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Evaluating The Interaction Between Oxidative Stress Management and Insecticide Resistance in *Chironomus dilutus*

GRANT OISHI
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Reviewed and approved* by the following:

Adam Simpson
Assistant Teaching Professor of Biology
Thesis Supervisor

Michael Campbell
Distinguished Professor of Biology
Honors Adviser

* Electronic approvals are on file.

ABSTRACT

In the environment, organisms encounter both natural and synthetic stressors. Exposure to these stressors may or may not cause acute toxicity; regardless, the metabolism of toxins/toxicants often creates reactive oxygen species, which can induce oxidative stress. Oxidative stress alters a variety of physiological processes, which in turn may impact an organism's susceptibility to other environmental stressors. Although oxidative stress is known to induce the activity of enzymes that are involved in xenobiotic metabolism, the direct influence of this process on insecticide susceptibility is unknown. This study explores the relationship between oxidative stress and susceptibility to a common-use neonicotinoid insecticide, imidacloprid. Using lab cultures of *Chironomus dilutus*, the *in vitro* activity of glutathione-S-transferase (GST) was quantified before and after the induction of oxidative stress via an organic inducer, paraquat, and an inorganic inducer, cadmium (Cd). Paraquat exposure in second-instar *C. dilutus* resulted in reduced GST activity from 2.655 (± 0.372) mmol/min/mg to 2.053 (± 0.142) mmol/min/mg. In fourth-instar *C. dilutus*, GST activity was increased following paraquat exposure from 2.182 (± 0.362) mmol/min/mg to 2.456 (± 0.085) mmol/min/mg. Imidacloprid and imidacloprid+paraquat toxicity tests of second-instar *C. dilutus* yielded median lethal concentrations (LC_{50}) of 1.58 and 1.33 ng/mL, respectively. The synergistic ratio of 1.19 indicates that co-exposure to paraquat increases the toxicity of imidacloprid. Exposure to a range of Cd concentrations in fourth-instar *C. dilutus* did not show a dose-response GST activity. The information gathered from this thesis has emphasized the complexity of the metabolic responses involved in multiple-stressor exposure scenarios.

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Chapter 1

Introduction

1.1 Origin of Insecticides

One of the most prolific, and significant, human inventions is the development of agriculture. Originating 12,000 years ago, humans began changing their ways from a hunter-gatherer society to the planted and cultivated methods of farming we see today (National Geographic Society, 2019). This shift in our approach to obtain food and resources enabled us to focus on other aspects of our development and evolution as a species, such as building settlements, exploring the world around us, and thinking about more than just where our next meal would come from. Over the thousands of years since animal and plant farming began, society has gone through one revolution after another in terms of technology and sophistication. However, one plague that has followed agriculturists throughout the thousands of years has been the attack of their crops by pests.

To combat the dynamic epidemic that are agricultural pests, humans have used various methods of prevention. One of the most common methods of protection from crop pests is the use of agrochemicals, or insecticides on crops. Terms such as “pesticide” and “insecticide” are common in today’s age; however, the use of chemicals to ward off agricultural pests is not a new practice. The Sumerians were awarded the first recorded use of insecticides, 4500 years ago (Unsworth, 2010). Having issues with mites, the Sumerians used sulfur-containing compounds to deter pests from their crops (Unsworth, 2010). Similarly, the Chinese used compounds such as

mercury and arsenic to treat pediculosis, or body lice 3200 years ago (Unsworth, 2010). As these practices were effective, compounds such as sodium chlorate and sulfuric acid were used to combat agricultural pests up until the 1940's (Unsworth, 2010). The 1940's are also when new insecticides were introduced that have changed agriculture forever: synthetic organic insecticides (Unsworth, 2010).

Synthetic insecticides are anthropogenic organic compounds created to kill insects in agricultural, industrial, and residential settings (Forgash et al., 1984). In 1946, the first synthetic insecticide, dichlorodiphenyltrichloroethane (DDT), was introduced; and for a century, synthetic insecticides have been employed across the United States in an effort curb the deleterious effects pest species have on human establishments (Forgash 1984). While initially the new synthetic insecticides showed high efficacy and were cost-effective, there was an unforeseen cost to be paid in the future: insecticide resistance. Insecticide resistance is the development of a tolerance to any given insecticide (Hemingway et al., 2004). The heavy application of synthetic insecticides created a selective pressure on pest species treated with these agrochemicals, resulting in the proliferation of resistant lineages that survive the initial exposure. The issue of resistance dates back before synthetic insecticides to 1897; however, the years following 1946 showed the largest increase of insecticide resistance ever recorded (Forgash 1984).

In the past few decades, new class of insecticide—neonicotinoids—emerged with the release of the prototype drug imidacloprid in 1991 (Bass et al., 2015). Neonicotinoids act as agonists of nicotinic acetylcholine receptors in insects, irreversibly binding these receptors, resulting in an initial spontaneous discharge, followed by the inhibition of neuronal propagation (Dalefield, 2017). This novel class of insecticides now comprises more that 25% of insecticides purchased globally (Bass et al., 2015). However, the popularity of neonicotinoids has contributed

to the development of neonicotinoid-resistance, with over 500 peer-reviewed publications citing pest resistance to these compounds (Bass et al., 2015). The majority of papers published involve imidacloprid, on which over 330 cases have been reported (Bass et al., 2015). Among the reported resistant species are members of the insect orders Diptera and Lepidoptera (Bass et al., 2015), which include the study organisms utilized in this thesis: *Chironomus dilutus* and *Endopiza viteana*, respectively.

1.2 Insecticide Resistance

Insecticide resistance poses public health, agricultural, and economic threats to agriculturists and the industries they support. With the use of insecticides, an estimated $70 billion USD are lost annually to crop pests globally (Food and Agriculture Organization of the United States, 2022). As stated, prior, insecticide resistance is the development of a tolerance to any given insecticide (Hemingway et al., 2004). To be more specific insecticide resistance can occur via one of two general mechanisms: metabolic resistance or target-site resistance (Hemingway et al., 2004). Target-site resistance involves the change in shape of the receptor responsible for binding the insecticide, causing the cascade of effects which render the toxicant harmful to the pest (Hemingway et al., 2004).

