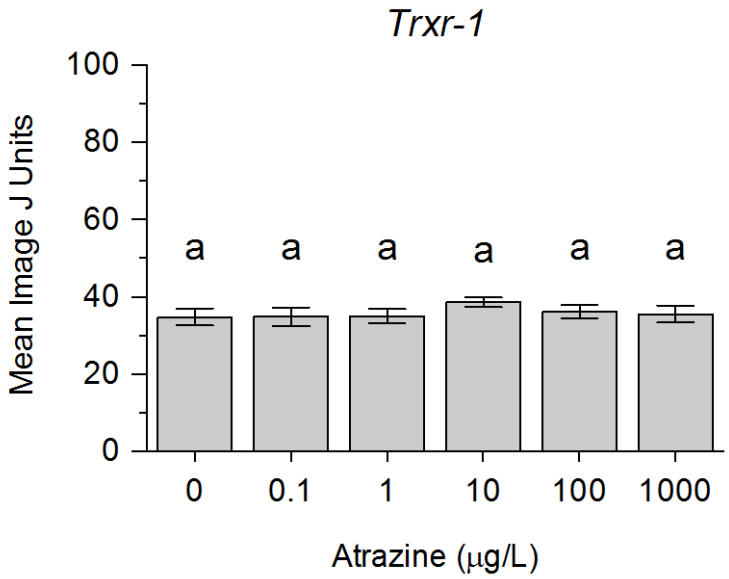
100
1000
Atrazine (μg/L)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100
1000
Atrazine (μg/L)

