AN EXAMINATION OF IMIDACLOPRID INDUCED STRESS AND HORMESIS IN THE BENEFICIAL INSECT PREDATOR, *PODISUS MACULIVENTRIS* (SAY) (HEMIPTERA: PENTATOMIDAE)

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
November 2022

To my Father

May the road

rise up to meet you.

May the wind

be always at your back.

May the sun

shine warm upon your face,

and rains fall

soft upon your fields.

And until we meet again,

may God hold you

in the palm of His hand.

-Irish Blessing-

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Abstract

Traditional models of toxicology have failed to capture the biological complexity of low dose effects of toxicological agents on organisms and the environment. The hormetic model, whereby low dose exposures to chemical or other stressors stimulate biological processes, has been shown to be a common occurrence in the toxicological literature. Stimulatory effects on life-history traits such as growth, reproduction, longevity, survival, and increased stress tolerance occur across the biological spectrum and are ubiquitous in insects. I examined the effects of low doses of imidacloprid on the beneficial insect predator, *Podisus maculiventris*, on survival and reproductive traits in insects exposed as nymphs or adults, and subsequent effects across generations. I also examined whether the same concentrations could induce hormesis on predatory behavior and predation. Finally, I further explored how hormesis manifests in insects at the molecular level through a systematic literature review and through a more specific transcriptome analysis of *P. maculiventris*. I observed that reproduction may be stimulated in *P. maculiventris*, without major effects on behavior and predation, but effects vary with age, generation, sex, and bioassay design. Molecular responses associated with hormesis included changes in the expression of genes associated with DNA damage response and repair, antioxidantion, detoxification, and growth, development, and reproduction. Further, analysis of the literature revealed that while patterns in the molecular responses associated with hormesis are robust, coordination of molecular responses is influenced by such things as stressor, life stage, time and generation, and sex of the individual. Thus, establishing stress response profiles may be necessary to determine the overall mechanism of hormesis.

List of Abbreviations

µg/L	micrograms per litre
mg/L	milligrams per litre
mL	millilitres
g/L	grams per litre
cm	centimetres
AI	active ingredient
LOAEL	lowest observable adverse effects level
NOAEL	no observable adverse effects level
NOEL	no observable effects level
LNT	linear no-threshold
LD_{50}	lethal dose of 50% (median lethal dose)
LD_{20}	lethal dose of 20%
°C	degrees Celsius
P	parental generation
F1	first generation offspring
SE	standard error
IPM	integrated pest management
RH	relative humidity
L:D	light:dark
ANOVA	analysis of variance
LSD	least significant difference
GLM	general linear model
PROC	procedures
OMAFRA	Ontario Ministry of Agriculture, Food, and Rural Affairs
ROS	reactive oxygen species
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
M	molar concentration
g	gravitational force
FDR	false discovery rate
FC	fold change
adj	adjusted
bp	base pairs
GO	gene ontology
MF	molecular function
CC	cellular component
BP	biological process
qRT-PCR	quantitative real-time polymerase chain reaction
RNA-seq	ribonucleic acid sequencing (transcriptome sequencing)
TCA	tricarboxylic acid
ETC	electron transport chain (mitochondrial respiratory system)

Acknowledgements

My time at the Faculty of Agriculture has been truly wonderful. I have had the great privilege of growing to know many faculty, staff, and fellow students who have greatly impacted my life. I would especially like to thank Dr. Randy Olson, Dr. Dave Burton, Dr. Robert France, Dr. Kathleen Kevany, Dr. Tarjei Tennessen, Dr. Raj Lada, Dr. Vilis Nams, Dr. Gordon Brewster, Dr. Gefu-Wang Pruski, Dr. Steven Dukeshire, Dr. Janine Gray, and Dr. Tess Astatkie for their incredible support, kindness, wisdom, and humour. I also wish to thank members of our Faculty laboratory staff, Tanya Muggeridge, for providing assistance and procuring supplies, and Debbie Mellish and Kalyani Prithiviraj, for always thinking of me when needing additional help with a lab class or grading student assignments. I would like to thank Gisele Mazerolle and Margaret Savard for their extraordinary administrative help throughout my studies, and warm chats in the PFES office. In addition, Mandi Wilson, whose stash of exotic office sweets leaves even the most refined candy connoisseurs in awe. I thank the administration at the Graduate Studies offices in both Truro and Halifax for their additional help throughout my graduate work. Their willingness to happily respond to even the most miniscule questions is uplifting. I also wish to thank the lovely ladies of Chartwells for the many lunches, coffees, and muffins you have provided to me and many others on campus.

To the many friends and mentors I have acquired at the Faculty, thank-you for the many lab laughs, coffee chats, nights at the Nook and Cranny, and Covid Zoom game nights. Thank-you all for your support!

My work would also not be possible without the support of my family. Thank-you to my parents who instilled in me a love for reading, learning, and exploring, and for

encouraging me to think about research. Thank-you to the many aunts, uncles, and cousins who have always taken interest in my endeavours. To Uncle Rob and Aunt Ellie, thank-you for your constant encouragement. Thank-you Aunt Linda and Uncle Gary for the many childhood summers at the lake exploring the outdoors, and thanks for always sending pictures of your household insect problems. Thank-you to my Uncle Ralph whose eagerness to talk about your garden insect pests is a constant joy, and my Aunt Jackie whose bewilderment at these bug chats provides constant amusement. Thank-you Uncle Donnie and Aunt Kathy for the best BBQ's and for always sharing the expensive wine. Thank-you Uncle Ray and Aunt Betty for the incredible seafood filled summers. Thank-you Aunt Betty for your fascination with my work; it means the world to me. Thanks to my Uncle Don. I am glad to make the Rix family proud. Thank-you to Rob for being the best younger brother I could ask for. Your hardworking spirit is always an inspiration. Thank-you Nat and James for always asking "Have you finished your thesis yet?", and to your girls and Mitch, who are pearls of my world. Thanks Todd and Brandi for the best laughs. Thank-you Jess and Araz for all your love. Thanks Jon, Mike, and Dave for all the amusing stories about dad before he was "dad" (he was definitely the cool uncle).

Special thanks go to my closest friends who "love my whimsical nature" and find me "a true joy to be around". With you I can be myself. No greater gift could be found in this world.

I sincerely thank the members of my advisory committee Dr. Cynthia Scott-Dupree, Dr. Shelley Adamo, Dr. Marty Leonard, and Dr. Sarah Stewart-Clark for your mentorship, kindness, support, and suggestions. I would also like to thank Dr. Fraser

Clark for providing laboratory space and for his additional advisory role with regard to my transcriptomics. Thank-you for both teaching and challenging me to problem solve through all my questions. I would also like to thank the Entomological Society of Canada and the Acadian Entomological Society for providing me the many opportunities I have had to present my research, volunteer, and serve my professional society.

Most importantly, I would like to thank my advisor, Dr. Chris Cutler. It is difficult for me to acknowledge all you have done for me in simply a few sentences. I owe my achievements to your incredible mentorship and encouragement. Your guidance has influenced the researcher, and the person I have become. You have helped and supported me in navigating many challenges. Under your tutelage, I have become stronger, wiser, and have gained confidence in myself that I never imagined I would have. The best decision I have ever made was joining your lab. I will be forever grateful for the profound impact you have had on my life.

The following thesis is formatted as a series of manuscripts that have been or will be published in peer reviewed journals, therefore there is some repetition of subject material across chapters.

CHAPTER 1 INTRODUCTION

1.1 Toxicology and the Dose-Response

Historically, toxicology was known simply as the "*science of poisons*" (Costa and Teixeira, 2014). Knowledge of toxic substances, poisons and venoms, dates to antiquity, gained from an accumulation of empirical experiences over the centuries. Detailed records of venomous animals and poisonous plants are found in historic literature, from medical to mythological (Hayes and Touwaide, 2014; Tsatsakis et al., 2018). The historic focus of toxicology has primarily been the adverse effects of toxins on living organisms, namely mortality, but as the father of toxicology, Paracelsus (1493–1541 CE) rightly pointed out, "*All things are poison and nothing is without poison, only the dose permits something not to be poison*" (Kendig et al., 2010). Therefore, the study of toxicology is not confined simply to adverse effects of poisons or toxicants, but to all effects (Hodgson, 2004).

This statement, the dose makes the poison, formed the basis for the modern concept of the dose-response, that is, the magnitude of the response or effect of a poison/toxin is related to the dose of the poison/toxin. Research into these features of the dose-response began in the 19th century (Calabrese, 2016a), with the work of Robert Koch, who assessed the bactericidal effects of chemical disinfectants on bacterial cultures (Koch, 1881), following which Kronig and Paul consistently showed that there existed a logarithmic relationship between the number of surviving bacteria and disinfectant concentration (Kronig and Paul, 1897). In the 1920's, Trevan expanded on this, arguing that dose-responses should be measured based upon the central tendency of a group response, leading to the establishment of the LD_{50} (lethal dose 50%, or median lethal

dose), the dose that is lethal to 50% of a population (Trevan, 1927). This concept was built on the idea that the dose-response represents the distribution of population variability in susceptibility to a toxin. He further established that the response to dose was not limited to mortality, and that it could include any biological response of interest. The subsequent efforts of Bliss, Gaddum, and Finney established statistical methods of analysing the dose-response (Bliss, 1935b; Bliss, 1935a; Bliss, 1940; Finney, 1944; Gaddum, 1945; Finney, 1952), culminating in the development of the probit analysis in the 1930's and 1940's, which employs mathematical transformation of the sigmoidal dose-response function into a linear function to easily estimate dose parameters associated with measured biological responses. Probit continues to be a standard method of toxicological data analysis (Postelnicu, 2011).

The concept of dose-response became pivotal in the 20th century, as the development and use of pharmaceuticals and pesticides became widespread. Reports of detrimental effects on humans and wildlife from exposures to such things as heavy metals, petroleum products, and pesticides began to emerge, giving rise to regulatory agencies and laws instituted to protect humans and wildlife from the adverse effects of chemical exposure (Rattner, 2009). Research showed that dose-responses could be influenced by factors such as the form of the chemical (eg. liquid, powder, gas), route of exposure (eg. oral, topical/dermal, inhalation), and number of exposures (acute, chronic), and that an array of effects could be induced by chemical substances, from the least to most severe, influencing the regulatory aspects of risk assessment (Dourson and Pournourmohammadi, 2014). Regulatory agencies evaluate toxicity based upon the dose-response identifying, based on available experimental evidence, the highest exposures at

which no statistically or biologically significant effects are detected, the "no observable effects level" (NOEL) or the "no observable adverse effects level" (NOAEL), and the similar metric, the "lowest observable adverse effect level" (LOAEL), the lowest exposure at which there is a significant increase in observable adverse effects (Figure 1.1).

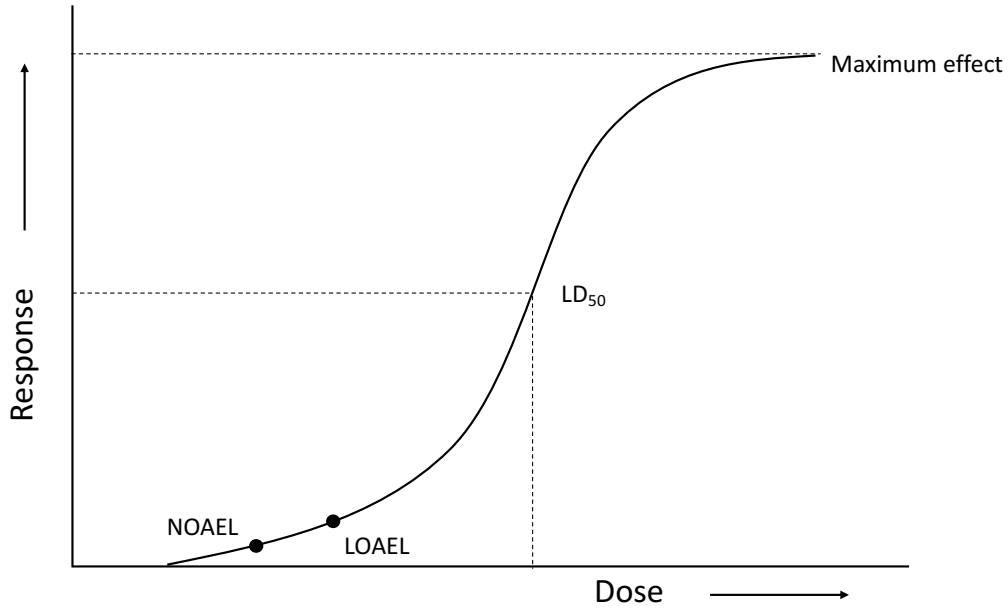

Figure 1.1: Dose-response curve showing the LD_{50} (lethal dose or exposure that kills 50% of the population), the LOAEL (lowest observable adverse effects level; the lowest dose or exposure observed to have an adverse effect), and the NOAEL (no observable adverse effects level; the highest dose or exposure at which no adverse effect can be observed). Adapted from Tsatsakis et al. (2018).

The NOAEL is a more conservative measure, assumed to approximate the threshold level, below which the effects of a toxin are unlikely to occur (Tsatsakis et al., 2018). These metrics have formed the basis for study in toxicology and toxicological risk assessment (National Research Council US Committee on Risk Assessment of Hazardous Air Pollutants, 1994; National Research Council US Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology, 2007).

1.2 Toxicological Models and Historic Context of Hormesis

Modern toxicology as a discipline has been primarily focused on the inhibitory effects of substances across a range of doses (Calabrese and Agathokleous, 2020). For over a century, it had been largely accepted that the threshold model fundamentally described the dose-response in chemical toxicology. Under this model it is assumed that only those doses above the threshold dose (NOAEL) are likely to cause biological effects on an organism (Figure 1.2a) (Calabrese, 2005). Similarly, the linear no-threshold (LNT) model (Figure 1.2b) whereby no dose, however low, is without effect, was accepted as describing the effects of chemical carcinogen and radiation exposure (Calabrese, 2005). These models were thought to be well supported. Thousands of observations corroborated the threshold model (Calabrese, 2005). Hermann Muller's work showing that mutation rates in irradiated fruit flies were without a radiation threshold, and the mutagenic effects were cumulative over the fruit fly lifespan (Muller, 1927), was extraordinarily compelling (Calabrese, 2004b). This work was, however, erroneous, focusing on doses far above what would be expected to naturally occur, while ignoring the findings of those who studied lower doses (Calabrese, 2011; Calabrese, 2019) and has subsequently been heavily scrutinized (Cardarelli and Ulsh, 2018; Yanovskiy et al., 2019). The acceptance of the threshold and LNT models has had profound effects, influencing the assessment of risk and establishment of safe limits for chemicals and carcinogens (Beck et al., 2007; Siegel et al., 2018). However, the modern move to focus on low doses has resulted in renewed research on hormesis, a dose-response phenomenon whereby low doses stimulate biological processes, whereas high doses inhibit biological processes (Calabrese and Baldwin, 2002; Calabrese, 2005).