Metabolic resistance can take many forms as metabolism is a multi-phase process of biotransformation. Insects generally rely on three enzyme families that confer metabolic resistance to toxicants via detoxification: carboxylesterases, cytochrome P450 monooxygenases (CYP450), and glutathione-S-transferases (GST) (Kerkut & Gilbert, 1985; Despres et al., 2007). These families of enzymes function primarily in phase I and phase II metabolic reactions. Phase I

metabolism involves the cleavage of toxicants via enzymes into less toxic metabolites which can be excreted from the organism (e.g., esterases hydrolyzing the ester bonds in carbaryl, producing inactive metabolites that are harmless to the organism and readily excreted) (Hemingway et al., 2004). Phase II metabolism involves conjugation, or the addition of a polar functional group to a toxicant that makes it more readily excretable (e.g., glutathione conjugation via glutathione-*S*-transferase) (Hemingway et al., 2004). Both cleavage and conjugation alter the chemical properties and shape of the compound, altering its toxicity, and/or producing more readily excretable metabolites (Hemingway et al., 2004). A third phase of metabolism can be involved in insecticide resistance, which involves excretion of the compound from the cells of an organism via transport proteins (ATP-binding cassettes) (Gott et al., 2017). Of these three catabolic categories, phase I and phase II metabolism are more commonly implicated as mechanisms of insecticide resistance.

Glutathione-*S*-transferases (GST) have a long history of conferring insecticide resistance by detoxifying xenobiotics, modifying endogenous metabolites, and managing products of oxidative stress (Enayati et al., 2005; Sanil et al., 2014). A few species that have shown upregulated GST activity in DDT, organophosphate, carbamates, and pyrethroid-resistant strains include *Anopheles stephensi* (Sanil et al., 2014; Enayati & Ladonni, 2006), *Anopheles gambiae* (Chen et al., 2003; Prapanthadara et al., 1993; Ding et al., 2003; Ding et al., 2005; Ranson et al., 2000; Ranson et al., 2001), *Anopheles funestus* and *Anopheles arabiensis* (Oliver & Brooke, 2016), *Aedes aegypti* (Lumjuan et al., 2005), *Spodoptera frugiperda* (Yu, 1992), *Nilaparvata lugens* (Vontas et al., 2001), *Plutella xylostella* (Huang et al., 1998), *Phlebotomus argentipes* (Hassan et al., 2019), *Hyalella azteca* (Weston et al., 2013), *Tenebrio molitor* (Kostaropoulos et al., 2001), and *Musca domestica* (Fournier, et al., 1992). While some species (e.g., the *Anopheles*

genus) have greater implications as vectors for disease and other arthropods (e.g., *Spodoptera frugiperda*) are more agriculturally relevant as crop pests (Oliver & Brooke, 2016; Sanil et al., 2014; Chen et al., 2003; Ranson et al., 2001; Yu, 1992), insecticide resistance in all pest species is problematic.

1.3 Metabolic Resistance & Oxidative Stress

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidant inhibitors (McCarthy et al., 2004). ROS are created through numerous pathways, one of which is the metabolism of toxicants via enzymatic and non-enzymatic processes (Abdollahi et al., 2004). Commonly used drugs (e.g., acetaminophen, ciprofloxacin, phenobarbital, and tricyclic antidepressants), pollutants (e.g., asbestos, carbon monoxide, and paraquat), radiation (UV light, x-rays, gamma radiation), metal ions (iron, copper, cadmium, nickel, chromium, and mercury), bacteria (E.g., *E. coli*), and pesticides (E.g., herbicides, insecticides, fungicides, bactericides) can all act as sources of exogenous ROS (Abdollahi et al., 2004; Hayaoka & Dauterman, 1982; Yamamoto et al., 2011). Exposure of insects to herbicides – which do not have acutely toxic effects on insects unless applied in superfluous amounts – is one avenue by which oxidative stress can be induced non-lethally (Abdollahi et al., 2004). The problem with ROS is that they are not idle molecules: they interact with cellular components and can damage lipids, proteins, and DNA (Hülya, 2005). The effects of ROS can result in necrosis or cellular apoptosis (McCarthy et al., 2004). When ROS are formed, antioxidants are utilized to detoxify and rid the organism of harmful compounds.

One of the main intracellular antioxidants is glutathione (GSH), a highly polar tripeptide which is conjugated to toxicants via GST (Pavlidi et al., 2018). Once GSH is bound to the toxicant or ROS, it can dissolve into hemolymph and be excreted as waste (Pavlidi et al., 2018). This polarization and excretion of undesirable molecules minimizes the damage they can inflict, as well as the duration of their exposure, drastically reducing toxicity of a toxicant (Shou-Min, 2012; Hassan et al., 2019; Rinaldi et al., 2002; Owuor & Kong, 2002; Hayes & McLellan, 1999; Zou et al., 2000). Upregulated GST in arthropods has been noted as contributing directly to resistance to all major classes of insecticides (Pavlidi et al., 2018). The mechanism by which GST neutralizes the toxicant is two-fold: GST can conjugate GSH to the toxicant, resulting in its elimination from the organism, or GST can conjugate GSH to ROS that are produced by the chemical activity of the toxicant, which can be just as harmful as the pesticide itself (Yan et al., 2013; Udomsinprasert et al., 2005; Singh et al., 2001; Sawicki et al., 2003).