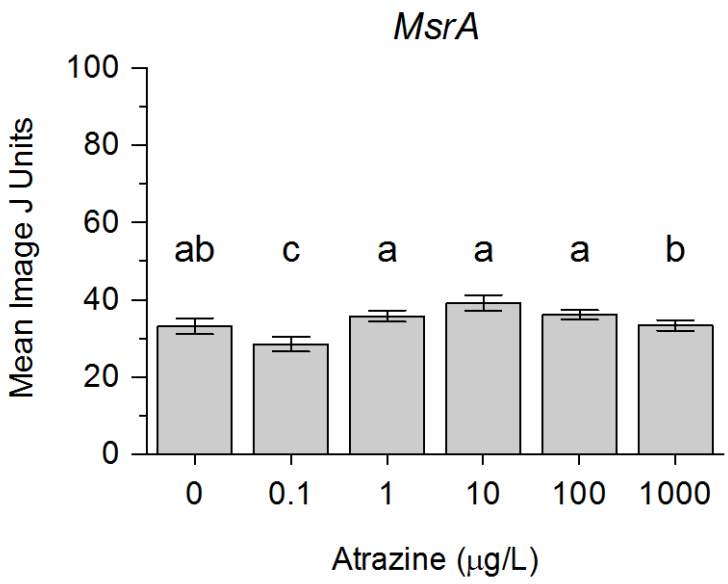
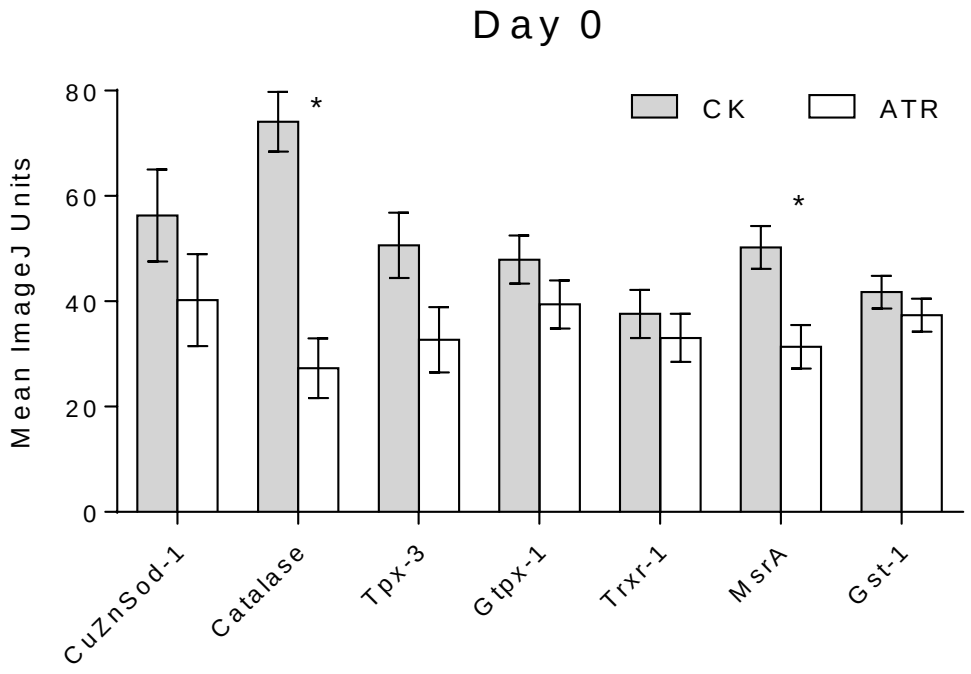
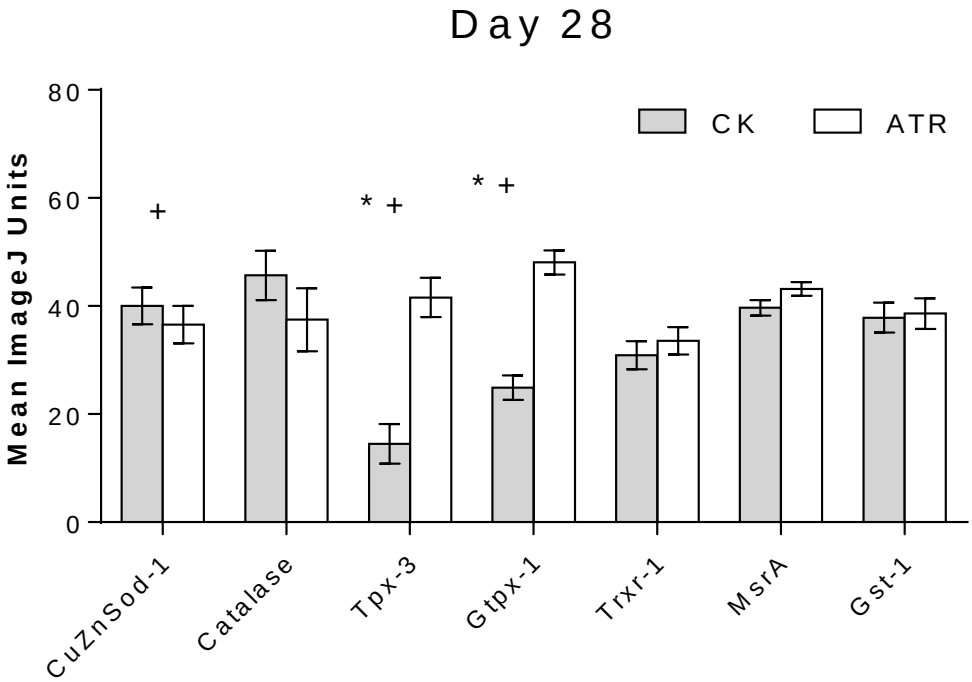


Figure 2.4. Semi-quantitative reverse transcription (RT)-PCR amplification of antioxidant-encoding genes in honey bees untreated and treated with atrazine in the laboratory. Bees were treated with 0.1, 1, 10, 100, and 1,000 µg/L (parts per billion or ppb) atrazine for 24 h. Mean Image J units are presented as the mean ± standard error (*n* = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$). β-actin was used as a standard to indirectly calculate the ratio of intensity of the antioxidant genes for each atrazine treatment.


Day 0
Mean ImageJ Units
80
60
40
20
0
CK
ATR
*
*
CuZnSod-1
Catalase
Tpx-3
Gtpx-1
Trxr-1
MsrA
Gst-1


Day 28
Mean ImageJ Units
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60
40
20
0
CK
ATR
+
* +
* +
CuZnSod-1
Catalase
Tpx-3
Gtpx-1
Trxr-1
MsrA
Gst-1

Figure 2.5. Semi-quantitative reverse transcription (RT)-PCR of antioxidant-encoding genes in honey bees collected from hives before (Day 0) and after (Day 28) atrazine exposure. Relative RT-PCR experssion levels are presented as the mean ± standard error (n = 40). A mixed -model analysis of covariance was used to determine the treatment and time effects at day 28, while accounting for variation between treatments at day 0. Significant differences in treatment are indicated with an asterisk and differences due to time are indicated with a plus sign using a least squares means student's t-test ($\alpha = 0.05$).