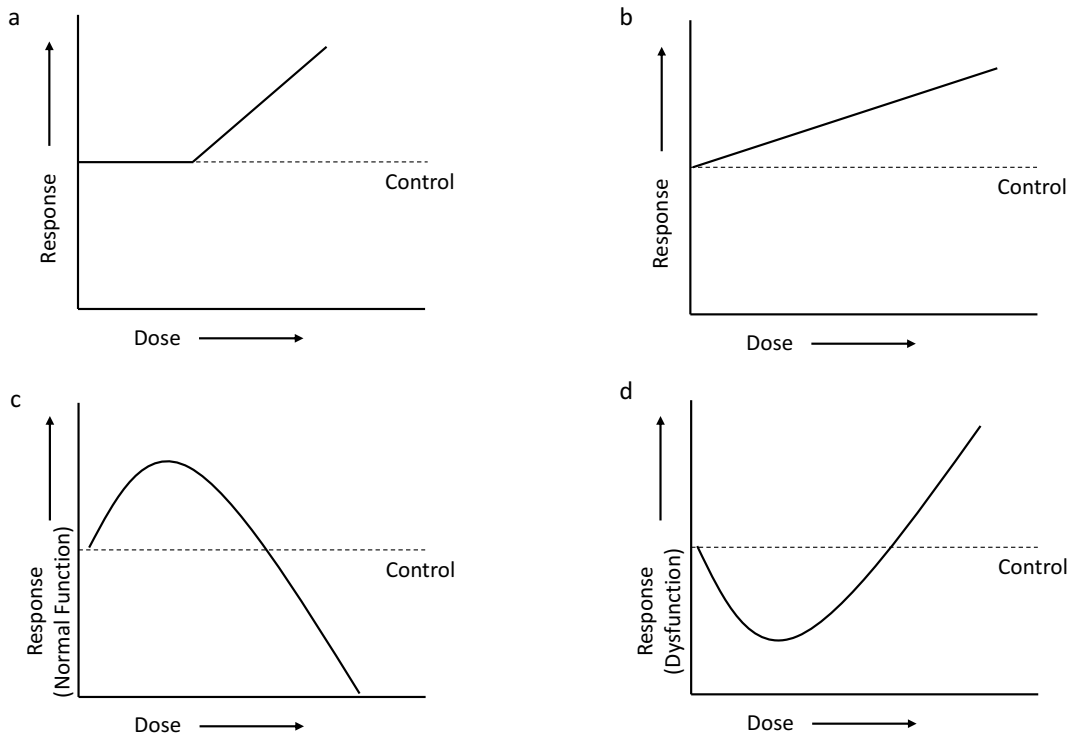

Figure 1.2: Curves representing dose response models. (a) The threshold model which assumes there is a dose-response threshold above which exposure to a stressor will induce a biological effect, and below which none occurs; (b) The linear no-threshold model assumes at low doses there is a linear relationship between dose and effect terminating at zero; (c) The inverted-U-shaped hormetic model whereby low doses stimulate biological processes, and high doses inhibit biological processes; (d) The J-shaped hormetic model where low doses reduce the negative effects of background stress (decrease in dysfunction) and high doses enhance the negative effects of background stress (increase in dysfunction). Adapted from Calabrese (2004a).

The stimulatory effects of low doses of stress were first brought to light in the 1880's by Hugo Schulz (Calabrese, 2015a). While studying the effects of disinfectants on yeast, Schulz unexpectedly observed that yeast exposed to low doses of disinfectant had enhanced metabolism (Schulz, 1887; Schulz, 1888). While Schulz's experiments were highly robust, his unfortunate decision to link hormesis with controversial concepts in homeopathy lead to the repudiation of his work by the medical community (Henschler, 2006). Some research in the early decades of the 20th century also noted similar

stimulatory effects in bacteria, fungi, yeast, plants, and insects (Calabrese and Baldwin, 2000a; Calabrese and Baldwin, 2000b; Calabrese and Baldwin, 2000c). In particular, the phenomenon was noted in work on the effects of radiation on biological organisms, however, was dismissed as inconsequential (Calabrese, 2015a). As such, the hormesis concept remained largely forgotten until it was revived by Southam and Ehrlich, who coined the term hormesis in 1943 to describe the stimulatory effects on growth of fungal species by extracts of the red cedar tree (Southam and Ehrlich, 1943). They subsequently noted that numerous other studies had demonstrated similar biphasic dose-responses (Calabrese et al., 2016).

Hormesis is now considered in many cases to represent the dose-response more accurately than the threshold and LNT models (Calabrese and Baldwin, 2003; Calabrese, 2005; Calabrese and Blain, 2005), having been documented in plants (Jia et al., 2013; Brito et al., 2018), micro-organisms (Flores and Garzon, 2012; Nancharaiah and Francis, 2015), mammals (Costantini et al., 2012; Di and Qin, 2018), and invertebrates (Cohen, 2006; Calabrese, 2013c; Cutler, 2013). Hormesis has been documented not only in response to chemical toxins, but also in response to other environmental factors such as radiation, hypoxia/anoxia, exercise, dietary restriction, and temperature, and as such is considered an adaptive response to low doses of stress (Calabrese and Baldwin, 2002; Mattson, 2008; Costantini et al., 2010; Calabrese and Blain, 2011; Costantini, 2019). Hormesis is thought to result from either overcompensation or direct stimulation of metabolic and repair mechanisms following stress. The key feature of overcompensation stimulation is that the overcompensation of repair mechanisms occurs in response to a disruption in homeostasis caused by a stressor (Calabrese, 2001; Calabrese and Baldwin,

2002). Under this framework, a response is elicited to repair minor damage associated with the stress and re-establish homeostasis, but in modest excess of that needed to repair the damage from a low dose, resulting in hormesis (Calabrese and Baldwin, 2002). This modest overcompensation likely evolved to ensure that damage was repaired quickly and adequately, and to ensure resources were available in the event of a subsequent stress in a limited time frame (Calabrese and Baldwin, 2002). Direct stimulation hormesis on the other hand, is an adaptive response that operates under the normal metabolic functions of the organism, and thus does not occur in response to damage associated with a disruption in homeostasis (Calabrese and Baldwin, 2002; Calabrese, 2008b). This is more evident in pharmacology where an agonist may have dual receptor affinity, activating both stimulatory and inhibitory receptors depending on agonist concentration (Szabadi, 1977).

Hormesis is typically represented as either an inverted-U-shaped or J-shaped dose-response model. The inverted-U-shape characterizes the typical hormetic response of stimulation at low doses, and inhibition of response at high doses (Calabrese and Baldwin, 2002) (Figure 1.2c). J-shaped dose-responses show a hormetic response whereby decreases in dysfunction occur following exposure to low doses of stress, and an increase in dysfunction at high doses (Figure 1.2d). This relatively under-studied response typically manifests as a reduction in disease incidence following low dose stress, or reduced carcinogenesis following low dose exposure to a mutagen (Calabrese and Baldwin, 2001; Ricci et al., 2012).

Hormesis may also manifest in additional ways. Pre-conditioning hormesis occurs when exposure to a low dose stressor or toxic agent results in enhanced ability to survive subsequent and higher levels of additional stress. The response to pre-conditioning doses

follows the characteristic inverted U-shape, with low pre-conditioning doses providing a protective stimulatory response following subsequent stress and high pre-conditioning doses an inhibitory response (Calabrese, 2008a; Calabrese, 2016b; Calabrese, 2016c). Some of the first known work demonstrating pre-conditioning was on lentil plants where prior exposure to X-rays protected plants from injury against an additional more challenging X-ray exposure (Calabrese, 2016b). Pre-conditioning, however, was brought to the forefront in a study by Murry et al. (1986) who showed that a modest ischemic stress in a dog resulted in subsequent reduction in heart damage by up to 70-80% following a second more intense, prolonged ischemic stress. Post-conditioning hormesis occurs when protection is incurred from a mild to moderate conditioning dose that is administered after the challenging dose (Calabrese, 2018). This has similarly been shown in a cardiovascular therapeutic context where damage from an induced myocardial infarction is reduced when a low dose hypoxic stress is administered following the myocardial infarction (Calabrese et al., 2007). Post-conditioning may also include situations where disease incidence is reduced in organisms with moderate to high levels of background disease following exposure to a low dose of a stressor or toxin, demonstrating a J-shaped response (Calabrese, 2018).

While hormesis can present in a number of ways, its general features are largely predictable. Doses or levels of stress that typically induce hormesis are below the NOAEL, where one might predict responses to be no different from the control. This range of doses or stress levels usually averages 10-20-fold below the NOAEL, but may be very broad, ranging anywhere from 100-1000-fold below the NOAEL (Figure 1.3). In most cases the level of stimulation is modest, between 30-60% greater than the control,

but can be upwards of 100% greater than the control (Figure 1.3) (Calabrese, 2013a; Lushchak, 2014). As mentioned previously, hormesis has been observed across the taxonomic spectrum, but also across all levels of biological organization from the cellular, organ, and whole organism level (Calabrese, 2014), and may even occur across generations (Costantini, 2019). The types of responses typically observed include increases in longevity or survival, fecundity or fertility, growth, metabolism, behavior, stress resistance, and immunity or disease resistance (Calabrese and Blain, 2011). Hormesis is thought to represent the extent of phenotypic plasticity, the change in phenotype in response to the environment, in an organism (Costantini et al., 2010; Calabrese and Mattson, 2011; Costantini, 2019). As such, this demonstrates that hormesis is fundamental to both survival and evolution.

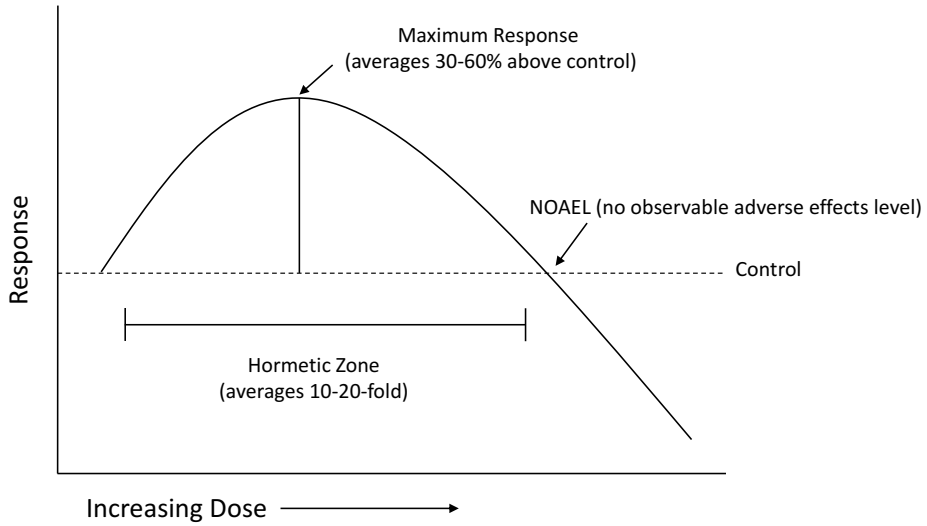

Figure 1.3: Dose-response curve showing the quantitative features of hormesis.

1.3 Hormesis, Insects, and Agricultural Pest Management

The significance of hormesis, from an agricultural perspective, is its potential impact on pest management. This has prominently been considered in the context of pesticide

induced hormesis in insects (Cutler et al., 2022; Guedes et al., 2022). Pesticide use in agriculture became wide scale in the 1940s and pesticides have since played a key role in enhancing food production and global food security. Thousands of tonnes of pesticides are produced every year with an estimated annual value in excess of $35 billion dollars (Carvalho, 2017). The effects of pesticides on the environment have long been known. *Silent Spring*, published by Rachel Carson in 1962, demonstrated that indiscriminate use of pesticides was ecologically detrimental and damaging to human and animal health (Carson, 1962; Epstein, 2014). The introduction of integrated pest management (IPM) in agriculture has made for a more multifaceted strategy, but the primacy of chemical use in pest management remains (Cooper and Dobson, 2007; Guedes et al., 2016). Modern reduced risk insecticides with more desirable human and environmental safety profiles have replaced older generations of insecticides (Guedes et al., 2016); however, these compounds have short term persistence, exposing insects to variable and low doses more frequently. For example, Cutler et al. (2005) showed that foliar applications of imidacloprid on potato broke down rapidly to sublethal levels for Colorado potato beetle, *Leptinotarsa decemlineata* (Say) in under 10 days. The half-life of thiamethoxam on rice foliage was 5 days, and lethal residual activity to a brown stink bug parasitoid, *Telenomus podisi* Ashmead, was reduced in 10 days with no effects observed at day 20 (Rakes et al., 2021). This means insects are likely to be exposed to hormetic levels of pesticides in an agricultural setting (Guedes et al., 2016).

Hormesis in insects exposed to low doses of pesticides often presents as stimulated or increased reproductive parameters, such as fecundity, fertility, or stimulated oviposition (Cutler, 2013). This has been widely observed in aphids and other pest

Hemiptera (Morse and Zareh, 1991; Yu et al., 2010; Ayyanath et al., 2013; Haddi et al., 2016; Rix et al., 2016; Santos et al., 2016; Sial et al., 2018), and has also been seen in Diptera (Zhao et al., 2018; Shaw et al., 2019), Coleoptera (Guedes et al., 2010; Mallqui et al., 2014), and Lepidoptera (Deng et al., 2016; Malbert-Colas et al., 2020), amongst others (Cutler, 2013). This has led some to speculate that pest resurgence and secondary pest outbreaks may be caused by insecticide-induced hormesis rather than suppression of natural enemies, in some instances (Dutcher, 2007; Guedes and Cutler, 2014; Cutler et al., 2022). Cordeiro et al. (2013) provided evidence this could be the case, showing that low doses of deltamethrin stimulated a large population increase in southern red mite, *Oligonychus ilicis* (McGregor), but a predatory mite, *Amblyseius herbioulus* (Chant), was more tolerant to deltamethrin than its southern red mite prey, and thus not suppressed. Evidence has also been brought forth that low dose pesticide exposure may hasten the development of tolerance or resistance to pesticides or other environmental stressors by increasing mutation frequencies, which could additionally impact pest management (Gressel, 2011). Exposure to herbicides increased tolerance to two pyrethroids and an organophosphate insecticide in tobacco cutworm, *Spodoptera litura* (Fabricius) (Liu et al., 2019a). Low dose triazophos increased thermotolerance in brown planthopper, *Nilaparvata lugens* (Stål) (Ge et al., 2013). Sublethal exposure to imidacloprid that increased fecundity in green peach aphid, *Myzus persicae* (Sulzer), also enhanced tolerance to food/water stress and subsequent imidacloprid exposure (Rix et al., 2016; Rix and Cutler, 2018).

While hormesis in pest insects could have negative impacts on agriculture, stimulation of beneficial insects could have the opposite effect. The implementation of

IPM programs has highlighted the value of and need for robust populations of natural enemies. The production of natural enemies for biological control is a multimillion-dollar industry, and the implementation of natural enemies into IPM programs has saved industries hundreds of millions of dollars in control costs and crop losses (Naranjo et al., 2015). Successful integration of insect natural enemies into a pest management program, whether through augmentative, classical, or conservation biological control, requires that they be able to survive and reproduce in sufficient numbers to provide meaningful pest control. They must also be able to search out, locate, and subdue prey. Hormesis in any of these traits could be beneficial for pest management. There is precedent for hormesis to be induced in beneficial arthropods. Reproductive hormesis has been robustly demonstrated in predatory stink bugs, *Podisus spp.*, and *Supputius cincticeps* (Stål), exposed to pyrethroids (Zanuncio et al., 2005; Zanuncio et al., 2013), as well as survival and longevity (Pereira et al., 2009; Zanuncio et al., 2011). Exposure to low doses of malathion increased longevity and egg laying per day in female parasitoids, *Pimpla turionellae* (L.) (Buyukguzel, 2006). Behavior and predatory capabilities have also been increased upon exposure to low doses of pesticides. The cumulative number of killed fruit flies, *Drosophila melanogaster* (Meigen), was increased over controls in an agrobiont wolf spider *Pardosa agrestis* (Westring) exposed to glufosinate-ammonium herbicide (Korenko et al., 2016). Female parasitoids, *Trichogramma pretiosum* Riley, exposed to tolfenpyrad treated corn earworm, *Helicoverpa zea* (Boddie), eggs drummed, stung their host, and fed with higher frequency than other treatments (Khan et al., 2015). Thiamethoxam and alpha-cypermethrin temporarily stimulated locomotion in the Carabid, *Platynus assimilis* (Paykull) (Tooming et al., 2014; Tooming et al., 2017).