A prime example of an oxidative stress inducer is paraquat, one of the most used herbicides in the world, which is known to trigger an increase in GST activity following exposure (Ahmad et al., 2014; Black et al., 2008; Yang & Tiffany-Castiglioni, 2005; Figueiredo-Fernandes et al., 2006; Martinez-Lara et al., 1996). Cadmium (Cd) is a heavy metal that is also known to induce oxidative stress; however, current research indicates that the effect of Cd on GST activity varies. Some studies showed upregulated GST activity following Cd exposure (Nair & Choi, 2011; Ognjanovic et al., 2003; Wan et al., 2009; Zhang et al., 2019; El-Sabbagh et al., 2022; Kim et al., 2010), while others demonstrated the opposite (Islam et al., 2019; Jahangir et al., 2005; Xu et al., 2015). Some studies even showed downregulated GST activity at low concentrations of Cd, and upregulated activity at a concentration twice as high (Cunha et al., 2007).

Because of the shared physiological mechanisms underpinning the detoxification of ROS and xenobiotics, **understanding how these metabolic processes interact may illuminate novel adaptive responses to multiple stressors**. My thesis aims to investigate this knowledge gap using a traditional model system (*Chironomus dilutus*), which was used to address a series of experimental objectives. The first objective was to establish baseline toxicity of a common-use insecticide in second and fourth-instar midge larvae (*C. dilutus*). Following initial toxicity tests, I determined baseline GST activity in *C. dilutus*, which was compared to the GST activity of larvae that were exposed to sublethal doses of an oxidative stress inducer. If the results indicated a treatment effect on GST activity, I compared the baseline insecticide toxicity to that of a mixture toxicity test (insecticide + inducer) to determine if altered GST activity influenced the acute toxicity of the insecticide. This workflow was conducted with both an organic and inorganic inducer of oxidative stress.

Chapter 2

Materials and Methods

2.1 Study Organisms

The model organism used in this study was *Chironomus dilutus*, colloquially known as the midge. Midges are semi-aquatic benthic arthropods, which cycle through the following life stages: egg sac, larvae (instars 1-4), pupae, and adult males/females (Oliver, 1971). For this study, I focused my efforts on the larval life stage, specifically the second- and fourth-instar. The semi-aquatic nature and relative sensitivity of midges makes them an ideal model for aquatic toxicity tests. Because they are still undergoing growth and development, younger *C. dilutus* larvae (first and second instar) are more sensitive to stressors than older larvae (third and fourth instar) (Ingersol et al., 2015). For these reasons, second-instar larvae are commonly used in standardized protocols (USEPA, 2000). Fourth-instar larvae were also included in the study due to their higher tissue mass and because they are developmentally representative of late-stage Grape Berry Moth larvae. Terrestrial toxicity tests were initially attempted in the summer of 2021 with Grape Berry Moth (GBM; *Endopiza viteana*) – one of the major crop pests in the Northeast United States – however, methodology proved difficult. As such I switched to *C. dilutus* as a representative model, which were maintained in sustainable laboratory cultures.

While *C. dilutus* is an ideal species for toxicity testing, it is not an agricultural pest species (USEPA, 2000). As mentioned previously, the Grape Berry Moth is a ubiquitous grape pest throughout the mid-Atlantic United States (Tobin et al., 2003). As an insect with a complex life cycle (complete metamorphosis), GBM shares similar life history characteristics with *C. dilutus*. Furthermore, GSTs are widely conserved across divergent taxonomic groups, including animals, plants, yeasts, and bacteria (Sheehan, et al., 2001; Frova, 2006). Consequently, it is reasonable to assume that GST function is similar between *C. dilutus* and GBM, as they both belong to the same taxonomic Class (Insecta).

Where GBM and *C. dilutus* differ is in their larval life history. Midges are deposited as egg sacs in the water and metamorphose into red larvae due to the iron in their hemolymph (commonly known as "blood worms") (Reyes-Maldonado et al., 2021). Remaining in fresh water as larvae, they grow until gaining enough energy to undergo the next stage in metamorphosis, pupation (Oliver, 1971). As pupae, midges transform into black, tadpole-like organisms which use their tail to propel themselves through the water and to the surface, so that they can emerge from their pupal casings as adults (Oliver, 1971).

GBM, on the other hand, start their lifecycle as adult females oviposit on grape berries, developing buds, or flower clusters (Nagarkatti et al., 2002; Tobin et al., 2003). During summer months eggs hatch within 3-5 days, after which the larvae eat their surrounding grape enclosure to grow (Nagarkatti et al., 2002). The larvae then burrow out of their grape nursery, falling to the ground below (as grape vines are typically suspended) (Tobin et al., 2003). Immense damage can be done to grape clusters through this process, as direct injury leaves grapes open to pathogens (Isaacs et al, 2012). Then the larvae wrap themselves in whatever foliage is available (often grape leaves) and undergo pupation (Tobin et al., 2003). Depending on the season, GBM may form pupae to undergo the next transition in metamorphosis, or for protection during overwintering (Tobin et al., 2003). GBM emerge from overwintering pupae as adult moths in varying distribution with genetic factors and temperature playing a large role (Isaacs et al, 2012). Adults have a variable lifespan, surviving for 4-23 days (Isaacs et al., 2012). Generally, two to four generations, with lower geographic latitudes correlating to more generations (Tobin et al., 2003). Nagarkatti and colleagues (2002) noted resistance of GBM to carbaryl in the Lake Erie grape belt that had never been documented before, foreshadowing a troublesome future for managing future generations of insecticide resistant GBM.

2.2 *In vitro* Enzyme Assays

Protein Isolation and Quantification

Tissue collection involved siphoning sediment from laboratory cultures, then sieving through sediment to isolate larvae. Individuals were selected by hand using the large mouth of a Pasteur pipette based on size (second- or fourth-instar). Midges were immediately placed in their respective treatment units (toxicity testing) or flash-frozen in liquid nitrogen (enzyme assays).

Following tissue collection—either fresh from lab cultures or after an incubation treatment—the samples were homogenized in 1 mL of phosphate buffer (PBS) with sterile disposable pestles for 30 seconds. The protein homogenates were then centrifuged for 30 minutes at 4°C (12,000 rpm). The resulting supernatant was transferred into fresh 1.5 mL centrifuge tubes and refrigerated for same-day use.