2.4 DISCUSSION

Reactive oxygen species (ROS) are highly reactive complexes which are continually and conservatively produced in aerobic cells. These ROS can work beneficially in the system by acting as scavengers to neutralize xenobiotics and pathogens, or they can accumulate and inflict detrimental intracellular reactions, including cellular membrane damage and the oxidation of proteins and DNA. Oxidative stress results from the uncontested buildup of ROS in an aerobic organism. The health of honey bees is affected by their capacity to minimize oxidative stress. The oxidative stress theory states that the accumulation of ROS elicits oxidative stress and physiological damage that compromises the health and lifespan of an insect (Williams et al. 2008). All aerobic organisms possess antioxidant systems, which function to prevent oxidative damage. The antioxidant activity of a honey bee can neutralize or mitigate the damage caused by oxidative stress, but at a physiological cost (Williams et al. 2008). Alaux et al. (2011) reported that increased oxidative stress in queen and worker honey bees cause physiological changes that impact colony health and honey bee longevity.

Atrazine, a commonly-used triazine herbicide, is a known inducer of oxidative stress in numerous vertebrate and invertebrate species. Recent studies have shown that atrazine has the potential to induce oxidative stress in fish (Jin et al. 2010, Xing et al. 2012, Blahová et al. 2013), rats (Singh et al. 2008, Pogrmic-Majkic et al. 2012), and cause endocrine disruption (Oka et al. 2008, Salaberria et al. 2009, Jin et al. 2013) in different organisms. Atrazine was reported to be genotoxic by causing single and double strand breaks in DNA through the formation of ROS (Song et al. 2009). Singh et al. (2008)

demonstrated that atrazine induced oxidative stress by increasing malondialdehyde (MDA) production in rats. MDA is the byproduct of lipid peroxidation and the most common measure of cellular damage caused by oxidative stress. Numerous studies also link atrazine exposure to increased oxidative stress and deleterious effects in invertebrates, including insects (Anderson et al. 2008, Thorton et al. 2010, Vogel 2013). However, relatively little information exists regarding atrazine exposure to honey bees or changes in antioxidant composition or function. The hypothesis of this study was that exposure of honey bees to atrazine would increase production of ROS which, in turn, would elicit oxidative stress. This study examined the induction of ROS and oxidative stress in honey bees exposed to the commonly-used agricultural herbicide atrazine. The antioxidant enzyme activities and antioxidant-encoding gene expression levels were assessed in honey bees following laboratory and in-hive exposures to atrazine.

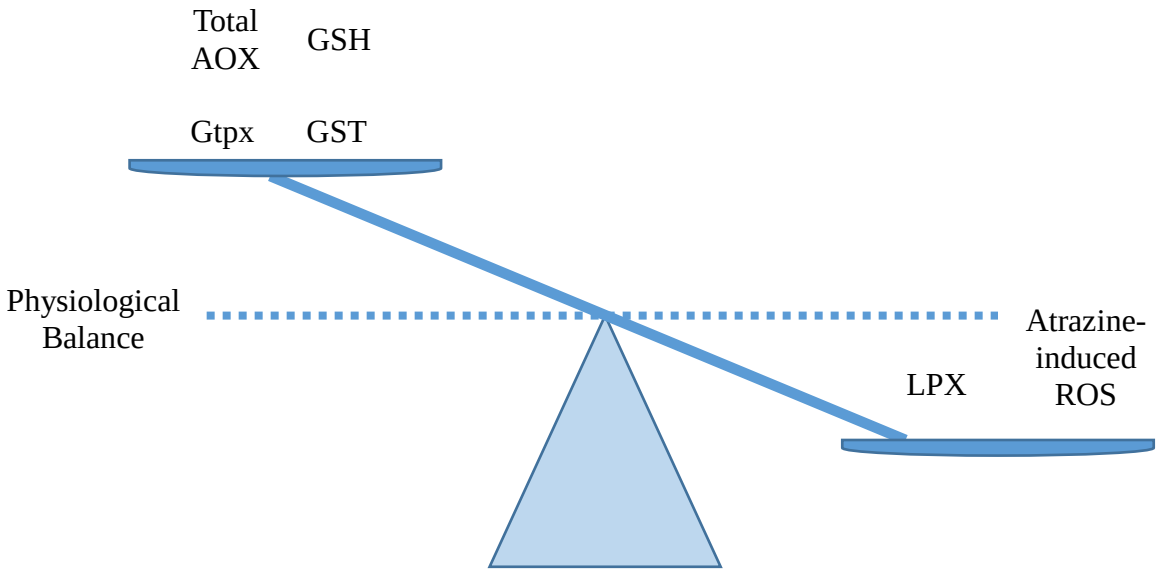
The antioxidant enzymes of honey bees treated with atrazine at 0.1, 1, or 10 µg/L for 24 h in the laboratory were assessed. Enzymatic antioxidant levels measured in this study include total antioxidants, total glutathione, glutathione peroxidase, glutathione *S*-transferase, and lipid peroxidase. Four of the five enzyme activities were decreased after exposure to atrazine. Compared to control honey bees, total antioxidant activities decreased *ca*. 40%, total glutathione content decreased *ca*. 26%, glutathione peroxidase activities decreased *ca*. 58%, and glutathione *S*-transferase activities decreased *ca*. 24%. The lipid peroxidase activities of honey bees exposed to atrazine increased *ca*. 55% compared to the untreated honey bees.