Harlequin lady beetles, *Harmonia axyridis* (Pallas), exposed to sublethal beta-cypermethrin walked faster and for longer duration and distance than untreated beetles. They also flew more frequently for longer duration and distance (Xiao et al., 2017).

While stimulatory effects following low doses of pesticides or other stressors are well documented, it is not entirely clear to what extent trade-offs may occur under hormesis. Energetic constraints are thought to limit the ability of multiple traits to be stimulated at once. It is therefore thought that while hormesis could be beneficial for organism fitness in some instances, it is more likely for hormesis to be neutral or mildly detrimental to organism fitness (Forbes, 2000; Stearns, 2000). Reproduction, and longevity or survival are frequently traded-off against one another both in insect and other model organisms (Flatt, 2011). In other instances, multiple traits may be stimulated, but with mild to moderate trade-offs, such as observed by McClure et al. (2014), who saw enhanced longevity, fecundity, and heat stress tolerance in *D. melanogaster*, but at the expense of immunity, or in some instances without observed trade-offs, as was seen with *M. persicae* where reproduction, longevity, and stress tolerance were increased following imidacloprid exposure (Cutler et al., 2009; Ayyanath et al., 2013; Rix et al., 2016). Life-history traits may also be stimulated in tandem with various aspects of behavior. Flight, mating performance, and survival were increased in Caribbean fruit fly, *Anastrepha suspensa* (Loew) exposed to anoxia and radiation (Lopez-Martinez and Hahn, 2012). Chronic exposure to rutin stimulated climbing capabilities in *D. melanogaster*, and increased larval development and lifespan (Chattopadhyay et al., 2017).

1.4 Molecular Responses Associated with Hormesis in Insects

Hormesis is now recognized as being ubiquitous in insects, with common stimulatory effects such as reproduction, survival and longevity, growth and development, and preconditioning for stress tolerance occurring across a diversity of insects, at varying life stages, and in response to a multitude of stressors including chemical pesticides, oxidative stress, temperature stress, dietary restriction, and radiation (Cutler, 2013; Berry and Lopez-Martinez, 2020). This has led to an increased interest in understanding the molecular responses and mechanisms behind hormesis. For hormesis to demonstrate such robust predictability in patterns of manifestation across stressors and organisms, it is thought it must be mediated by coordination of conserved elements of the stress response (Hoffmann, 2009). Studies have identified a number of responses to be associated with hormesis. DNA damage response and repair systems are thought to play key roles in hormesis. Among these, heat shock proteins, which act as chaperones, mediating protein folding, localization, degradation, and DNA repair are essential for repair and recovery following stress (King and MacRae, 2015; Dubrez et al., 2020). Stress generates molecular damage. Changes in cytoskeletal, cytoplasmic, and cell surface structures can occur. In addition, changes in protein stability can result in the accumulation of abnormally folded proteins. Heat shock proteins, by repairing protein and cellular damage, promote survival and also protect organisms from stress (Rattan, 2006). Research on heat shock proteins and hormesis has found that mild heat shocks can increase longevity and heat tolerance in *Drosophila spp.* and has been associated with increased expression of *Hsp70* (Khazaeli et al., 1997; Hercus et al., 2003; Scannapieco et al., 2007; Sarup et al., 2014). Given their function as universal stress response proteins,

heat shock proteins have also been shown to be involved in stimulatory effects induced following exposure to other stressors. *Hsp70* was shown to be important for lifespan extension following irradiation in *D.* melanogaster (Moskalev et al., 2009). Exposure of *M. persicae* to sublethal concentrations of imidacloprid that increased fecundity and starvation/desiccation tolerance also resulted in increased expression of *Hsp60* (Rix et al., 2016).

Antioxidation is also thought to play a major role in the development of hormesis (Costantini, 2014b). Antioxidants work to neutralize reactive oxygen species (ROS), which can induce cellular injury through protein oxidation, lipid peroxidation, and damage DNA. ROS are produced under normal cellular metabolism, but under changing or stressful conditions, ROS production is increased (Garcia-Caparros et al., 2020). Increased antioxidant capacity is linked to higher reproductive outputs, survival, and longevity in insects (Arking et al., 2000; DeJong et al., 2007; Tasaki et al., 2017). Low doses of stress can induce the expression of antioxidant enzymes and has been associated with hormesis in insects. For example, dietary restriction resulted in increased antioxidant enzyme activity, and was associated with increased lifespan in Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Chen et al., 2013). Exposure to malathion induced increased antioxidant activity and was associated with increased longevity and fecundity in the *P. turionellae* parasitoid (Buyukguzel, 2006).

Detoxification is also thought to play a role in the hormetic response in insects given that exposure to insecticides and other chemicals consistently induces hormesis in insects (Cutler, 2013; Guedes and Cutler, 2014). Exposure of fungus gnat, *Bradysia odoriphaga* Yang and Zhang, to low doses of chlorfenapyr induced the activity of

carboxylesterases and glutathione-s-transferases (Zhao et al., 2018). Cotton aphid, *Aphis gossypii* Glover, exposed to sublethal doses of thiamethoxam had faster pre-adult development, increased longevity, and increased fertility, associated with increased expression of five cytochrome P450 genes (Ullah et al., 2020). Similarly, increased fecundity and survival to dietary/water stress was observed in *M. persicae* exposed to low doses of imidacloprid. This corresponded to dynamic changes in the expression of *E4-esterase* and cytochrome P450 *CYP6C43* (Rix et al., 2016).

Further work has sought to understand the regulatory mechanisms of hormesis and some signalling pathways involved in the molecular stress response have been thought to mediate some hormetic effects. For example, heat shock factor (HSF), given its role in regulating the expression of heat shock proteins, has been thought to play a key role in the hormetic response (Son et al., 2010). The Nrf2/ARE/Keap-1 signalling pathway regulates the expression of antioxidants and detoxification genes in insects (Son et al., 2010; Misra et al., 2011) and has been shown to promote longevity (Pitoniak and Bohmann, 2015). FOXO signalling also mediates expression of some aspects of the oxidative stress response, such as the expression of superoxide dismutases and catalases, and mediates autophagy through the insulin signalling pathway, all involved in stress resistance and lifespan extension (Son et al., 2010; Maruzs et al., 2019), and have been up-regulated in longevity hormesis in *D. melanogaster* (Chattopadhyay et al., 2017).

1.5 *Podisus maculiventris*

Podisus maculiventris (Say), the spined soldier bug, is a medium sized predatory stink bug, native throughout North America (Heppner et al., 2008; Richman and Mead, 2017). *Podisus maculiventris* is an economically important insect natural enemy, feeding on the

eggs and larvae of over 100 species of Coleoptera and Lepidoptera pests (Kneeland et al., 2012). It is found in a variety of agroecosystems such as alfalfa, apple, corn, potato, tomato, soybean, and various *Brassica* crops. It may also be found in shrubland, hemlock, pine, and deciduous forest (Herrick and Reitz, 2004). *Podisus maculiventris* is hemimetabolous, going through three life stages, egg, nymph, and adult, with five nymphal instars between egg and adult. Development can occur at temperatures as low as 13 °C, but survival is minimal or non-existent beyond 36 °C (Legaspi, 2004; Baek et al., 2014). Development time from egg to adult can be anywhere from around 20-50 days and depends on temperature, and food source and availability (Coudron et al., 2002; Baek et al., 2014). Adults overwinter in underground debris or tree bark and can be found from March to September depending on their distribution (Herrick and Reitz, 2004). Adult females may lay several hundred eggs throughout their lifetime, and adults can live for upwards of 80 days (Coudron et al., 2002; Legaspi, 2004).

Podisus maculiventris is sold commercially as a biological control agent. Its ability to successfully reduce pest populations in both greenhouse and field operations, wide range of prey species, and voracious appetite make it an effective predator for biological control (Ables and McCommas, 1982; Biever and Chauvin, 1992; De Clercq et al., 1998; Tipping et al., 1999; Heppner et al., 2008; Montemayor and Cave, 2012). *Podisus maculiventris* feeds throughout its lifecycle, thus continuous pest control can be maintained (Desurmont and Weston, 2008). Field studies have shown effective control of viburnum leaf beetle, *Pyrrhalta viburni* (Paykull) (Desurmont and Weston, 2008). *Leptinotarsa decemlineata* was controlled through augmentative release of *P. maculiventris* in small plots of tomato but was also effective by means of conservation

biological control using pheromone attractants in potato (Aldrich and Cantelo, 1999; Tipping et al., 1999). Tomato looper, *Chrysodeixis chalcites* (Esper), populations were reduced by 65% one week after releasing of fourth instar *P. maculiventris* in a greenhouse (De Clercq et al., 1998).

1.6 Objectives and Hypothesis

Natural enemies provide valuable services to IPM programs. Robust individuals that can survive, reproduce, and predate under stress are highly desirable, thus hormetic stimulation of traits that could enhance natural enemy success may be beneficial for agriculture. However, trade-offs that may accompany hormesis could neutralize any potential positive effects, or even have overall negative effects (Forbes, 2000; Stearns, 2000). The primary aim of my thesis was to examine insecticide-induced hormesis in beneficial insects, on a phenotypic and molecular level, using the insect predator, *P. maculiventris*, and the insecticide, imidacloprid, as an insect-stressor model, and to characterize hormetic responses and associated molecular and biochemical responses across insect species.

The first half of my thesis examined the phenotypic effects associated with exposure to low dose concentrations of imidacloprid in *P. maculiventris*. The first objective was to determine whether exposure to low dose concentrations of imidacloprid increased survival, and reproductive traits (oviposition, fecundity, fertility) in *P. maculiventris* exposed as nymphs or adults, and whether hormetic effects carried over into the next generation. Given that previous work in both pest and beneficial insects has demonstrated stimulation of multiple traits associated with reproduction, and survival and/or longevity, and that hormesis can carry over generations, I hypothesized that

exposure of *P. maculiventris* to low dose concentrations of imidacloprid would result in stimulatory effects on oviposition, fecundity, fertility, and survival, and that reproductive stimulation would also carry over into a second generation of exposed insects.

The second objective of my thesis was to determine whether patterns in common behaviors associated with locomotion and movement (walking/antennating, stationary, and preening), searching (antennating and probing), and predation were stimulated by low dose exposures to imidacloprid. Given that low dose insecticide exposure has previously been shown to enhance locomotion, prey searching and finding, and predation, and that behavior may be stimulated in tandem with life-history traits, I hypothesized that low doses of imidacloprid that stimulate aspects of reproduction and/or survival in *P. maculiventris* would also stimulate locomotory and searching behaviors that could influence prey finding including walking/antennating, antennating, and probing, and as such would also increase predation.

Stress responses are under genetic control and act on cellular and systemic levels to coordinate responses that divert energy away from non-essential functions during homeostatic challenges (Johnson and White, 2009). The stress response thus acts to reconfigure the organism's system such that it maximizes the organism's ability to cope with the stress (Johnson and White, 2009; Johnson, 2017). Regulation and expression of genes involved in the stress response is also implicit in the stimulatory effects associated with hormesis (Mattson, 2010; Son et al., 2010). Molecular and biochemical studies on hormesis in insects have largely focused on only on a small subset of genes or proteins. A full analysis of molecular and/or biochemical responses associated with the stimulatory

effects observed under hormesis is needed to better characterize the patterns and manifestation of hormesis in insects.

The second half of my thesis aimed to analyze and contextualize the common molecular and/or biochemical responses associated with stress induced stimulatory and hormetic effects across insects. The third objective of my thesis was to conduct a systematic review of the literature to comprehensively analyze the relationships between the phenotypic stimulatory effects observed under exposure to low doses of stress and the molecular and/or biochemical effects in insects. In coordination with the systematic review, the fourth objective of my thesis aimed to more specifically examine the molecular responses associated with hormesis in *P. maculiventris* through whole transcriptome analysis using RNA-seq. Given that hormesis is thought to manifest from coordinated regulation of conserved aspects of the stress response, I hypothesized that molecular and/or biochemical responses associated with stimulatory effects would have commonality across stressors and insect species, consisting of processes such as detoxification, antioxidation, and general stress and repair processes (such as chaperones). I also hypothesized that these responses would be coordinated by both increased and decreased expression. I hypothesized that this would also be reflected in the transcriptomic response of *P. maculiventris* to low doses of imidacloprid.

CHAPTER 2 LOW DOSES OF A NEONICOTINOID STIMULATE REPRODUCTION IN A BENEFICIAL PREDATORY INSECT

A version of this chapter was published in the *Journal of Economic Entomology*. Details associated with copyright permission can be found in Appendix 1. Rix, R.R. developed and conducted experiments, analyzed data, and wrote the manuscript. Cutler, G.C. aided in conceiving experiments, contributed to writing, and provided reviews and edits to manuscript drafts.

Rix, R.R., and Cutler, G.C., 2020. Low doses of a neonicotinoid stimulate reproduction in a beneficial predatory insect. Journal of Economic Entomology. 113, 2179 - 2186.

2.1 Introduction

Insecticides are invaluable tools for pest management in agriculture. They are applied at concentrations intended to kill targeted pests, but temporal and spatial changes in insecticide concentrations in the environment over time eventually expose insect populations to a range of sublethal concentrations. In addition to pest species, sublethal insecticide concentrations often impact beneficial insects, like pollinators and natural enemies. Reduced survival, longevity, and reproduction following exposure to sublethal doses of insecticide have been well documented for decades (Ripper, 1956; Croft and Brown, 1975; Desneux et al., 2007; Muller, 2018).

It is now well recognized that certain low doses of insecticide along the dose-response continuum – sublethal doses below those that cause inhibitory effects – can stimulate biological processes and functions in insects. The biphasic dose-response model, characterized by stimulation at low doses of stress and biological inhibition at high doses of stress, is called “hormesis”. In general, irrespective of the organism or

stressor, hormetic responses are thought to enhance survival and resistance to environmental stress, regulating physiology and the allocation of resources to maintain organismal stability under adverse conditions (Calabrese et al., 2010). With insects, manifestations of insecticide-induced hormesis are usually documented as increased reproduction, although other effects such as enhanced longevity, immunity, stimulated behavior, and preconditioning for stress, have been documented (Cutler, 2013; Guedes and Cutler, 2014; Cutler and Guedes, 2017).

Research on insecticide-induced hormesis in insects has focused predominantly on pest species (Cutler, 2013; Guedes and Cutler, 2014). This makes sense given the obvious linkages between insecticide use and insect pest incidence. However, beneficial insects may also be exposed to hormetic concentrations of pesticides or other environmental stressors, which may manifest as stimulatory responses in field situations. Moreover, culturing and mass rearing of beneficial insects for pollination and biological pest control is a multi-billion-dollar industry (Kampmeier and Irwin, 2009), and insects that are produced in large quantities may be subject to disease, competition, predation, and parasitism (Cutler and Guedes, 2017). Benefits of hormetic stress for human health are well documented (Mattson and Calabrese, 2010), and we similarly might be able to apply hormetic principles during mass culture of insects to improve insect longevity, reproductive outputs, or stress tolerance (Cutler, 2013). Key traits for field success such as survival and colonization, tolerance to environmental stress, longevity, and fecundity can be impaired following successive generations of mass rearing and cultivation (Blackburn et al., 2016). Hormetic stress could potentially be strategically applied during rearing to mitigate some of these challenges.