The Bicinchoninic Acid (BCA) assay was used to quantify the protein concentration of each tissue homogenate. A standard curve of bovine serum albumin (BSA) ranging from 25-750 ug/mL was prepared fresh each day, as well as the BCA reagent. The BCA assay was performed using a microplate spectrophotometer set at 562 nm; five biological replicates were included for each unknown protein homogenate. Once the protein concentration of each sample was estimated from the BSA curve, all homogenates were diluted to 50 ug/mL in preparation for the GST assay.

Glutathione-S-Transferase Assay

The GST assay is a kinetic spectrophotometric assay that quantifies the production of a metabolite over a 10-minute period. Methods were adapted from Habig and colleagues (1974) for use in a microplate reader. This assay was performed using standardized protein homogenates of 50 ug/mL; preliminary testing indicated that this specific concentration yielded the highest GST activity. The protein samples were incubated with 10.35 mM reduced glutathione (GSH; conjugate) and 200 mM CDNB (substrate). As GST conjugates glutathione to CDNB, absorbance is emitted at 340 nm. The microplate

was incubated in the microplate reader for 15 minutes at 30°C, shaken every 3 minutes to ensure adequate mixing. During the assay run, absorbance was measured every 30 seconds for 10 minutes. GST specific activity was calculated from the slope of each reaction using the known extinction coefficient of the GSH-CDNB conjugate (9.6 $mM^{-1}cm^{-1}$) standardized by the protein concentration.

BCA and GST assays were repeated to quantify 1) baseline specific activity in second and fourth-instar *C. dilutus*, 2) following paraquat exposure in second- and fourth-instar *C. dilutus*, and 3) following Cd exposure in fourth-instar *C. dilutus*.

2.3 Oxidative Stress Induction

Oxidative stress was induced in second and fourth instar *C. dilutus* over a 16-hour period via exposure to sublethal concentrations of an inducer. The organic and inorganic inducers of oxidative stress used in this study were the synthetic herbicide paraquat, and cadmium, respectively. To determine the maximum sublethal concentrations of each inducer, preliminary 24-hour exposure tests were performed with a range of concentrations.

Organic Oxidative Stress Induction: Paraquat

The paraquat range-finder test involved three 600-mL glass beakers per treatment containing a superficial layer of sand and 500 mL of moderately hard water (MHW). Ten second-instar *C. dilutus* larvae were transferred into each beaker from the lab culture. Each beaker was then spiked with 1 mL of paraquat ranging from 0.625-5.0 ug/mL, with three biological replicates per treatment. Controls were spiked with an equivalent volume (1 mL) of moderately hard water to reduce the risk of disturbance as a confounding variable. Beakers were placed in an incubator set to 23°C on a 16/8 light/dark cycle for 24 hours, after which mortality was scored.

Following the range-finder test for paraquat, a 16-hour paraquat exposure with second and fourth-instar *C. dilutus* was performed. A paraquat concentration of 5 ug/mL was deemed safe for sublethal exposure, and was the concentration used for the 16-hour exposure. The aim of the 16-hour exposure was to induce oxidative stress in the midges without causing lethality, as these tissues would be harvested for GST quantification. For this procedure there were two treatments for each instar: the control and paraquat treatments. Six replicates with 10 larvae per beaker were used for both treatments for a total of 24 beakers (six control and six paraquat second-instar beakers; six control and six paraquat fourth-instar beakers). The control groups only contained moderately hard water, while the paraquat beakers were brought to 5 ug/mL with a 1 mL spike of paraquat stock. Beakers were placed in an incubator set at 23°C on a 16/8 light/dark cycle. Following the 16-hour incubation period, surviving midges were extracted from each treatment and immediately flash-frozen with liquid nitrogen in cryotubes. Midges were kept frozen at -80°C to minimize tissue degradation.

Inorganic Oxidative Stress Induction: Cadmium

Cadmium is traditionally introduced into water as a salt ($CdCl_2$), which immediately dissociates as it enters solution. Unlike the paraquat treatments described above, the Cd treatments were focused exclusively on fourth-instar *C. dilutus* and required the use of steel containers as opposed to glass beakers. Because glass is not an inert surface, it could potentially interfere with chemical distribution and exposure. Fourth-instar midges were selected for the initial round of toxicity tests and assays due to their higher suspected tolerance and biomass. Although the original plan was to conduct Cd treatments with second-instar larvae, time constraints limited the experiment to fourth instar only. Because literature concerning the effect of Cd on GST activity is mixed, a range of Cd concentrations was used for the pre-treatment exposure and subsequent GST assays to look for a dose-response.

In short, fourth-instar midges were exposed to a range of Cd concentrations (5, 10, and 20 mg/L) for a duration of 16 hours. Six replicates were used for each concentration, with four larvae per container.

The units were placed in an incubator set to 23°C on a 16/8 light/dark cycle for 16 hours. Following the induction period, surviving larvae were extracted from each treatment and immediately flash-frozen with liquid nitrogen in cryotubes. Midges were kept frozen at -80°C to minimize tissue degradation.

Per the results, a second 16-hour Cd exposure was conducted with higher concentrations (25, 50, 100) mg/L following the same procedure.

2.4 Toxicity Tests

Second-instar Chironomus dilutus

Initial toxicity tests were performed with second instar *Chironomus dilutus* collected from existing laboratory cultures. Midges were exposed to a range of concentrations of the neonicotinoid insecticide imidacloprid (0.4, 1.0, 2.5, 6.25, and 15.6) ng/mL for a duration of 48 hours. At this point in the study, glass 500-mL beakers were still used with a thin layer of sand and 500 mL of moderately hard water, with three replicates per treatment containing 10 larvae. Beakers were spiked with 50uL of their respective imidacloprid stocks (50 uL of moderately hard water in controls) and then placed in an incubator set to 23°C on a 16/8 light/dark cycle for 48 hours. After the 48-hour incubation, the toxicity test was scored for mortality to calculate the median lethal concentration (LC50).