Total antioxidant and lipid peroxidase activity was similarly altered in bees exposed to atrazine at 10 ppb in the field over 28 days. Both treatment and time were discovered to have significant effects on total antioxidants in post-exposure bees. After accounting for the covariate, or the antioxidant levels in bees at day 0, field bees treated with atrazine experienced a 46.6% decrease in total antioxidant levels compared to untreated bees after day 28 of atrazine exposure. Treatment with atrazine was found to be a significant effect in lipid peroxidase activity in field treated bees. After accounting for the covariate (day 0 lipid peroxidase levels), atrazine-treated bees in the hive experienced a 38.6% increase in lipid peroxidase activity compared to control bees on day 28.

Overall, total antioxidant activity was decreased in bees exposed to atrazine in both laboratory and in-hive conditions. In the laboratory, four of the five chosen measures of antioxidant activity were decreased compared to the control at an exposure of 1 ppb atrazine. In other laboratory studies of vertebrate species, exposure to atrazine at markedly higher concentrations led to an increase in antioxidant activity (Elia et al. 2002, Chen et al. 2015, Abarikwu 2014). Other laboratory studies involving vertebrates have found that exposure to atrazine can increase antioxidant response only to be reversed to untreated-control levels by the feeding of known antioxidant compounds such as Vitamin E (Singh et al. 2010). The alteration of enzymatic antioxidant activities after exposure to atrazine appears to be consistent between studies, but in many cases the overall trend shows increasing antioxidant activity. While atrazine has been shown to affect a range of insect species in other aspects and measures of oxidative stress (Anderson et al. 2008, Thorton et al. 2010, Vogel et al 2013), few studies to date examine enzymatic antioxidant

activity in insects exposed to atrazine. Helmer et al. (2015) studied honey bees exposed to atrazine in the laboratory at environmentally relevant doses. Their study examined seven exogenously (diet) derived compounds in addition to lipid peroxidation levels. Levels of five of these seven compounds were not found to differ from the untreated control. However, detection of the remaining two compounds did decrease with increasing atrazine exposure.

It is possible that the exposure to atrazine in the hive and laboratory overwhelmed the endogenous antioxidant capacity leading to a decreased antioxidant defense in honey bees. Figure 2.6 illustrates a possible mechanism by which the overall antioxidant function of the honey bee was overwhelmed by atrazine exposure. While this study did not examine every facet and enzyme of the antioxidant system, I believe that the physiological balance which is generally struck between antioxidant defense and ROS production in aerobic organisms was "tipped" in the favor of ROS production, or an overall decrease in antioxidant activity. The decrease in four essential antioxidant enzyme activities and concurrent increase in lipid peroxidase activity after exposure to atrazine in the laboratory and the hive was sufficient to decrease overall antioxidant function and indicate an induction of oxidative stress.



Total AOX: Total antioxidants. Cumulative enzymatic effort to prevent or neutralize ROS production and damage

GSH: Total glutathione. Electron donor; serves as substrate for ROS conjugation for excretion from system

GST: Glutathione *S*-transferase. Phase II isozyme which links ROS to glutathione for detoxification

Gtpx: Glutathione peroxidase. Protects system from oxidative damage by reducing H_2O_2 to H_2O

Fig. 2.6. Hypothetical physiological balance of honey bees after exposure to atrazine. Antioxidant defense on one side of the scale and build-up of reactive oxygen species (ROS) on the other. Legend on the right elucidates function of each antioxidant enzyme.