The spined soldier bug, *P. maculiventris*, is a predatory stink bug found throughout North America (Heppner et al., 2008; Richman and Mead, 2017). *Podisus maculiventris* is an important insect natural enemy, feeding on eggs and larvae of over 100 species of Coleoptera and Lepidoptera pests (Ables and McCommas, 1982; Biever and Chauvin, 1992; De Clercq et al., 1998; Tipping et al., 1999; Kneeland et al., 2012; Montemayor and Cave, 2012). *Podisus maculiventris* inhabits a wide range of agricultural ecosystems, engaging in substantial natural predation (Hazzard et al., 1991; Perdikis et al., 2011). Augmentative release of *P. maculiventris* has proven effective in suppressing both Coleoptera and Lepidoptera pests (Biever and Chauvin, 1992; Tipping et al., 1999; Desurmont and Weston, 2008; Perez-Alvarez et al., 2019), but rearing and maintaining adequate field numbers for pest control remains a challenge due to rearing and release costs, inconsistent survival, and reductions in fecundity resulting from storage prior to release (Thorpe and Aldrich, 2004; Perdikis et al., 2011; Montemayor and Cave, 2012).

I wanted to examine whether low amounts of chemical stress would induce stimulatory responses in *P. maculiventris*. Previous work has demonstrated enhancement in reproduction and survival in predatory stink bugs following insecticide exposure (Guedes et al., 2009; Pereira et al., 2009). I tested low doses of the neonicotinoid imidacloprid for its ability to stimulate reproduction in *P. maculiventris*, when treated at different life stages and across a second generation. I predicted that exposure to low-dose concentrations of imidacloprid would result in stimulatory effects on reproductive traits and survival, and that stimulation would be carried over into the next generation.

2.2 Materials and Methods

2.2.1 Insects

A *P. maculiventris* colony was established Dalhousie University Faculty of Agriculture (Truro, NS, Canada) from eggs obtained from the Agriculture and Agri-Food Canada Research and Development Centre (London, ON, Canada). Eggs (100-200) were placed in 10 cm diameter plastic Petri plates lined with filter paper (Fisher Scientific, Ottawa, ON, Canada) with a fresh cabbage (*Brassica oleracea* L.) leaf for newly hatched nymphs. Second instar nymphs from those eggs were placed in transparent plastic containers (Dart Canada, Scarborough, ON, Canada) lined with filter paper, with a cabbage leaf and several mealworm (*Tenebrio molitor* L.) larvae (PetValue, Truro, NS, Canada) as water and food sources. A 5 cm diameter portion of the container lid was removed and covered with cloth mesh for ventilation. Cabbage foliage and *T. molitor* larvae were replaced as needed.

When *P. maculiventris* nymphs became late third to early fourth instars, groups of 50-60 were moved, for the remainder of their life cycle, to larger ventilated plastic containers (37 × 24 × 14 cm) lined with paper towel that contained a 2-3-week-old cabbage plant and *T. molitor* larvae. Insects were transferred to clean containers with a fresh cabbage plant and paper towel once per week and provided *T. molitor* twice per week as needed. Eggs laid on paper towel, plants or on sides of containers were collected once per week and transferred to Petri plates as described above for continued rearing. All insects were housed in a growth chamber held at 23 ± 0.3 °C, 55 ± 5.0% RH, and 16:8 hours (L:D).

2.2.2 Insecticide

Imidacloprid (Admire® 240 SC; 240 g/L of active ingredient (AI); Bayer CropScience Canada, Calgary AB, Canada) was suspended in deionized water to make 1000 mg/L AI stock solutions that were diluted to appropriate concentrations for experiments. Fresh solutions were prepared for each experimental block.

2.2.3 Dose-Response Experiments

Dose-response assays were done on third instar nymphs and 2-day old adult males and females. Nymphs were treated in groups of five with 0, 5.0, 10.0, 15.0, 30.0, 45.0, 60.0, 80.0, 100.0 or 120.0 mg/L of imidacloprid. Male and female adults were treated separately in groups of five with 0, 20.0, 50.0, 70.0, 80.0, 100.0, 300.0, 500.0, or 1000.0 mg/L of imidacloprid. All treatments were administered topically using a Potter spray tower (Burkard Scientific Ltd., Uxbridge, United Kingdom). Mortality was assessed after 48 hours. Five replicates were completed for each concentration in each experiment (total of 250 nymphs treated; total of 225 adult males, and 225 adult females treated). Mortality data was analysed using a probit analysis in SAS (SAS, 2013).

2.2.4 Reproductive Hormesis Experiments

Dose-response bioassays described above determined concentrations of imidacloprid that caused lethal effects, those that caused deleterious sublethal effects, and exposure concentrations that were around the NOAEL. Because insecticide-induced hormesis tends to occur following exposure to concentrations below the NOAEL (Calabrese, 2013a), I wanted to measure *P. maculiventris* reproduction at several imidacloprid concentrations below and above the NOAEL. In my reproduction experiments, I treated third instar nymphs with 0, 0.015, 0.15, 1.5, 5.0, or 10.0 mg/L imidacloprid, and I treated 2-day old

adults with 0, 0.5, 1.0, 5.0, 10.0, and 100.0 mg/L imidacloprid. When treated nymphs developed into adults, or directly following treatment of adults, males and females exposed to the same concentration of imidacloprid were paired and allowed reproduce for up to 70 days. Insect pairs were housed in Petri plates (10 cm diameter, 2.5 cm deep) lined with filter paper and were provided *T. molitor* larvae, a cabbage leaf, and a soaked cotton dental roll as an additional source of water. Food and water were replaced as needed. Experiments were conducted as completely randomized block designs with 3-6 replicates of each treatment within each block, and a total of 6 blocks. Time to oviposition, total number of eggs laid (fecundity), proportion of eggs hatched (fertility), and survival data were analyzed using analysis of variance (ANOVA), followed by multiple means comparison using Fisher's LSD in SAS (PROC GLM), where appropriate.

2.2.5 Trans-Generational Hormesis Experiments

Given the long lifespan and high maintenance of the reproductive experiments described above, I modified my methods to test whether adult insecticide-induced hormesis can occur trans-generationally in *P. maculiventris*. I observed that average daily reproductive outputs in adult insects treated with a low concentration (0.5 mg/L) of imidacloprid began to decrease after 30-40 days following oviposition (see Results), and therefore insects in my trans-generational experiment were examined for 30 days following oviposition.

Adult male and female insects (2-day old) were treated with a control and a hormetic concentration of imidacloprid (0 mg/L, 0.5 mg/L), as determined in the previous experiment. There were 10 replicates of each treatment. Following treatment, males and females of the same treatment were paired and allowed to reproduce for 30 days

following first oviposition. Reproductive output data were recorded for the parental (P) generation and analyzed with analysis of variance to re-confirm hormesis in the P generation.

Fifty eggs laid by each female from the two treatments were placed in Petri dishes lined with moist filter paper. Following hatch, F1 insects were placed in clear plastic containers with mealworms and soft cabbage leaves for food and water, as described above. When second instars developed into F1 adults, mating pairs were crossed for each treatment combination. Thus, there were 4 combinations of treatments: 1) untreated-untreated, i.e., insects treated with control solution in both generations; 2) untreated-treated, i.e., insects treated with control solution in the first generation and a hormetic concentration in the second generation; 3) treated-untreated, i.e., insects treated with a hormetic concentration in the first generation and a control solution in the second generation; 4) treated-treated, i.e., insects treated with a hormetic concentration in both generations (Figure 2.1). Following treatment, mating pairs were held for 30 days following first oviposition, as described previously. Data on reproductive outputs from the second generation were analyzed as a 2x2 factorial design in SAS (SAS, 2013), the main effects being treatment in the first generation, and treatment in the second generation. Multiple means comparison was conducted using Fisher's LSD where appropriate.

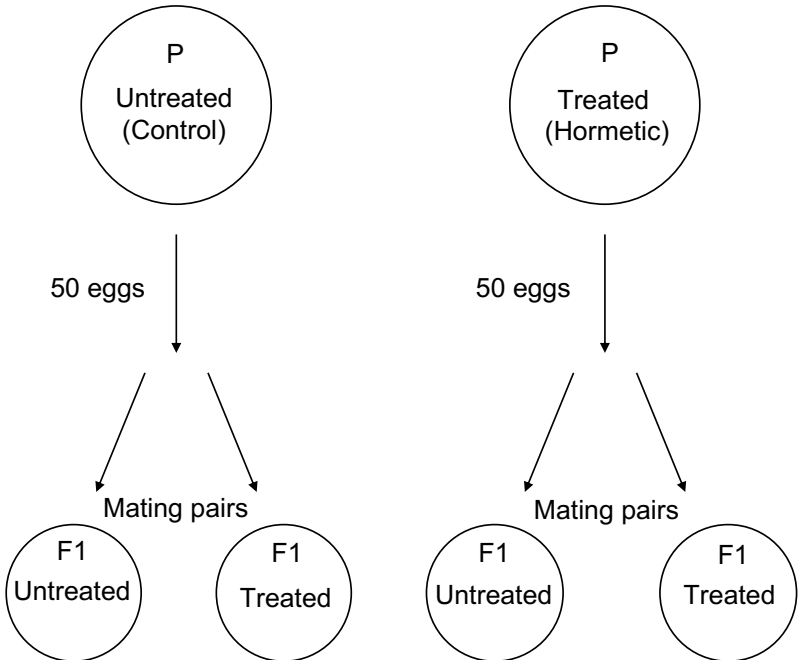

Figure 2.1: Schematic for transgenerational hormesis experiment. Fifty eggs from control or hormetic parents were collected and mating pairs that developed from these eggs were crossed for four treatment combinations:1) untreated-untreated - insects treated with control solution in both generations; 2) untreated-treated - insects treated with control solution in the first generation and a hormetic concentration in the second generation; 3) treated-untreated - insects treated with a hormetic concentration in the first generation and a control solution in the second generation; 4) treated-treated - insects treated with a hormetic concentration in both generations.

2.3 Results

2.3.1 Dose-Response Experiments

Imidacloprid was more toxic to nymphs than adult males and adult females. The LC_{50} for third instar nymphs was 2.5-fold lower than for adult females (Table 2.1). The imidacloprid NOAEL for all life stages and sexes was less than 10.0 mg/L (Table 2.1).

Table 2.1: Contact toxicity (48 hours) of imidacloprid to third instar nymphs and adult male and female *Podisus maculiventris*.

Insects	*n*	Slope (± SE)	LC1 (mg/L) (95% CI)	LC50 (mg/L) (95% CI)	χ^2 (*P*)
Nymphs	250	2.77 (± 0.34)	4.58 (2.11 - 7.48)	31.64 (24.25 - 39.26)	26.64 (0.98)
Adult Females	225	2.28 (± 0.30)	7.63 (2.94 - 13.68)	80.06 (58.61 - 106.49)	32.96 (0.70)
Adult Males	225	2.26 (± 0.33)	6.13 (1.90 - 12.07)	65.66 (44.84 - 89.23)	49.06 (0.11)

2.3.2 Reproductive Hormesis Experiments

For insects treated as third instar nymphs, treatment had a significant impact on the number of eggs laid (fecundity), proportion of eggs hatched (fertility), and survival across the 70-day experimental duration, but not time to oviposition (Table 2.2). For insects treated as adults, the number of fecundity and survival were affected by treatment, but treatment had no effect on time to oviposition or fertility (Table 2.2).

Table 2.2: Statistics for effects of imidacloprid on *Podisus maculiventris* reproductive endpoints and survival over 70 days. Insects were acutely exposed control or imidacloprid treatments, ranging from 0.015-10.0 mg/L (nymphs), or 0.5-100.0 mg/L (adults).

Insects	Time to oviposition	Eggs Laid	Proportion Hatched	Percent Survival
Nymphs	$F_{5,25} = 0.55$; $p = 0.73$ Block: $F_{5,25} = 23.04$	$F_{5,25} = 14.45$; $p < 0.001$ Block: $F_{5,25} = 2.55$	$F_{5,25} = 13.24$; $p < 0.001$ Block: $F_{5,25} = 15.62$	$F_{5,25} = 4.25$; $p = 0.0062$ Block: $F_{5,25} = 4.15$
Adults	$F_{4,20} = 0.43$; $p = 0.79$[a] Block: $F_{4,20} = 4.18$	$F_{5,25} = 28.04$; $p < 0.001$ Block: $F_{5,25} = 2.84$	$F_{4,20} = 0.05$; $p = 0.99$[a] Block: $F_{4,20} = 6.08$	$F_{5,25} = 12.35$; $p < 0.001$ Block: $F_{5,25} = 1.06$

[a]Adults treated with 100.0 mg/L imidacloprid did not lay eggs, although a small number of individuals did survive. Time to oviposition and proportion hatched were thus analyzed without the 100.0 mg/L treatment.

A significant hormetic effect on fecundity was observed when nymphs were treated at the lowest concentration of imidacloprid (0.015 mg/L) and at an intermediate low concentration (1.5 mg/L) (Figure 2.2a; Figure 2.3b). Average daily reproductive outputs for hormetic concentrations peaked at 30-40 days (Figure 2.2a). A significant reduction in fecundity was observed in insects treated with 10.0 mg/L (Figure 2.3b). Whereas nymphs treated with 0.015 mg/L imidacloprid had increased fecundity with no reduction in time to oviposition, fertility, or survival relative to control treatment nymphs, nymphs from the 1.5 mg/L treatment had increased fecundity accompanied by reduced fertility and reduced survival (Figure 2.3a-d). Time to oviposition was not impacted by any of the imidacloprid treatments (Figure 2.3a). Considerable variability in endpoints measured was observed across all treatments (Figure 2.3a-d).

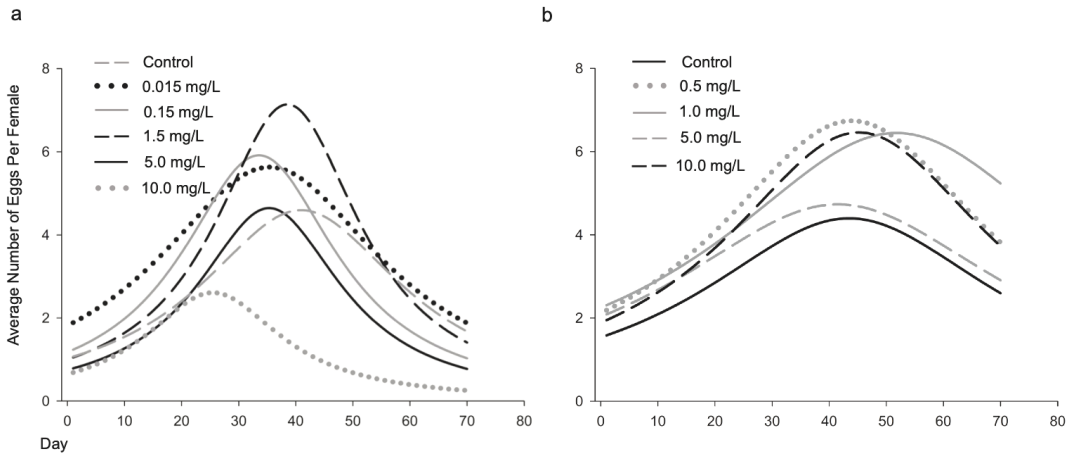

Figure 2.2: Average number eggs per day laid by *Podisus maculiventris* females treated with imidacloprid as third instar nymphs (a) or adults (b). Lines represent fit curves.