This process was duplicated for mixture toxicity tests involving the same range of imidacloprid concentrations (0.4, 1.0, 2.5, 6.25, and 15.6) ng/mL with concurrent exposure of 5 ug/mL paraquat for 48 hours. After the 48-hour incubation, the toxicity test was scored for mortality to calculate the LC50 of the chemical mixture.

Fourth instar Chironomus dilutus

Toxicity tests with fourth-instar *C. dilutus* were attempted using the same procedure as conducted with second-instar larvae with some modifications. Steel containers were used instead of glass beakers

and five replicates were used per treatment (four larvae per container). The testing conditions and imidacloprid concentrations were identical to the second-instar tests (0.4, 1.0, 2.5, 6.25, 15.6 ng/mL); however, results were inconclusive. For the subsequent toxicity test, a higher imidacloprid concentration range was used (2.5, 10, 40, 160 ng/mL).

Chapter 3

Results

3.1 *In vitro* Enzyme Assays

Paraquat-Induced Oxidative Stress

GST activity of second- and fourth instar *Chironomus dilutus* was quantified in paraquat-exposed and untreated midges. Exposure to paraquat reduced GST activity in second-instar midges from 2.66 (± 0.372) mmol/min/mg to 2.05 (± 0.142) mmol/min/mg (Figure 1). Conversely, in fourth-instar midges the activity of GST was increased in the paraquat-exposed group from 2.18 (± 0.362) mmol/min/mg to 2.46 (± 0.085) mmol/min/mg (Figure 1).

Cadmium-Induced Oxidative Stress

GST activity of fourth-instar *C. dilutus* was quantified in unexposed midges (control) and midges exposed to a range of Cd concentrations (5, 10, 20 mg/L). Results showed slight increases in GST activity in the 5 mg/L treatment, as well as slight decreases in the mid-range concentrations; however, a significant dose-response was not observed, so in a subsequent GST assay I exposed fourth-instar *C. dilutus* to a higher range of Cd concentrations (25, 50, 100 mg/L) (Figure 2). Effect sizes are as follows for the respective Cd concentrations: 5, 10, 20, 25, 50, and 100 mg/L Cd showed a 1.06, 1.0, 0.93, 0.93, 0.96, and 0.99x effect as compared to the control specific activity of GST (Table 2). These results indicate increased GST activity with exposure of fourth instar *C. dilutus* to 5 mg/L Cd, equal activity at 10 mg/L, and decreased activity for all Cd concentrations greater than 10 mg/L relative to the control (Table 2).

3.2 Toxicity Tests

Initial toxicity tests were performed with second instar *Chironomus dilutus* collected from existing laboratory cultures. Midges were exposed to the neonicotinoid insecticide imidacloprid at concentrations of 0.4, 1, 2.5, 6.25, and 15.6 ng/mL. An LC50 of 1.58 (1.08-2.34) ng/mL was estimated using SPSS (Table 1).

The mixture toxicity test performed with second-instar midges involved imidacloprid and paraquat. The resulting LC50 of this mixture toxicity test was 1.33 ng/mL. Comparing the mixture LC50 to that of the imidacloprid alone yielded a synergistic ratio (SR) of 1.19. A synergistic ratio is the direct comparison between baseline toxicity and mixture toxicity, calculated by dividing the LC50 of the imidacloprid toxicity test by the imidacloprid + paraquat LC50 (Wheeler et al., 2006). This SR indicates that exposure to imidacloprid with concurrent oxidative stress induction (via paraquat) increases its overall toxicity by a factor of 1.19 (Table 1).

Toxicity tests were attempted using imidacloprid on fourth-instar *C. dilutus*; however, results were inconclusive and an LC50 could not be generated from the data. A median effect concentration (EC50) of 2.42 (0.997-4.97) ng/mL was calculated based on insecticide-induced impairment using SPSS.

Chapter 4

Discussion

GST has been shown to confer metabolic resistance to insecticides in arthropods, as well as manage reactive oxygen species (ROS) that are produced from insecticide exposure (Pavlidi et al., 2018). The aim of this thesis was to understand the potential interactions between these two functions of GSTs – detoxification of organic toxicants and ROS in the model organism *Chironomus dilutus*. This was to be accomplished using three objectives. The first objective was to establish toxicity of a common-use insecticide in second and fourth-instar midge larvae. Next, constitutive GST activity was determined for second and fourth-instar midge larvae as well as following exposure to sublethal doses of organic and inorganic oxidative stress inducers. To examine the organism-level effects of modulated GST activity, the baseline toxicity of an insecticide (imidacloprid) was then compared to the toxicity of a mixture including imidacloprid and an inducer of oxidative stress (organic and inorganic). This would determine if up or downregulated GST activity conferred synergistic or antagonistic effects with the insecticide.

The first and second objectives were accomplished using paraquat, a known inducer of oxidative stress and one of the most frequently used herbicides in the world. GST assays showed minor variation between control and oxidative stress-induced midges for the second and fourth instar life stages. At this point, the mechanisms of GSTs as a family of detoxifying enzymes need to be considered. GSTs principally eliminate organic toxicants from the organism, meaning it was possible that the observed changes in GST activity were due to the presence of paraquat as an organic toxicant rather than its contribution to ROS production in the organism. This potential interaction presented a confounding variable that could not be discounted. As such, I decided to alter the course of the study and repeat the tests with a known inorganic inducer of oxidative stress: cadmium. As GST does not metabolize inorganic compounds, using an inorganic inducer of oxidative stress would in theory eliminate this confounding variable, and only show GST modulation as a result of ROS production.