The findings from this study do conflict with studies from vertebrate species; however, this may be due to a more evolved and robust antioxidant capacity in vertebrates compared to insects. Honey bees in particular have fewer genes for innate immunity and detoxification enzymes than other insects, and far more genes encoding for odorant receptors. This decreased number of immunological and detoxification genes may be due in part to the social structure of honey bee evolutionary history, which favors social immunity and collection of food resources for the betterment of the hive over individual immunity (Bassett 1997, Brockman 2001, Bromham 2005, Weinstock et al. 2006). Another possibility for variation between these laboratory results, and those of other species, may rely on the honey bee as a social insect species that require interaction with their colony for normal behavior, physiology, and behavior. Pheromones are transmitted by queen and worker bees in a hive setting which regulate behavior and physiological traits, in addition to conditions vital to social and innate immunity in honey bees. Jandt et al. (2015) reported that experimentation of the social insect species, *Polistes fuscatus*, negatively affected gene expression, physiology, behavior, and colony dynamics when wasps were reared in a laboratory versus the field.

Activity of one component in the antioxidant system, lipid peroxidase, was increased in both the hive and laboratory conditions. MDA, the byproduct of lipid peroxidation, is the most common measure of cellular injury in studies of oxidative stress. An increase in lipid peroxidase activity is indicative of increased cellular damage caused by ROS. The overwhelming majority of studies investigating lipid peroxidation in both vertebrate and invertebrate species found an increase in lipid peroxidase activity after exposure to

atrazine (Elia et al. 2002, Jin et al. 2010, Singh et al 2011, Farombi et al. 2013). This study affirms a similar increase in lipid peroxidase activity in honey bees treated with atrazine in both field and laboratory settings, even after accounting for variation between enzyme levels in day 0 bees.

Semi-quantitative gene expression levels of seven antioxidant-encoding genes were measured in honey bees were treated with five increasing concentrations of atrazine for 24 h in the laboratory. The gene expression levels after exposure to atrazine were altered differentially according to gene, and no singular trend was observed. Expression of *CuZnSod-1* and *Catalase* increased with exposure to atrazine up to the highest concentration. The expression of some genes increased at lower exposure levels only to decrease at higher concentrations, such as *MnSod-2* and *Gtpx-1*. *MsrA* expression levels decreased at low concentration levels but saw no changes in expression compared to the control at higher concentration. Expression levels of *Tpx-3* and *Trxr-1* did not change after exposure to any atrazine treatment. The concentration range of atrazine in the laboratory exposure exceeded the previously reported environmentally relevant concentrations of 1-25 ppb (Gilliom et al. 2006). These exaggerated concentrations (i.e. 100 and 1000 ppb) were designed to elicit an acute and discernable response in relative gene expression as compared to the untreated control and thus do not possess true biological significance.

After 28 days of exposure to 10 ppb atrazine in the hive, the expression levels of seven antioxidant encoding genes were assessed. Expression levels of c*atalase*, *Trxr-1*, *MsrA*,

and *Gst-1* remained consistent between control and atrazine treatments after day 28. Exposure time was found to have an effect on expression levels of three genes including *CuZnSod-1*, *Tpx-3*, and *Gtpx-1* and treatment with atrazine had an effect on expression in *Tpx-3* and *Gtpx-1*. Expression of *Tpx-3* and *Gtpx-1* in atrazine-treated bees on day 28 increased 186.5% and 93.2%, respectively, compared to the untreated bees.

Many studies investigate differential gene expression levels in different species after exposure to atrazine, but relatively few look at antioxidant-encoding genes. These studies look at various endpoints of atrazine exposure such as genes related to: degradation (Devers et al. 2004, Monard et al. 2010), various hormones (Hecker et al. 2005, Pogrmic et al. 2009, Yang et al. 2010), various stressors (Anderson et al. 2008, Langerveld et al. 2009, Yang et al. 2010, Xing et al. 2013), and numerous other genes of interest. Jin et al. (2010) studied the oxidative stress response of zebrafish exposed to atrazine by way of antioxidant-encoding genes in different tissue samples. It was found that *CuZnSod*, *MnSod*, and *catalase* genes were differentially expressed in different tissues and no overall pattern of up- or down-regulation of genes occurred. Corona et al. (2005) investigated expression of the same antioxidant-encoding genes used in this study in honey bees, but looked at differences in expression between castes in relation to senescence, not after exposure to a known oxidative stress inducer. To date, there are no studies that have investigated antioxidant-encoding gene expression levels in honey bees exposed to atrazine.