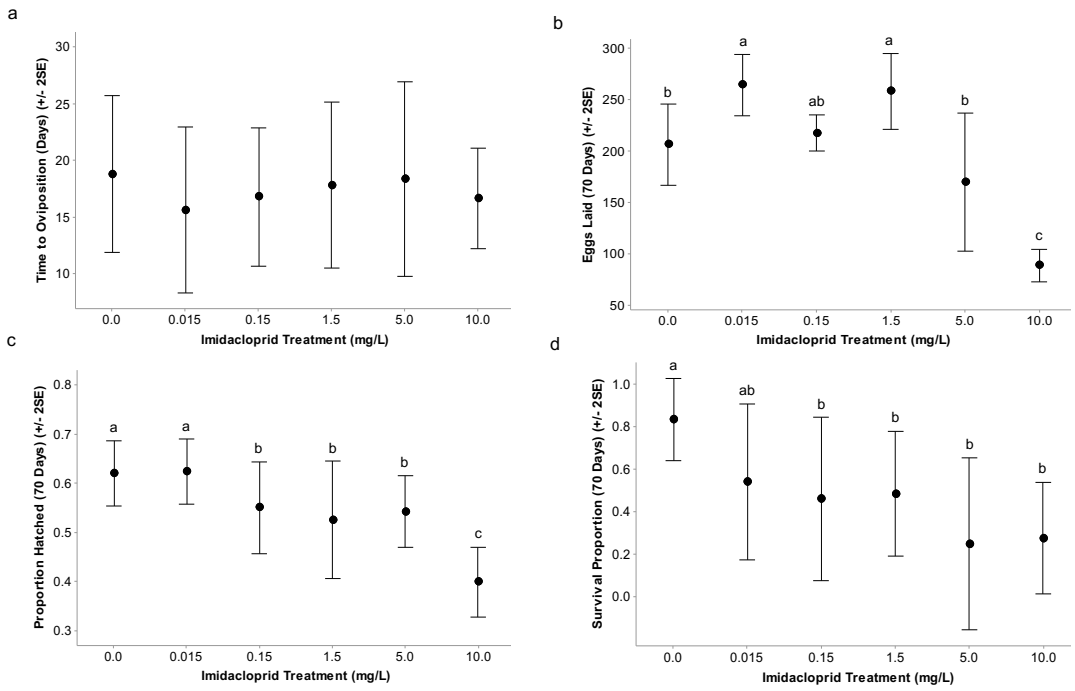

Figure 2.3: Time to oviposition (a), eggs laid (b), proportion hatched (c), and percent survival (d) for *Podisus maculiventris* treated with imidacloprid as third instar nymphs. Insects were treated once with imidacloprid, and the experiment was run for 70 days. Means with different letters are significantly different.

In insects treated as adults, a significant increase in fecundity was observed for those treated with the lowest imidacloprid concentrations (0.5 mg/L and 1.0 mg/L) (Figure 2.2b; Figure 2.4b). I observed that average daily reproductive outputs for hormetic concentrations in adults appeared to peak between the first 30-40 days of reproduction in insects treated with 0.5 mg/L of imidacloprid, but in insects treated with 1.0 mg/L, average daily outputs peaked slightly later between 40 and 50 days of reproduction (Figure 2.2b). No differences in time to oviposition, fertility, or survival were observed in the 0.5 mg/L and 1.0 mg/L treatments, relative to the control treatment (Figure 2.4a,c,d). Fecundity and survival were significantly reduced only in insects treated with the highest imidacloprid concentration (100.0 mg/L) (Figure 2.4b,d).

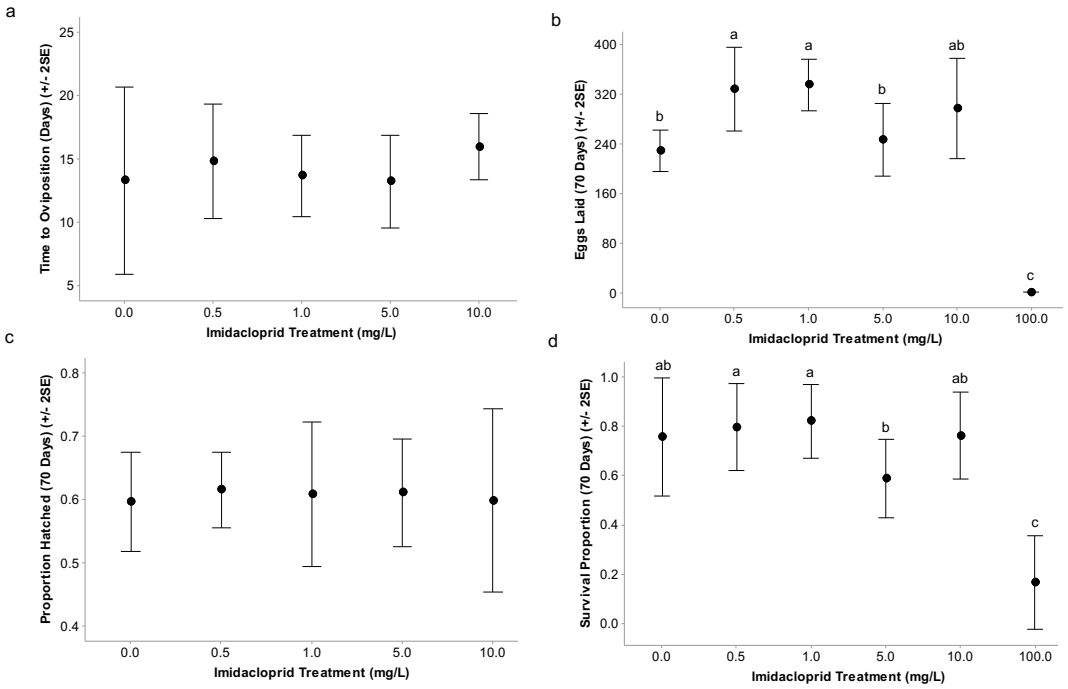

Figure 2.4: Time to oviposition (a), eggs laid (b), proportion hatched (c), and percent survival (d) for *Podisus maculiventris* treated with imidacloprid as adults. Insects were treated once with imidacloprid, and the experiment was run for 70 days. Means with different letters are significantly different.

2.3.3 Trans-Generational Hormesis Experiments

Reproductive hormesis was established in the P generation in insects treated with 0.5 mg/L imidacloprid in the first 30 days of reproduction post-oviposition ($F_{1,18} = 8.39$, $P = 0.010$; 0 mg/L = 168.7 ± 29.0 total eggs; 0.5 mg/L = 232.9 ± 33.4 total eggs). Similar to the previous experiment, there was no effect on fertility ($F_{1,18} = 0.010$, $P = 0.91$; 0 mg/L = 0.69 ± 0.075 proportion hatch; 0.5 mg/L = 0.69 ± 0.059 proportion hatch) or time to oviposition ($F_{1,18} = 1.46$, $P = 0.24$; 0 mg/L = 11.30 ± 4.32 days; 0.5 mg/L = 8.50 ± 1.69 days).

When offspring from 0 and 0.5 mg/L P generation insects were crossed in my 2x2 factorial experiment (Figure 2.1) there were no generational main effects or interaction effects of treatment on time to oviposition (Table 2.3). There was a significant interaction between imidacloprid treatment and fecundity across the P and F1 generations (Table 2.3). When the P generation was not treated with imidacloprid, F1 insects exposed to the imidacloprid treatment laid more eggs than untreated F1 insects (Table 2.4). In addition, when P generation insects were treated with imidacloprid but F1 insects were not, those untreated F1 insects nonetheless had increased egg laying relative to insects that were untreated in both generations (Table 2.4). On the other hand, when both generations of insects were treated with imidacloprid, there was no significant increase in egg laying in the F1 generation relative to insects that were untreated in both generations. F1 insects from parents treated with 0.5 mg imidacloprid L^{-1} had significantly reduced fertility (Table 2.3, Table 2.4).

Table 2.3: Statistics for the effects of imidacloprid on *Podisus maculiventris* reproductive endpoints in F1 adults. Parental (P) and F1 generation adults were treated with either 0 (control) or 0.5 mg/L imidacloprid (hormetic concentration) in a 2x2 factorial design (two levels of generation, two levels of imidacloprid).

Effect	Time to oviposition	Eggs Laid	Proportion Hatched
Treated in P	$F_{1,36} = 0.060$; P = 0.81	$F_{1,36} = 0.29$; P = 0.60	$F_{1,36} = 5.24$; P = 0.028
Treated in F1	$F_{1,36} = 0.19$; P = 0.66	$F_{1,36} = 3.06$; P = 0.089	$F_{1,36} = 3.68$; P = 0.063
P * F1 Interaction	$F_{1,36} = 0.020$; P = 0.88	$F_{1,36} = 7.81$; P = 0.0083	$F_{1,36} = 1.45$; P = 0.24

Table 2.4: Least squares means of time to oviposition, eggs laid, and proportion eggs hatched in second generation *Podisus maculiventris* adults from parents treated with 0 (control) or 0.5 mg/L imidacloprid. Four treatment combinations were tested; 1) untreated adults in both generations (c-c); 2) untreated parental (P) generation and treated F1 generation (c-trt); 3) treated P generation and untreated F1 generation (trt-c); and 4) treated in both generations (trt-trt). Data are from the first 30-days of reproduction following oviposition. Letters and symbols represent significant differences within columns.

Treatment Combination	Time to oviposition[a] (± 2SE)	Eggs Laid (± 2SE)	Proportion Hatched[b] (± 2SE)
c-c	8.40 (± 2.50)	174.50 (± 11.24)b	0.7434 (± 0.066)a
c-trt	8.10 (± 1.53)	236.60 (± 26.20)a	0.7158 (± 0.053)a
trt-c	8.80 (± 2.20)	220.00 (± 37.20)a	0.7014 (± 0.073)b
trt-trt	8.20 (± 1.81)	205.70 (± 28.20)ab	0.5808 (± 0.10)b

[a] No significant main effects or interaction effects of treatment were observed
[b] Only the main effect of treatment in the P generation was significant for the proportion of eggs hatched.

2.4 Discussion and Conclusions

Enhancement of life history traits such as development, longevity, and reproduction in pest insects following exposure to low doses of pesticides and other commonly encountered stressors can be detrimental for agriculture. Most study of insecticide-induced hormesis has therefore predominantly been on pest insects, with the phenomenon being associated with pest outbreaks, pest resurgence, and increased stress tolerance (Cutler and Guedes, 2017). On the other hand, these same hormetic effects induced in beneficial insects could be advantageous for agriculture. For insect biological control to be successful, natural enemies must be able to survive and colonize, and possess traits such as high reproduction, survival, and longevity, especially when under stress. On a commercial scale, cultured maintenance of these key traits for adequate biological control continues to be a challenge in beneficial insects, like *P. maculiventris* (Thorpe and

Aldrich, 2004; Perdikis et al., 2011; Montemayor and Cave, 2012). I therefore examined whether *P. maculiventris* functional traits could be stimulated following exposure to low concentrations of a chemical stressor.

Stimulation of reproduction is one of the most commonly observed hormetic responses in insects exposed to low doses of insecticides (Cutler, 2013). In my first experiments, I observed that acute exposure of nymphs and adults to concentrations of imidacloprid below the NOAEL stimulated their reproduction. I observed that average daily reproductive outputs peaked between approximately 30-40 days for insects treated with 0.5 mg/L and between 40-50 days for insects treated with 1.0 mg/L of imidacloprid, after which, average daily reproductive outputs began to decline towards control levels. Age specific reproductive stimulation has been observed in *Podisus spp.*, with peak reproduction around 30-40 days following reproductive maturity (Guedes et al., 2009; Pereira et al., 2009; Zanuncio et al., 2013), with both age and survival as contributing factors to subsequent reductions in fecundity (Pereira et al., 2009; Zanuncio et al., 2013).

Trade-offs in resource allocation among different physiological processes are often expected to occur under stress. Energy allocation may be increased in some physiological processes and decreased in other processes (e.g. growth, reproduction), in order to maintain fitness under stress (Forbes, 2000). These trade-offs may result in neutral or even negative overall organism fitness. However, trade-offs may not underlie hormetic responses. In my study, fecundity was stimulated in insects treated as adults without affecting time to oviposition, survival, and fertility, which were no different from that of controls. Only the highest concentrations of imidacloprid resulted in reduced fecundity, fertility, and survival. Other studies with stink bugs have observed stimulation

of multiple traits within the same generation following exposure to low doses of insecticide, without major trade-offs. Sublethal exposure of *Podisus distinctus* (Stål) to permethrin increased net reproductive rate and intrinsic rate of population increase, in addition to a slight increase in survival time (Guedes et al., 2009). *Supputius cincticeps* had increased fertility, life expectancy, and shorter generation time following a sublethal permethrin exposure (Zanuncio et al., 2005). Following exposure to gamma-cyhalothrin, increased egg viability, survival, and longevity were observed in *Podisus nigrispinus* (Dallas) (Pereira et al., 2009).

The capacity of an organism to deal with stress can vary depending on the nature and duration of the stress, life stage at which the stress is experienced, and the individual (Metcalfe and Alonso-Alvarez, 2010). Stress prior to sexual maturity can have consequences for adult fitness (Costantini et al., 2012). In *P. maculiventris* treated as nymphs, I observed intragenerational trade-offs when nymphs were exposed to a higher hormetic concentration, but not a lower hormetic concentration. Reproduction was stimulated by exposure to low concentrations of imidacloprid, with no significant impact on time to oviposition, survival, and fertility with exposure to the lowest hormetic concentration (0.015 mg/L). However, when nymphs were exposed to a higher reproductively hormetic concentration (1.5 mg/L), subsequent fertility and survival of these individuals were significantly reduced. This suggests reproductive stimulation may trade-off with survival or fertility within the same generation under some hormetic conditions. Similarly, exposure of female *P. turionellae* to 0.1 ppm malathion increased longevity and stimulated oviposition, but egg hatch was reduced, whereas a 0.01 ppm

malathion treatment resulted increased longevity and stimulated oviposition without a decrease in egg hatch (Buyukguzel, 2006).

The effects of environmental stress can also carry over multiple generations. An organism's phenotype may not only be influenced by stressors it encounters, but also by the stressors encountered by its parents, particularly maternal stress (Mousseau and Fox, 1998). I therefore also examined whether hormetic effects observed in *P. maculiventris* treated as adults were transgenerational. I observed that stimulation of reproduction in adults exposed to a low concentration of imidacloprid carried over into F1 generation insects not treated with insecticide. However, when imidacloprid was applied to both P and F1 insects, reduced fecundity in F1 insect was observed. In contrast, Margus et al. (2019) found that *L. decemlineata* whose mothers had enhanced survival following low-dose deltamethrin exposure also had higher survival when subsequently exposed to the same low dose insecticide exposure. Offspring of hormetic mothers were thus more resilient to subsequent insecticide exposure. This was not the case for my experiment, where offspring of reproductively hormetic parents had lower fecundity than their parents when subsequently treated with imidacloprid, although fecundity was not below that of controls.

Hormesis in the P generation traded-off with fertility in the F1, but the affect appeared greatest in those additionally treated F1 individuals, although no interaction was observed. Previous work with *M. persicae* used a multigenerational approach, across a range of insecticide concentrations, with continuous or one-time exposure to assess patterns of hormesis and trade-offs. Trade-offs were shown between generations, but overall reproductive fitness across all generations was not compromised (Ayyanath et al.,

2013). My 2x2 factorial design also showed that reproductive stimulation can occur without major compromises to fertility or survival. Although fertility was statistically reduced under hormesis in my generational experiment, the average proportion of hatched individuals was still upwards 70% and was compensated by increased fecundity. On average, there were 130 hatched eggs per female in the control-control exposure and 154 hatched eggs per female in the treated-control exposure. I also observed no significant effects of treatment on time to oviposition, meaning reproductive outputs were not delayed by the hormetic imidacloprid treatments.