Toxicity data for Cd in isolation or in combination with imidacloprid was not readily available, therefore I proceeded to start with the GST assays using fourth-instar midges (Figure 2). No dose-response in GST following Cd exposure was evident in the data, which suggests that GST activity is not strongly modulated in fourth-instar *C. dilutus* at the tested concentrations (5-100 mg/L). The fact that I used only fourth-instar midges could have masked potential treatment effects. As animals grow, their ability to bioaccumulate potentially harmful chemicals typically increases. Therefore, it is possible that fourth-instar midges were more tolerant to imidacloprid and Cd at the individual and physiological level. In other words, the ability of Cd to induce oxidative stress and GST activity might disappear at more mature life stages. Alternatively, it is possible that the Cd concentrations used were insufficient to elicit a ROS-mediated GST response. Williams and colleagues (1987) found the 24-hour LC50 of fourth instar *Chironomus riparius* exposed to Cd was 950 times greater than the first instar LC50 (2,400 mg/L compared to 2.1 mg/L Cd). Given that the Cd concentrations used in this experiment were low compared to Williams et al. (1987), it is likely that increasing the range would result in more promising results. Conducting mixture toxicity tests with imidacloprid and Cd would also help clarify this dilemma. Lekvongphiboon and Praphairaksit (2020) exposed *Tubifex tubifex*, an aquatic oligochaete, to mixtures of imidacloprid and Cd. Synergistic toxicity with imidacloprid and Cd exposure was found after 24 and 48-hours of exposure, as well as Cd accumulation in tissues and elevated acetylcholinesterase activity (Lekvongphiboon and Praphairaksit, 2020). This suggests that co-exposure to Cd does alter metabolic detoxification mechanisms, although GST may not be among them.

In this thesis and in the literature, there are conflicting results as to whether exposure to Cd up or downregulates GST activity. Numerous studies displayed an upregulation in GST activity following Cd exposure at concentrations comparable to those used in this thesis (Nair & Choi, 2011; Wan et al., 2009; Zhang et al., 2019; El-Sabbagh et al., 2022; Kim et al., 2010). Nair & Choi (2011) studied the effects of Cd and paraquat exposure to *Chironomus riparius* on GST and found upregulation in both cases. These results were found in similar organisms to those studied in this thesis (e.g., *C. riparius*), as well as more

distantly related aquatic species (e.g., *Haliotis discus, Procambarus clarkii, Oreochromis niloticus,* and *Takifugu obseurus*) as well as several terrestrial species (*Rattus norvegicus*) (Ognjanovic et al., 2003; Nair & Choi, 2011; Wan et al., 2009; Zhang et al., 2019; El-Sabbagh et al., 2022; Kim et al., 2010).

Conversely, many papers report a decrease in GST activity following exposure to Cd. Organisms in these studies also varied across taxonomic groups, including terrestrial vertebrates and invertebrates (*Mus musculus, Antheraea assamensis, Spodoptera litura*) as well as marine gastropods (*Monodonta lineata* and *Nucella lapillus*) (Jahangir et al., 2005; Islam et al., 2019; Xu et al., 2015; Cunha et al., 2007). Cd exposure concentrations were often less than those used in studies that noted upregulated GST activity (upregulated GST activity Cd concentrations ranged from 0.5-100mg/L; downregulated GST activity Cd concentrations ranged from 0.0016-1.6).

Cunha and colleagues (2007) used Cd concentrations of 0.35, 4.55, 0.592, 0.769, 1.0, and 1.3 mg/L in *Monodonta lineata* and *Nucella lapillus* – two marine gastropods. They found a decrease in GST activity from 0-0.6 mg/L Cd, which then increased to baseline from 0.6-0.9 mg/L Cd, then exceeded baseline GST activity from 0.9-1.4 mg/L Cd (Cunha et al., 2007). Similar to the results from this thesis, nonlinear trends were observed, raising the question of whether Cd exposure results in a dose-dependent non-uniform GST response (Cunha et al., 2007).

To better gauge the full extent of paraquat and Cd-induced oxidative stress on *C. dilutus*, a broader range of paraquat and Cd treatments could be applied to second and fourth-instar midges. This would provide a more complete profile of oxidative stress-mediated GST activity.

While GST is known to be upregulated in the presence of oxidative stress, it is possible that another metabolic mechanism is at play in these organisms for managing oxidative stress (e.g., catalase, glutathione peroxidase, superoxide dismutase, paraoxonase) (Finaud et al., 2006; Srivastava et al., 2017). It could also be that certain GSTs were up or downregulated, while the net GST activity remained relatively unchanged. As Nair & Choi (2011) demonstrated, exposure to the same concentrations of Cd resulted in mixed up and downregulation of 13 different GSTs. Sheehan and colleagues (2001) note there

are three major families of GSTs: cytosolic (alpha, mu, pi, theta, sigma, omega, and zeta), mitochondrial (kappa), and microsomal. It is possible that the activity of microsomal GST activity (not measured in this study) was modified by paraquat or Cd exposure and that the methodology was not specific enough to detect those changes.

A potential explanation as to why there was not a notable change in GST activity from baseline in the fourth-instar midges is that they are simply larger organisms. Typically, animals that are smaller are more sensitive to internal and external stressors. Larger animals have a greater capacity to regulate toxicants and manage oxidative stress due to greater lipid mass and enhanced ability for bioaccumulation, as well as more total protein content and enzymes to breakdown toxicants (Williams et al., 1986). I found evidence to support this in a previous study, which examined how xenobiotic metabolism changes across metamorphosis in *C. dilutus* and *Endopiza viteana* (Oishi et al., *in prep*). I determined that acetylcholinesterase and *a*-esterase activity vary between the second- and fourth-instar stages, as well as across metamorphosis. To determine if this is the case, my methods should be repeated using both second and fourth-instar midges.

Culture contamination is an extraneous variable that was unaccounted for. During the process of my experiment, laboratory *C. dilutus* cultures became contaminated with *Hyallela azteca*. This could present an additional stressor for midges, as they now had to compete with another species for resources (e.g., nutrients, space, oxygen). That being said, all *C. dilutus* were extracted from the same cultures, so this level of contamination would have been present in all samples; however, it still could have affected the overall trends in GST activity.

As described above, I encountered several obstacles and challenges over the course of my data collection. If I had more time to collect data, I would make several changes to my experimental approach. I would repeat all toxicity tests and GST assays with both second- and fourth-instar larvae to reduce confidence intervals and acquire repeatable data. I would increase the concentrations of Cd and paraquat to see if a dose-response is present at much higher ranges. I would use more specific protein isolation

procedures to extract specific GSTs (e.g., microsomal) for downstream analysis (Nair & Choi 2011). I would also be interested in exploring other attenuators of oxidative stress (e.g., carboxylesterases or CYP450 monooxygenases). Finally, I would select another test species to determine if the same results are seen in another model (e.g., *Hyalella azteca*, *Danio rerio*).