No uniform upward or downward trend in gene expression levels was noted in this study, which may be due in part to the different role each gene plays in the antioxidant system. Gene expression levels in laboratory-treated bees may have experienced a fluctuating trend due to atrazine exposure in conjunction with higher inherent stress related to being removed from the field. Additionally, the honey bees used in both hive and laboratory portions of this study may have undergone unknown and unrelated stress prior to the experiments, which could have led to variable antioxidant gene expression levels. Hives in the field portion were outfitted with new frames and foundation in an attempt to limit exposure to other pesticides or pathogens found in the field, but this preventive measure does not exclude exposure after hive establishment.

This study aimed to investigate enzymatic and biochemical responses of honey bees to atrazine exposure in both the laboratory and hive. Laboratory experiments were conducted on bees collected from the field, brought into the lab, and exposed to atrazine concentrations spanning from environmentally relevant to acute. This approach allowed for manipulation of conditions, which may have otherwise led to uncontrollable variation in the hive such as a more consistent evaluation of compound consumed by the bees, and a smaller chance of compound degradation due to a shorter (24-h) exposure period. The in-hive experiment allowed for processes to take place in a natural environment and in a manner more biologically relevant for the species at hand. Both experimental settings have their respective advantages and disadvantages, which may have caused deviation from “true” values, but no study can perfectly control or remove all extraneous environmental stressors. The purpose of this study was to elucidate oxidative stress

biomarkers in honey bees exposed to a commonly-used herbicide in the laboratory and hive. We found that honey bees exposed to the herbicide atrazine in the laboratory and the field experienced an overall decrease in antioxidant enzyme activities, and that relative expression levels of some antioxidant-encoding genes were differentially changed after atrazine exposure. We believe that exposure to atrazine in the laboratory and in the hive at environmentally relevant doses overwhelmed the endogenous antioxidant capacity of the honey bees with a resulting build-up of ROS and oxidative stress. The information gathered here serves to bridge a current knowledge gaps related to honey bees and atrazine exposure with the hope of protecting these important pollinators from any deleterious health effects related to unintended exposure to agricultural pesticides.

Chapter 3

SUMMARY

The pollination services provided by honey bees contribute to the food and fiber needs of our society. The decline of managed colony numbers in recent decades warrants an investigation into any factor which may contribute to a loss in honey bee health. Oxidative stress results from the uncontested buildup of destructive reactive oxygen species (ROS) in an aerobic organism. These ROS can accumulate and inflict detrimental intracellular reactions, including cellular membrane damage and the oxidation of proteins and DNA. Atrazine is the second most commonly applied pesticide in the U.S. and is a known inducer of oxidative stress in multiple species. Numerous studies link atrazine exposure to an induction of oxidative stress in other species, but limited knowledge exists in regards to its effects on honey bees. This study examined any induction of reactive oxygen species and oxidative stress in honey bees exposed to the agricultural herbicide atrazine. Levels of enzymatic antioxidant components and relative expression levels of antioxidant-encoding genes in bees exposed to this herbicide were assessed in both laboratory and in-hive conditions.

This study demonstrates that atrazine does elicit oxidative stress responses in honey bees in both laboratory and in-hive conditions. Overall enzymatic antioxidant activity decreased in honey bees exposed to environmentally relevant concentrations in the laboratory and in-hive, suggesting an induction of oxidative stress and a disruption of antioxidant defenses. Expression levels of seven antioxidant-encoding genes were assessed in honey bees exposed to multiple concentrations of atrazine in the laboratory and one concentration in the field. No singular upward or downward regulation trend in

antioxidant-encoding gene expression was observed in either laboratory or in-hive treated honey bees. It is not possible to implicitly state that oxidative stress caused by atrazine exposure brought about these differential changes in gene expression; however, past research studies have found that exposure to atrazine does change gene expression levels in multiple species. This study serves to bridge a current knowledge gap related to honey bees and atrazine exposure and investigates an additional facet of the multitude of stressors possibly related to honey bee decline.

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