Predatory insects may be highly susceptible to neonicotinoids or other insecticides (Douglas and Tooker, 2016; Martinez et al., 2019; Ramos et al., 2019). However, reproduction of pentatomids may be stimulated by pesticide exposure (Zanuncio et al., 2005; Zanuncio et al., 2011; Haddi et al., 2016; Santos et al., 2016), and it is well established that low levels of stress – chemical or otherwise – can enhance reproductive outputs in insects (Cutler, 2013). A detailed understanding of the manifestation of such hormetic responses in a given species under specific conditions is needed to effectively leverage hormesis in biological control agents. I observed that reproductive hormesis in *P. maculiventris*, an important natural enemy of a broad range of agricultural pests, can occur across two generations without major trade-offs to life history traits. This is significant because it suggests that, for example, biocontrol producers may be able to strategically apply low doses of stress to natural enemies during culturing, without compromising reproduction in subsequent generation. Further work is needed to confirm whether or not the trend holds across more generations. In addition to traits associated with reproduction, other traits are of course important for natural enemy success. Natural

enemies must possess the ability to withstand temperature and nutritional stress, maintain robust immunity to defend themselves against pathogens and parasites, and retain efficient predating behaviors. It will be important to conduct further experiments to determine whether induction of hormetic responses in beneficial insects results in trade-offs with any of these essential traits.

CHAPTER 3 NEONICOTINOID EXPOSURES THAT STIMULATE PREDATORY STINK BUG, PODISUS MACULIVENTRIS (HEMIPTERA: PENTATOMIDAE), REPRODUCTION DO NOT INHIBIT ITS BEHAVIOR

A version of this chapter was published in the *Journal of Economic Entomology*. Details associated with copyright permission can be found in Appendix 1. Rix, R.R. developed and conducted experiments, analyzed data, and wrote the manuscript. Cutler, G.C. aided in conceiving experiments, contributed to writing, and provided reviews and edits to manuscript drafts.

Rix, R.R., and Cutler, G.C., 2021. Neonicotinoid exposures that stimulate predatory stink bug, *Podisus maculiventris* (Hemiptera: Pentatomidae), reproduction do not inhibit its behavior. Journal of Economic Entomology. 114, 1575-1581.

3.1 Introduction

The use and conservation of insect natural enemies for biological control are integral to IPM programs. Full integration of biological control into IPM is often challenging due to continued reliance on insecticides in intensive agriculture. Even if natural enemies are not killed by insecticides, they can exhibit a wide range of sublethal effects that can compromise their effectiveness in biological control (Desneux et al., 2007). This can include behavioral changes in locomotion, navigation, predator avoidance, searching, predation, mate finding, and mating, all of which impact reproduction, survival, and functioning of natural enemies (Muller, 2018). For example, locomotion was reduced in the parasitoid wasp, *Spalangia endius* Walker, following exposure to imidacloprid (Burgess and King, 2020), and lambda-cyhalothrin exposure altered foraging behavior, which reduced predation rate in the zoophytophagous mirid, *Nesidiocoris tenuis* (Reuter)

(Soares et al., 2020). Similarly, female *Nasonia vitripennis* (Walker) parasitoid wasps exposed to a sublethal dose of imidacloprid were unable to locate hosts with olfactory cues and were less responsive to male sex pheromones, which also disrupted courtship behavior and mating (Tappert et al., 2017).

Although large or even small doses of insecticides can inhibit insects, exposure to mild doses of insecticide may also stimulate insects (Cutler, 2013; Guedes and Cutler, 2014; Cutler and Guedes, 2017). This biphasic response to a stressor, characterized by high-dose inhibition and low-dose stimulation, is called hormesis (Calabrese and Baldwin, 2002; Calabrese and Baldwin, 2003; Calabrese et al., 2016). In the context of pest management, insecticide-induced hormesis has been shown to stimulate pest insect population growth, and prime individual insects for subsequent stress (Cutler, 2013; Guedes and Cutler, 2014; Cutler and Guedes, 2017). Natural enemies also have hormetic responses following sublethal insecticide exposure, including stimulation of behaviors. For example, sublethal doses of chlorpyrifos enhanced kairomone perception (Delpuech et al., 2005), associative learning, host searching, and parasitism rate (Rafalimanana et al., 2002) of the parasitoid, *Leptopilina heterotoma* (Thomson). Changes in movement can influence predator-prey encounters, and insecticide exposure can enhance predator efficiency. For example, walking frequencies of seven-spot ladybird, *Coccinella septempunctata* L., increased when exposed to sublethal deltamethrin residues (Wiles and Jepson, 1994), and cypermethrin stimulated killing and feeding rates of the spotted wolf spider, *Pardosa amentata* (Clerck) (Toft and Jensen, 1998).

I previously showed that low doses of imidacloprid can stimulate reproduction of the spined soldier bug, *P. maculiventris* (Chapter 2) (Rix and Cutler, 2020), an important

zoophytophagus predator common across eastern North America (De Clercq et al., 1998; Kneeland et al., 2012; Montemayor and Cave, 2012). My previous results are significant because they suggest low-dose exposures to imidacloprid or other insecticides could stimulate reproduction of this natural enemy in the field, or that low-level chemical stress could be strategically applied to natural enemies like *P. maculiventris* in order to maximize their effectiveness in biological control. Although I observed that certain imidacloprid treatments stimulated *P. maculiventris* reproduction without effects on oviposition, fertility, or survival (Chapter 2) (Rix and Cutler 2020), additional experiments are needed to clarify whether insecticide-induced reproductive hormesis comes at the expense of other biological functions. Studies in multiple systems have shown that mild stress can have stimulatory, inhibitory, or no effects on biological functions (Le Bourg and Rattan, 2014). I therefore conducted experiments to test whether exposure to a reproductively hormetic concentration, and other mild treatments of imidacloprid would stimulate, inhibit, or have no effect on common *P. maculiventris* behaviors and predation. Based on the fact that sublethal exposure to insecticides may stimulate multiple functions without trade-offs (Cutler et al., 2009; Ayyanath et al., 2013; Rix et al., 2016), I hypothesized that exposure to a reproductively hormetic concentration of imidacloprid would stimulate at least some behaviors and predation in *P. maculiventris*, with no significant inhibitory effects on other measured endpoints.

3.2 Materials and Methods

3.2.1 Insects

Insect colonies were established and reared as previously described in Rix and Cutler (2020) (Chapter 2). Briefly, third- to fifth-instar nymph and adult insects in groups of 50-

60 individuals, were reared on 2-3-week-old cabbage (*B. oleracea*) plants in ventilated plastic containers (37 × 24 × 14 cm) lined with paper towel. Insects were transferred to clean containers and provided fresh cabbage plants once per week, and provided with *T. molitor* larvae (PetValue, Truro, NS, Canada) as a food source twice per week. Eggs laid by adults on plants and throughout containers were collected and placed in 10 cm plastic Petri plates lined with filter paper (Fisher Scientific, Ottawa, ON, Canada) in groups of 100-200 eggs. Newly hatched nymphs were provided a fresh cabbage leaf as a source of food and water, and following second instar emergence, were transferred to small ventilated, transparent plastic containers (Dart Canada, Scarborough, ON, Canada). A filter paper lining was inserted into the container and insects were provided a fresh cabbage leaf and *T. molitor* larvae as needed. Upon reaching late third to early fourth instar, insects were transferred to larger ventilated containers as described above, and the rearing cycle continued. Insects were kept in a growth chamber held at 23 ± 0.3 °C, 55 ± 5.0% RH, and 16:8 hours (L:D). Insects were held 24 hours without food and water before all experiments.

3.2.2 Insecticide

Imidacloprid (Admire® 240 SC; 240 g/L of active ingredient (AI); Bayer CropScience Canada, Calgary AB, Canada) was suspended in deionized water to make 1000 mg/L AI stock solutions that were diluted to experimental concentrations. Fresh solutions were prepared for each experiment. Because I previously reported that exposure to 0.5 mg/L of imidacloprid was reproductively hormetic to *P. maculiventris* (Chapter 2) (Rix and Cutler, 2020), I wanted to test imidacloprid treatments at, below, and above this

concentration on *P. maculiventris* behavior. Thus, in various experiments I exposed insects to 0.05, 0.5, 5.0, and 20.0 mg/L of imidacloprid.

3.2.3 Behavior in a Plastic Container Arena

I first wanted to test for potential effects on behavior in a simple setting, without additional stimuli. Cohorts of three young adult (<2-3-week-old males or females) bugs were treated topically with 2 mL of solution using a Potter Spray Tower (Burkard Scientific Ltd., Uxbridge, UK). Following treatment, individual male and female insects were placed in clear plastic containers (Dart Canada, Scarborough, ON, Canada), approximately 10 cm in diameter and 5 cm high, on a lab bench. After a 30-minute acclimation period, insect behavior was recorded every two minutes for a total of 90 minutes. In this experiment, instantaneous behaviors were recorded as "walking-antennating", "antennating", "preening", or "stationary" (Table 3.1). These categories of behavior were selected based upon preliminary observation of typical behaviors of *P. maculiventris* in this setting, and similar observations in previous studies with predaceous Hemiptera (Wiedenmann and Oneil, 1991; Bouagga et al., 2018; Vidal-Gomez et al., 2018). The experiment was conducted as a completely randomized blocked design consisting of 3 replicate male or female *P. maculiventris* per treatment, and the entire experiment was repeated 3 times (blocks in time).

Table 3.1: *Podisus maculiventris* behaviors measured in plastic container arenas and plant (*Brassica oleracea*) arenas with *Plutella xylostella* larvae. Depending on the experiment, *Podisus maculiventris* were exposed to 0, 0.05, 0.5, 5.0, and/or 20.0 mg/L of imidacloprid.

Behavior	Description
Walking-Antennating	Active searching activity characterized by continuous walking with simultaneous antennal movement.
Antennating	Stationary searching activity characterized by antennal movements.
Probing	Active searching activity characterized by the extension of the insect's proboscis and the probing of the surroundings

Behavior	Description
Plant Probing	Characterized by the extension of the insect's proboscis and the probing of the surrounding leaf with the labium and/or the insertion of the stylet into the plant
Preening	Grooming activity characterized by cleaning of the antennae and proboscis using the forelegs
Stationary	The insect remains at rest and motionless, carrying out no activity

3.2.4 Predation in a Plastic Container Arena

I next wanted to examine predation rates in the same simple arena. Treated young adults (<2-3-week-old males or females) were placed in plastic containers as described above, along with 6 mealworm larvae (*T. molitor*); approximately 1.5 cm in length, 11-12 instar) and a cotton dental wick soaked with water as a water source. The total number of larvae consumed at 24 and 48 hours was recorded. *T. molitor* were considered consumed if they were dead (no response to gentle prodding with a needle) and with internal contents fully or partially sucked by *P. maculiventris*. Cadavers of consumed larvae were removed and replaced with live larvae 24 hours after the start of the experiment. The experiment was conducted in a completely randomized blocked design consisting of 3 replicate male or female *P. maculiventris* per treatment, and the entire experiment was repeated 3 times (blocks in time).

3.2.5 Behavior in a Plant Arena

In another experiment, I examined effects of low-dose imidacloprid treatment on *P. maculiventris* predatory behavior in an arena with prey and a host plant. Experimental plants were 3–4-week-old cabbage (*B. oleracea*), 10-12 cm in height, growing in 7 cm pots filled with Promix®. Plants were trimmed such that 3 full leaves remained to give 3 areas (lower, middle, upper) of elevation in the canopy. Male and female adult *P. maculiventris* (<2-3-week-old) were treated topically with control (0 mg/L), 0.5, or 20.0

mg/L of imidacloprid, in a Potter spray tower as described above. Following treatment, individual insects were placed at the base of the meristem of experimental plants, and a 4th or 5th instar diamondback moth larva (*Plutella xylostella*) (L.), was placed on the upper-most leaf. Starting 30 minutes after insecticide treatment, *P. maculiventris* behaviors were recorded every two minutes for a total of 90 minutes. Behaviors were as described in Table 3.1. I also recorded the corresponding location of the insect on the plant, as follows: 1) Off-plant, on soil or plastic pot; 2) Stem region, consisting of any part of the plant that was not a leaf; 3) Lowest leaf; 4) Middle leaf; and 5) Upper leaf. The experiment consisted of 3 replicate male or female *P. maculiventris* and plants per treatment, and the entire experiment was repeated 3 times (blocks in time).

3.2.6 Predation in a Plant Arena

The experimental arena with a cabbage plant was as described above, with ten 4th or 5th instar diamondback moth, *P. xylostella,* larvae placed randomly throughout the canopy, and a treated insect (0, 0.5, or 20.0 mg/L imidacloprid applied in a Potter tower) placed at the base of the stem. Plants were placed in ventilated plastic containers (37 × 48 × 14 cm). The total number of larvae consumed at 24 and 48 hours was recorded, and consumed larvae were replaced with live larvae 24 hours after the start of the experiment. The experiment consisted of 3 replicate male or female *P. maculiventris* and plants per treatment and the entire experiment was repeated 3 times (blocks in time).

3.2.7 Statistical Analysis

Measures (frequency counts) of individual behaviors and predation were analyzed separately for females and males using ANOVA ($\alpha = 0.05$) with treatment as the factor of interest, and block as an additional factor. Where appropriate, post hoc tests were

conducted using Fisher's LSD. Where assumptions of normality and homogeneity of variance were not met, data were transformed using a logarithmic or cubic function. Back-transformed data are presented. All statistical analyses were completed using the General Linear Model procedure in Minitab (Minitab®, 2020).

3.3 Results

For both male and female *P. maculiventris*, only the 20.0 mg/L treatment had a significant effect on walking-antennating behavior. This treatment reduced walking-antennating 5- to 20-fold relative to other treatments (Figure 3.1a). Exposure to 0.05 mg/L imidacloprid resulted in 6-fold more antennating in females ($p < 0.05$) and 4-fold more antennating in males ($p < 0.1$), and the 5.0 mg/L treatment also significantly increased antennating in female bugs (Figure 3.1b). The probing frequency of females was not affected by any treatment, but this behavior was significantly stimulated in males exposed to 0.5 mg/L imidacloprid (Figure 3.1c). Treatment had no effect on *P. maculiventris* preening frequency (Figure 3.1d). Stationary frequency, although not significantly affected by treatment trended upwards at the highest concentrations (Figure 3.1e). Consumption of *T. molitor* was generally reduced with increasing imidacloprid concentrations (Figure 3.1f).

Under conditions of complex searching on cabbage plants with stimuli, imidacloprid treatments did not significantly affect any of the measured behaviors of females (Figure 3.2a-f). In males, treatment did not affect walking-antennating, antennating, or stationary behavior, but there was mild ($p < 0.1$) stimulation of plant probing and preening in the 0.5 mg/L imidacloprid treatment (Figure 3.2a-e). Frequencies of location switches on plants were also insignificant in females and males (Figure 3.2f).

Predation on *P. xylostella* was reduced 3- to 6-fold ($p < 0.05$) in males or females exposed to 20.0 mg/L imidacloprid (Figure 3.2g).