Chapter 5

Conclusion

Attempts to identify the origins of insecticide resistance involve the systematic elimination of possible mechanisms. One such mechanism, Phase II metabolism (e.g., GST), is a common culprit due to its broad detoxification capabilities. In this thesis, GST activity in larval *C. dilutus* was relatively unchanged following exposure to Cd and showed unexpected trends in response to paraquat exposure. Despite these negative results, different mechanisms of oxidative stress management could be explored, including superoxide dismutase and catalase (Finaud et al., 2006).

The importance of this research is multifaceted. With shifts in the environment due to climate change, the beginning of the growing season will occur earlier, and the end will occur later each year (Dobson et al. 2020). A longer period of hospitable conditions could eventually support an additional generation for crop pests (Isaacs et al, 2012; Chen et al., 2011; Tobin et al., 2008). Additionally, because these pests would likely be exposed to higher peak temperatures in the summer, this new stressor could act as a selective pressure that gives rise to even hardier pest lineages. Furthermore, with the continued application of new combinations of pesticides (fungicides, herbicides, insecticides, rodenticides, bactericides) in residential, industrial, and agricultural settings, many target and non-target species are exposed to a complex cocktail of chemical stressors (Bradley et al., 2019). Midges, for instance, are an example of a non-target species that is often affected by runoff containing pesticides (Bradley et al., 2019). And while *C. dilutus* lacks the charisma of other insect species, it plays a vital role in freshwater ecosystems (Covich, et al., 1999). Therefore, it is imperative to understand the mechanisms that dictate pesticide sensitivity and tolerance in both target and non-target species. While pesticide resistance is only one of seemingly infinite issues that face humankind and the ecosystems at large, understanding this

phenomenon is an important piece of the puzzle that has the potential to make large-scale changes that will impact the world.

Appendix A

Tables and Figures

Table 1: Acute 48-hour toxicity of imidacloprid and imidacloprid-paraquat mixture to second instar *Chironomus dilutus*.

Treatment	LC50 (ng/mL)	Synergistic Ratio
Imidacloprid	1.58	X
Imidacloprid + Paraquat	1.33	1.19

Table 2: Specific activity of GST in fourth instar Chironomus dilutus compared to baseline via fold change.

Cd Concentration (mg/L)	Specific Activity mmol/min/mg	Standard Deviation	Fold Change
Control	1.91	0.13	X
5	2.03	0.21	1.06
10	1.90	0.25	1.00
20	1.78	0.07	0.93
25	1.77	0.18	0.93
50	1.83	0.15	0.96
100	1.89	0.24	0.99

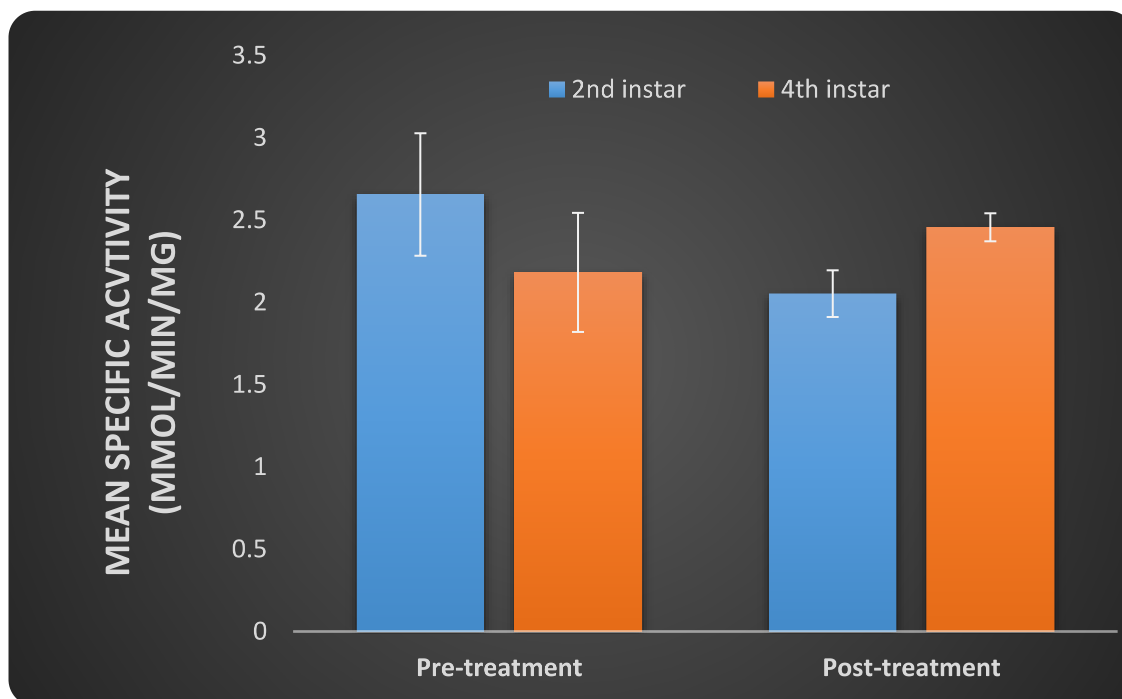

Figure 1: Specific activity of GST in second and fourth instar *Chironomus dilutus* before and after exposure to paraquat. Error bars represent standard deviation.