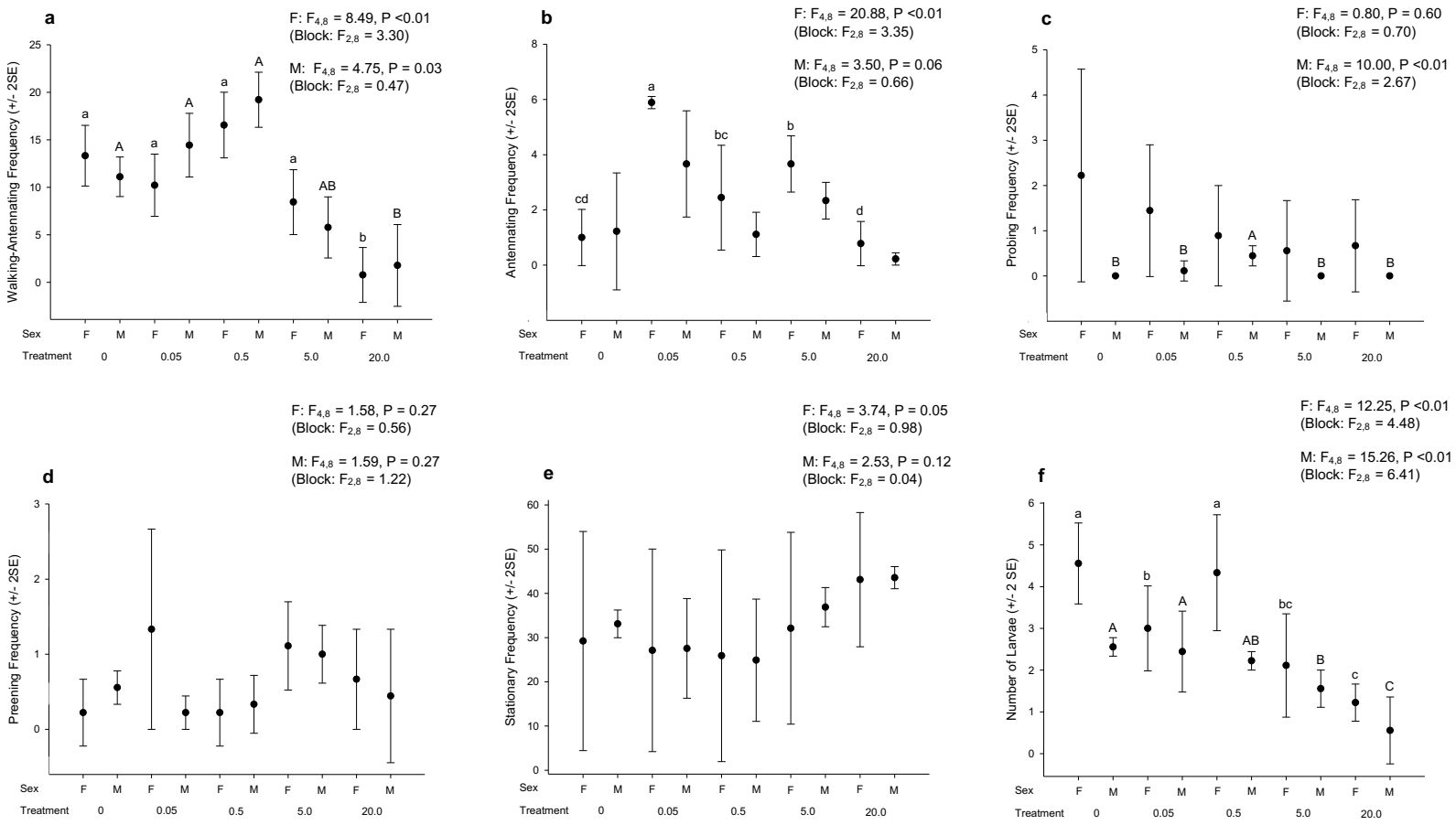

Figure 3.1: Frequencies of (a) walking-antennating, (b) antennating, (c) probing, (d) preening, (e) stationary behaviors, and (f) predation of female (F) and male (M) *Podisus maculiventris* in a simple plastic cup arena, following treatment with 0, 0.05, 0.5, 5.0, or 20.0 mg/L imidacloprid. Different letters of the same case represent significant differences between treatments within single sex.

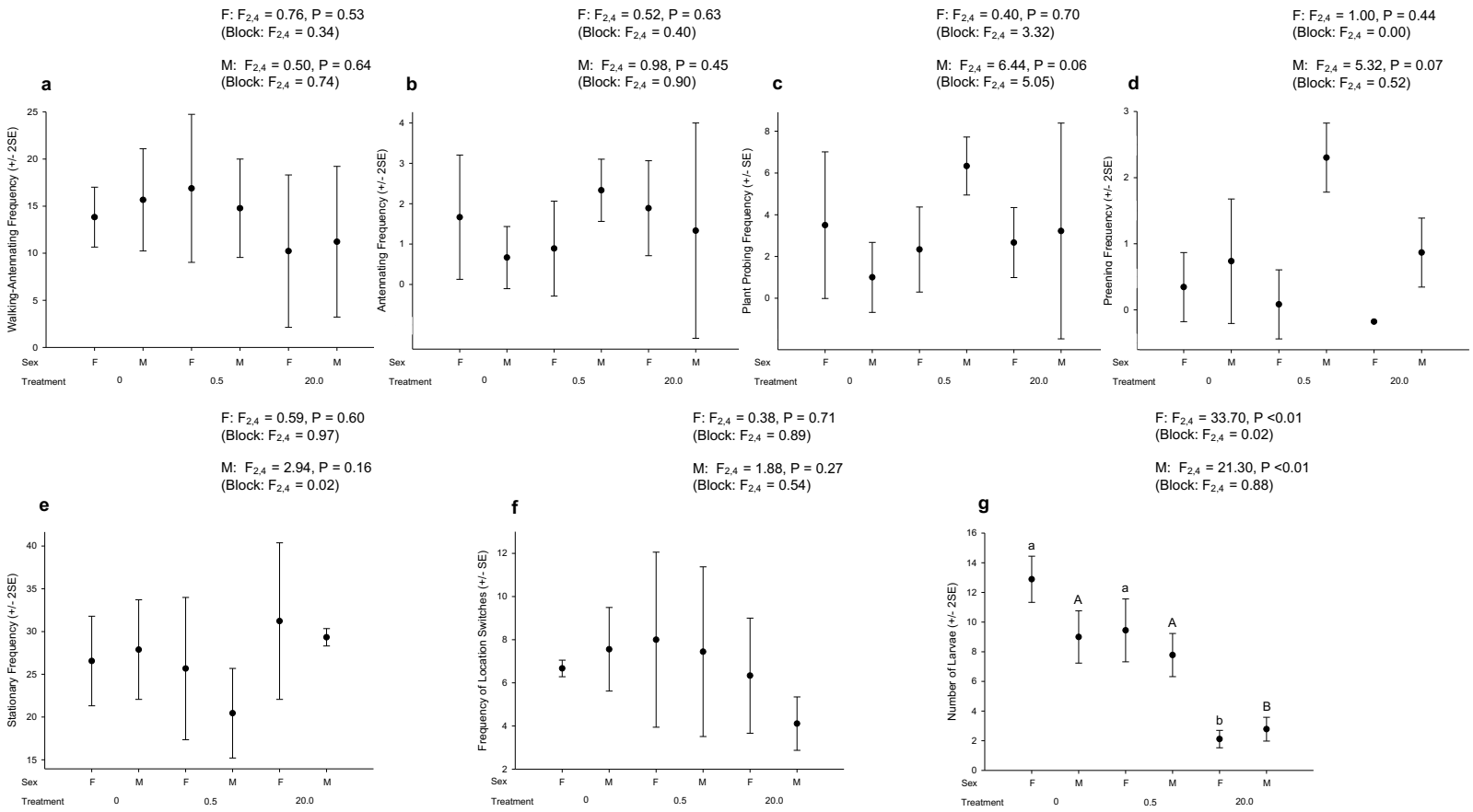

Figure 3.2: Frequencies of (a) walking-antennating, (b) antennating, (c) plant probing, (d) preening, (e) stationary behaviors, (f) frequency of location switches, and (g) predation of female (F) and male (M) *Podisus maculiventris* on plants, following treatment with 0, 0.5, and 20.0 mg/L of imidacloprid. Letters of the same case that are different represent significant differences between treatments within single sex.

3.4 Discussion and Conclusions

Overall, I observed that a treatment of imidacloprid previously reported to stimulate reproduction in *P. maculiventris* (0.5 mg/L) (Chapter 2) (Rix and Cutler, 2020) had no inhibitory effects on any behaviors measured, and that this treatment, a milder treatment (0.05 mg/L), and more intense treatment (5.0 mg/L) stimulated certain behaviors in male or female bugs, with few inhibitory effects. These finding support my initial hypothesis and suggest mild chemical stress can stimulate *P. maculiventris* reproduction without seriously compromising behaviors important for its functioning as a natural enemy.

Stimulatory effects were observed for three different behaviors, with some variability by treatment and sex. Antennating frequency was unaffected at the previously reported reproductively hormetic concentration (0.5 mg/L) (Chapter 2) (Rix and Cutler, 2020) but increased in females exposed to the 0.05 and 5.0 mg/L treatments in the plastic cup arena. Probing frequencies increased in males treated with a 0.5 mg/L imidacloprid concentration in both the plastic cup and plant experimental arenas, and stimulated ($P = 0.07$) preening behavior was observed in males with the same treatment in the plant set up. Stimulation of these behaviors may be reflective of imidacloprid's mode of action. Imidacloprid is a nicotinic acetylcholine receptor (nAChR) agonist that mimics acetylcholine, an important excitatory neurotransmitter in insect behavior (Matsuda et al., 2001; Grunewald and Siefert, 2019). Nicotinic acetylcholine receptors are distributed in sensory regions of the brain in some insects, including antennal lobes important in prey searching and identification (Thany et al., 2010; Eiri and Nieh, 2012). Stimulation of such behaviors has functional significance for this insect. For example, *P. maculiventris* will probe surroundings when searching for, locating, and attacking prey, and thus

stimulated probing could assist host searching and predation success (Kwon et al., 2006; Maekawa et al., 2011). There is precedent for mild imidacloprid exposure stimulating such behaviors of other natural enemies. For example, low-dose treatment of imidacloprid resulted in increased grooming by *S. endius* (Burgess and King, 2020).

Although I did not observe stimulated locomotion in my experiments, others have shown mild insecticide exposure can stimulate insect locomotion. For example, topical exposure of *H. axyridis* to low doses of beta-cypermethrin resulted in increased walking frequency, duration, distance, and speed (Xiao et al., 2017), and spiders treated with low concentrations of malathion had upwards of 30% stimulated locomotion (Tietien and Cady, 2007). Locomotion of stink bugs in my experiments tended to decrease with increasing concentrations of imidacloprid. Similarly, imidacloprid exposure induced paralysis and impaired walking in the ground beetle, *Harpalus pennsylvanicus* Degeer (Kunkel et al., 2001) and reduced walking and flying behavior in *L. decemlineata* (Alyokhin and Miller, 2015).

Predation was also not stimulated by any treatment but was inhibited at the highest (20.0 mg/L) imidacloprid treatment. This corresponded to the trends observed in decreased walking-antennating behavior (locomotion), which could reduce predator-prey interactions. Imidacloprid exposures at higher sublethal concentrations (field rates) were previously found to reduce predation in *Podisus spp.* (Malaquias et al., 2014; Resende-Silva et al., 2019). My results are also consistent with research showing reduced mobility and locomotion, and predation in other arthropods exposed to neonicotinoids (Tahir et al., 2014; Tooming et al., 2017; Barmentlo et al., 2019). It is well established that sublethal doses of insecticide can compromise insect behaviors (Ripper, 1956; Croft and Brown,

1975; Desneux et al., 2007; Muller, 2018). Consumption of larvae was also reduced in the 5.0 mg/L treatment – the only inhibitory effect found with this treatment in the current study. I previously found that a topical 5.0 mg imidacloprid treatment also reduced egg hatch and subsequent survival of *P. maculiventris* (Chapter 2) (Rix and Cutler, 2020). This suggests any stimulatory benefits of imidacloprid treatments ≥ 5.0 mg/L could be countered and negated by inhibitory effects on other critical biological functions.

I mostly observed similarities in behavioral patterns of males and females, with some exceptions, most notably stimulated probing of males under both experimental conditions, and stimulated preening of males when on plants. Other studies have shown behavioral differences in males and females. Male Neotropical brown stink bugs, *Euschistus heros* (Fabricius) exhibited greater walking activity following mild imidacloprid exposure (Haddi et al., 2016). Similarly, insecticide treated males of the parasitoid wasp, *Diachasmimorpha longicaudata* (Ashmead), had increased locomotory response, walking for longer distances (Andreazza et al., 2020). These differences of insecticides on behavior may be due to inter-sex differences in insecticide sensitivity. I previously observed that males were more sensitive to imidacloprid than females (Chapter 2) (Rix and Cutler, 2020). Differences in insecticide sensitivity may be attributable to size. Male insects are often smaller than female insects (Gergs et al., 2015; Boubidi et al., 2016), and males from *P. maculiventris* colonies used in this study were smaller than females (male weight = 48.5 mg +/- 8.0; female weight = 62.8 mg +/- 9.2; t_{38} = 5.26; $p < 0.01$). As my spray treatments would have given different doses to males vs. females (amount of active ingredient per body weight), it is possible that certain

treatments for probing and preening behavior were in the “hormetic zone” for males but not females.

Whatever the stimulatory concentration or dose for a particular biological endpoint, it is certainly conceivable my treatment concentrations would be encountered in the field by *P. maculiventris* and other insects. The field rate for a foliar spray of imidacloprid on cabbage is 48g/ha (Bayer CropScience. 2019. Admire® 240 flowable systemic insecticide. BayerCropScience, Calgary, AB, Canada). Insecticides like imidacloprid are typically applied with 135-400 L water per ha (Ontario Ministry of Agriculture, Food, and Rural Affairs. 2020. Publication 838. Vegetable Crop Protection Guide 2020-2021. OMAFRA, Toronto, ON, Canada.), meaning field concentrations applied to crops range 120-355 mg/L. Foliar applications of imidacloprid rapidly break down after application, entering the sublethal range of concentrations after only approximately a week (Cutler et al., 2005). This means insects could very well be exposed to sublethal and hormetic concentrations of imidacloprid a week or two after the product is applied to foliage in the field.

I acknowledge that there are many physiological, biochemical, immunological, and other behavioral endpoints not measured, and that biological trade-offs can accompany insecticide-induced hormetic responses in insects (Ayyanath et al., 2013; Santos et al., 2016). However, all considered – including the results of my previous study (Chapter 2) (Rix and Cutler, 2020) – my findings support the contention that the hormetic response is dynamic, with variation depending on a range of factors, including the strength of the stressor, biological endpoints measured, life stage, and sex (Parsons, 2001; Calabrese and Blain, 2005; Costantini et al., 2010; Tyne et al., 2015). The 0.5 mg

imidacloprid/L treatment that stimulated reproduction in my previous study (Chapter 2) (Rix and Cutler, 2020) also stimulated certain behaviors in the current study, but so did 0.05 and 5.0 mg imidacloprid/L treatments, depending on bioassay design and sex of the insect. In addition, with the exception of reduced predation efficiency at the 5.0 mg/L treatment, I found no evidence of inhibition of any behaviors at treatments that caused stimulation. This is significant because it shows that natural enemies like *P. maculiventris* can be exposed to mild chemical stress, up to a certain level, such that reproduction and behaviors can be stimulated without inhibiting other crucial biological functions. Beyond this 'hormetic zone', however, a sublethal treatment can stimulate some endpoints while inhibiting others, and greater doses of insecticide will only have inhibitory effects on all biological functions.

CHAPTER 4 REVIEW OF MOLECULAR AND BIOCHEMICAL RESPONSES DURING STRESS INDUCED STIMULATION AND HORMESIS IN INSECTS

A version of this chapter was published in *Science of the Total Environment*. Details associated with copyright permission can be found in Appendix 1. Rix, RR conducted literature searches and analysis, and wrote the manuscript. Cutler, G.C. provided suggestions and contributed to manuscript edits.