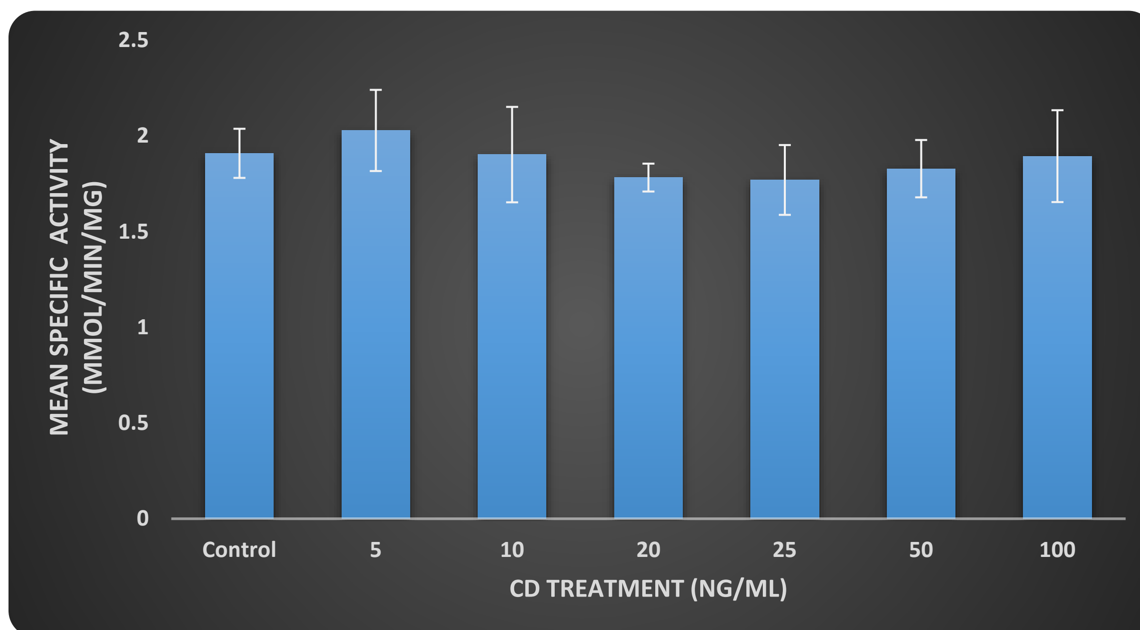

Figure 2: Specific activity of GST in fourth instar *Chironomus dilutus* following exposure to Cd. Error bars represent standard deviation.

Requiem for a Grape Berry Moth

On Gossamer Wings

Here I lie, but who am I?
Am I but a pest, in this familial land?
The fragrance of my quarry, a Concord dream
Sweet as a summer evening, and yet a bitter taste prevails
I sense the danger, I can smell it in my feet
And yet resist, I cannot
For oviposit, I must
With clusters so inviting, to an irresistible drive I succumb
I lay my future in the embrace of fickle fruits
Still green in their promise but also in their naivety
In my progeny, my future rests

New birth brings infinite potential, yet one inevitable fate
For this insipid danger I recalled, and ignored so long ago
Was not the sour aroma of tannins, bitter yet inconsequential
But something much more nefarious, and exceedingly foul
Unnatural it may seem, its odor is ambient
My young know no better, without the wisdom of their elders
Partaking in these forbidden fruits, as their ancestors did before them
But punished are they now, for venerable transgressions
Their first bite tainted by a noxious broth
Doomed from first instar to last
Doomed from first instar…to last

So here I lay, my identity now certain
My purpose, equally so
Continue I must, seeking the next cluster
On gossamer wings I fly
On gossamer wings I die

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ACADEMIA VITA

Grant Shiro Oishi
Email: grantoishi12@gmail.com

EDUCATION

Penn State Erie, The Behrend College || Schreyer Honors College	May 2022
Bachelor of Science in Nursing	
Honors in Biology	
Minor in Biology	

HONORS, AWARDS, AND ACCOMPLISHMENTS

Clark Family Scholarship	2021–2022
	2020–2021
	2019–2020
	Spring 2019
Penn State Behrend Undergraduate Student Academic Year Grant	2021–2022
	2019–2020
Class of 1922 Memorial Scholarship	2020–2021
Evan Pugh Scholar Award – Junior	Spring 2020
MCC Organization Awards:	
Most Outstanding Organization	Spring 2020
Outstanding Co-Sponsorship	Spring 2020
Penn State Behrend 2019 Undergraduate Student Research Fellowship	Summer 2019
Dean's List	Fall 2018–Present
Penn State Behrend Honors Program	Fall 2018–Present

WORK EXPERIENCE

University of Pittsburg Medical Center, Hamot	
Student Nurse Intern – Trauma Neuro ICU	May 2021–July 2021
Penn State Behrend	
Undergraduate Research Assistant – Simpson-Nutile Lab	May 2019–Present
Peer Tutor (Biology, Chemistry, Physics, Nursing)	Sept. 2019–Present
Peer Mentor – B.E.S.T Program	Aug.–Dec. 2020
Foxdale Village	
Certified Nursing Assistant	June 2020–Aug. 2020

RESEARCH EXPERIENCE

Undergraduate Honors Thesis	Fall 2021–Present
Penn State Behrend-Sigma Xi Undergraduate Conference	April 2022
SETAC North America 42nd Annual Meeting	Nov. 2021
Regional Science Consortium 17th Annual Research Symposium	Nov. 2021
Penn State Behrend-Sigma Xi Undergraduate Conference	
Second place in biology II session	April 2021
SETAC North America 41st Annual Meeting – SETAC SciCon2	Nov. 2020

LEADERSHIP AND DEVELOPMENT EXPERIENCE

Cultural Cooking Club	
Vice President	2021–Present
President	2019–2020
Secretary	2018–2019
Scrubs Club	
Treasurer	Fall 2020–Present
Penn State Behrend Welcome Week	
Guide	Fall 2020
Sigma Theta Tau International Honor Society of Nursing	
Member	2021–Present
MLK Day of Service	
Volunteer	Spring 2019
Mount Nittany Medical Center	
Patient Floors Volunteer	Summer 2018

LICENSES AND CERTIFICATIONS

ELNEC – Undergraduate/New Graduate	Fall 2020
Certified Nursing Assistant	Exp: June 2022
CPR Certification – AHA	Exp: June 2023