Rix, R.R., and Cutler, G.C., 2022. Review of molecular and biochemical responses during stress induced stimulation and hormesis in insects. Science of the Total Environment. 827, 154085.

4.1 Introduction

Dose-response relationships in toxicology have been traditionally characterized with a threshold model, whereby responses to progressively lower doses become indistinguishable from controls, or with a linear no-threshold model, which assumes at low doses there is a linear relationship between dose and effect that terminates at zero (Calabrese and Baldwin, 2003). These models were called into question by Hugo Schulz (Calabrese, 2015a), who when studying effects of disinfectants on yeast in the 1880s, noted enhanced metabolism at low doses and toxicity at high doses (Schulz, 1887; Schulz, 1888). This phenomenon later became known as 'hormesis' and has since been argued to be a better representation of dose-responses than both the threshold and linear no-threshold models (Calabrese and Baldwin, 2003; Calabrese, 2005; Calabrese and Blain, 2005; Calabrese, 2015b; Agathokleous et al., 2020; Calabrese and Agathokleous, 2020).

The classical depiction of hormesis is, depending on the endpoint measured, an inverted-U or J-shape curve, encompassing stimulation of a biological response at low

doses and inhibition of the response at high doses (Figure 1.2c,d) (Cutler and Guedes, 2017). This has been well described in plants, microbes, mammals, and invertebrates in response to chemical, physical, or biological stress (Calabrese and Blain, 2009; Calabrese and Blain, 2011; Cutler, 2013). In general, hormesis occurs from either a direct stimulation of cellular processes or defenses or, far more commonly, an overcompensation of cellular processes or defenses following exposure to a stressor or toxin (Calabrese and Baldwin, 2002). Direct stimulation occurs in the absence of damage, whereby stressors or chemical agents directly activate biological processes (Calabrese and Baldwin, 2001; Calabrese, 2013a). Overcompensation stimulation occurs in response to a disruption in homeostasis whereby resources for repair are produced in moderate excess (Calabrese, 2001; Calabrese and Baldwin, 2002). A number of molecular mechanisms seem to be involved in coordinating hormetic responses, such as the ERK1/2, p38, and JNK pathways involved in processes such as cell proliferation and development, and directing cellular responses to oxidative, heat, and radiation stress, and inflammatory responses and apoptosis (Cargnello and Roux, 2011; Calabrese, 2013b). A general mechanism has also been proposed to account for hormetic responses based upon the activation of the Nrf2 transcription factor, which regulates antioxidant and anti-inflammatory responses (Calabrese and Kozumbo, 2021a).

Hormesis has implications for agriculture and pest management, including insect pests (Guedes and Cutler, 2014). Insects are exposed to a wide range of stressors, from temperature and nutritional stress, to agrochemicals and disease. Given the adaptive importance of hormesis, it has been recognized as potentially influencing tolerance or resistance to stress and insecticides, and contributing to pest outbreaks and pest

resurgences (Guedes and Cutler, 2014). On the other hand, hormesis may stimulate beneficial insect populations and their performance (Cutler and Guedes, 2017). Increases in reproduction or longevity are among the most commonly observed stimulatory effects induced by low doses of stress in insects, however stimulated growth, behavior, and immunity have also been reported (Cutler, 2013). This is frequently in response to chemical stress, but also radiation, temperature, and dietary restriction (Calabrese, 2013c; Cutler, 2013; Cutler and Rix, 2015; Berry and Lopez-Martinez, 2020). Preconditioning or 'priming' hormesis, whereby a hormetic response accompanies increased tolerance to a subsequent stressor has been reported in studies with *D. melanogaster* in response to temperature and nutritional stress (Le Bourg et al., 2001; Le Bourg, 2007; Le Bourg et al., 2009; Le Bourg, 2013), but also in response to chemical exposure (Alptekin et al., 2016; Rix et al., 2016; Rix and Cutler, 2018). Agrochemical exposures that induce hormesis have more recently been shown to coincide with or enhance pest insect tolerance to insecticides and other stressors (Riaz et al., 2009; Ge et al., 2013; Rix et al., 2016; Rix and Cutler, 2018).

The stimulation of repair and recovery mechanisms that are central to hormesis allow organisms to deal with damage associated with low-dose stress (Calabrese and Mattson, 2011; Wiegant et al., 2012). The ubiquitous nature of hormesis, occurring irrespective of organism or stressor, suggests it should be moderated by primordial, highly conserved stress response pathways. The heat shock response, oxidative stress response, and detoxification response have all been implicated in the modulation of hormetic responses (Berry and Lopez-Martinez, 2020). Temperature, osmotic, nutritional, chemical, and oxidative stress, frequently experienced by insects, all induce the

transcription of heat shock proteins, which act as molecular chaperones, repairing damaged and improperly folded proteins (Rattan et al., 2004; King and MacRae, 2015). Stress also causes the buildup of ROS, resulting in production of antioxidant enzymes, such as superoxide dismutase, catalase, and peroxidase. These enzymes work in-tandem to neutralize ROS, which cause damage to DNA, proteins, and lipids (Garcia-Caparros et al., 2020). Increased heat shock protein and antioxidant gene expression or activity can protect insects against cellular damage under stressful conditions (Krishnan and Kodrik, 2006; Micheal and Subramanyam, 2013; King and MacRae, 2015), including low-dose or hormetic stress (Hercus et al., 2003; Calabrese, 2013c; Rix et al., 2016; Matsumura et al., 2017), and may be associated with enhanced longevity, reproduction, and stress tolerance (Arking et al., 2000; Morrow et al., 2004; Tasaki et al., 2017). Genes involved in chemical detoxification are thought to play a role during insecticide-induced hormesis (Cutler, 2013). Low doses of insecticides and other chemicals can induce expression of genes encoding detoxification enzymes, such as cytochrome P450s, glutathione-s-transferases, and esterases (Gu et al., 2013; Yang et al., 2016b; De Smet et al., 2017; Kang et al., 2018). Overexpression of detoxification genes is associated with increased insecticide tolerance and metabolic resistance (Bass and Field, 2011). Increased expression of detoxification genes has been observed during preconditioning hormesis, accompanied by stimulation of reproduction, longevity, fertility, and development (Deng et al., 2016; Rix et al., 2016; Ullah et al., 2020).

The phenotypic effects associated with hormesis in insects have been reviewed (Cutler, 2013), but few works have aimed to systematically examine the relationships between phenotypic responses and the molecular and biochemical changes that

accompany them. The aim of this chapter was to characterize commonly observed stimulatory responses following exposure to low doses of stress and associated molecular and biochemical responses to provide a clearer understanding of the patterns and manifestation of hormesis in insects. Given the ubiquity of the hormetic response, I hypothesized that there would be commonalities in phenotypic and molecular responses across stressors, and that phenotypic effects would most frequently be associated with conserved molecular and biochemical responses associated with preventing and mitigating the effects of stress. I expected papers to report up-regulation more than down-regulation of antioxidation, detoxification, heat shock, and developmental and reproduction genes during hormetic responses. I also expected given previous analyses on the general characteristics of hormetic responses (Calabrese and Blain, 2005; Calabrese and Blain, 2011; Calabrese, 2013a), that the majority of phenotypic responses would range within 30-60% increased above controls.

4.2 Methods

Web of Science (www.webofscience.com) was searched for papers reporting phenotypic stimulation (e.g. increased reproduction, increased heat tolerance) that was also associated with molecular or biochemical effects in response to a low or moderate dose of stress. Given the extensive amount of literature related to responses of insects to various stressors, an exhaustive search of the literature was not feasible. I therefore conducted strategic searches of the literature using combinations of terms related to hormesis or stress responses in insects, along with terms representative of commonly reported hormetic effects in insects, taxa frequently studied in the context of stress and hormesis, and general molecular or biochemical responses in insects associated with stress and

hormesis. These words/terms included: hormoligosis, hormesis, insect, Coleoptera, Diptera, Hemiptera, Heteroptera, Hymenoptera, Lepidoptera, *Drosophila*, stress, insecticide, reproduction, longevity, dietary restriction, heat, radiation, gene expression, induction, and detoxification. For example, hormesis 'and' Hemiptera; hormesis 'and' gene expression; insect 'and' detoxification 'and' insecticide; insect 'and' stress 'and' gene expression; *Drosophila* 'and' radiation 'and' gene expression, and others. Searches were restricted to the years 2000-2020 (last searches: September/October 2020). I received just under 8000 hits in Web of Science. The majority of these were filtered out following scanning of titles. Eliminated papers included those involved in sequencing and functional characterization of genes or identifying gene mutations, general gene expression or identifying genes associated with stress resistance (ie: pesticide resistance), those not directly examining a response to a stressor or stimulus (ie: those examining suites of genes in insects), and those not directly studying insects. After initial filtering, I examined the abstracts or texts of a remaining 450 papers, and of these identified fifty-five (55) papers that fit my *a priori* search criteria, which limited papers to only those demonstrating a stimulatory response in insects that was also associated with a molecular or biochemical response to that same stressor. Those papers only showing deleterious effects or no stimulatory effects, those only showing phenotypic effects, or those only showing molecular effects in response to stress were excluded. Studies that observed stimulatory effects but used insect populations following long term selection to a stressor, or those that used mutated lines of insects were also not included in the database. The following information was recorded in the database: type of stressor; magnitude of stress or dose; time points of data collection; phenotypic or molecular endpoints measured;

insect species, life stage, and sex; magnitude of phenotypic stimulation; and associated molecular or biochemical response. The magnitudes of phenotypic, molecular, or biochemical stimulation were determined through direct statements within the text or extrapolated from tables and graphs. The amount of phenotypic stimulation was recorded as being <25%, 25-50%, 50-100%, or >100% increased above controls, and expression of molecular or biochemical responses were recorded as being <2-fold, 2-5-fold, 5-10-fold, 10-100-fold, or >100-fold increased or decreased.

4.3 Results

Stressors, phenotypic responses, molecular or biochemical responses, response magnitudes, insect species, and references from the 55 papers analyzed are summarized in Table 4.1. A range of stress types eliciting stimulatory responses were identified, including chemical and pesticide stress, oxidative stress, radiation, temperature stress, crowding, and dietary restriction. Stimulatory responses to these stressors were wide ranging but could be categorized into responses involved in reproduction, survival and longevity, size and development, chemical and oxidative stress tolerance, temperature tolerance, and starvation and desiccation tolerance. Stimulatory effects ranged from <25% increased above controls to >100% increased above controls (Figure 4.1). Effects related to reproduction and temperature tolerance were observed most frequently, and those related to size and development, and starvation and desiccation resistance were among the least observed (Figure 4.1). The magnitude of most responses was <25% or 25-50% increased above controls, but almost half of the instances of temperature tolerance were >100% increased above controls. For all other effects, only a small portion of responses were >100% (Figure 4.1).

Table 4.1: Summary of molecular/biochemical responses and associated phenotypic stimulation in insects following exposure to stress.

	Molecular/Biochemical Response			**Phenotypic Response**			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
Heat	*Vg-1**	0 / 1 / 0	n/a	Fecundity	<25%	Melon fly (*Bactrocera cucurbitae*)	(Zhou et al., 2020)
	*Vg-2**	0 / 1 / 0	n/a	Shortened pre-oviposition period	25-50%		
	*Vg-3**	1 / 0 / 0	n/a	Ovary maturation	25-50%		
	*Vg-R**	1 / 0 / 0	n/a				
	JH-inducible protein*	1 / 0 / 0	n/a				
	JH-epoxide hydrolase*	0 / 1 / 0	n/a				
Heat	*Hsp70**	1 / 0 / 0	n/a	Longevity	<25 %	Fruit fly (*Drosophila melanogaster*)	(Sarup et al., 2014)
Heat	*Hsp70*	1 / 0 / 1	<2 fold / --	Heat tolerance	<25-100%	Fruit fly (*Drosophila melanogaster*)	(Sorensen et al., 2007)
				Longevity	<25%		
Heat	*Hsp70*	1 / 0 / 0	<2 fold / --	Longevity	<25 %	Fruit fly (*Drosophila melanogaster*)	(Hercus et al., 2003)
				Heat tolerance	25-50%		
Cold	*Hsp20*	0 / 6 / 0	-- / <2 fold	Cold tolerance	>100%	Sweet potato whitefly (*Bemisia tabaci*)	(Wang et al., 2011)
	Hsp70	6 / 0 / 0	2-5 fold / --				
	Hsp90	0 / 0 / 6	-- / --				
Temperature fluctuation	*Hsp70*	4 / 0 / 0	2-10 fold / --	Heat tolerance	>100%	Springtail (*Folsomia candida*)	(Waagner et al., 2010)
Heat	*Hsp70*	3 / 0 / 3	5-100 fold / --	Heat tolerance	>100%	Springtail (*Orchesella cincta*)	(Bahrndorff et al., 2009)
Heat	*Hsp70*	2 / 0 / 0	2-5 fold / --	Heat tolerance	50-100%	Pea leafminer (*Liriomyza huidobrensis*)	(Huang et al., 2007)

	Molecular/Biochemical Response				Phenotypic Response		
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	Hsp20	2 / 0 / 0	2-5 fold / --				
Heat	*Hsp70*	2 / 0 / 0	<2 fold / --	Longevity	<25 %	Fruit fly (*Drosophila buzzatii*)	(Scannapieco et al., 2007)
Heat	*CAT*	5 / 0 / 3	<2-5 fold / --	Heat tolerance	>100%	Oriental armyworm (*Mythimna separata*)	(Matsumura et al., 2017)
	SOD1	4 / 0 / 4	2-5 fold / --				
	SOD2	0 / 0 / 8	-- / --				
	Hsp70	7 / 0 / 1	2-10 fold / --				
Heat	*Hsc70*	1 / 0 / 0	2-5 fold / --	Heat tolerance	>100%	Indian meal moth (*Plodia interpunctella*)	(Kim et al., 2017)
	Hsp90	1 / 0 / 0	2-5 fold / --				
	Piac25	0 / 0 / 1	-- / --				
Cold	*Hsp70*	5 / 0 / 0	<2 fold / --	Cold tolerance	<25 %	False codling moth (*Thaumatotibia leucotreta*)	(Boardman et al., 2015)
Cold	*Hsp90*	0 / 4 / 1	-- / <2 fold-100 fold	Cold tolerance	>100%	Migratory locust (*Locusta migratoria*)	(Cui et al., 2014)
	Hsp70	0 / 3 / 2	-- / 2-100 fold				
	Hsp40	0 / 3 / 2	-- / <2 -100 fold				
	Hsp20.5	0 / 3 / 2	-- / 5-100 fold				
	Hsp20.6	0 / 2 / 3	-- / 5-10 fold				
	Hsp20.7	0 / 2 / 3	-- / 5-10 fold				
Heat	*Hsp90*	1 / 0 / 1	2-5 fold / --	Heat tolerance	>100%	Tobacco cutworm (*Spodoptera litura*)	(Shen et al., 2014)
	Hsc90	2 / 0 / 0	<2-5 fold / --				
	Hsp70	2 / 0 / 0	<2-10 fold / --				
	Hsc70	1 / 0 / 1	<2 fold / --				
	EcRA	2 / 0 / 0	<2-5 fold / --				
	EcRB1	1 / 0 / 1	2-5 fold / --				
Cold	*Hsp90*	1 / 0 / 1	2-5 fold / --	Cold tolerance	>100%	Tobacco cutworm (*Spodoptera litura*)	(Shen et al., 2014)

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	Hsc90	1 / 0 / 1	2-5 fold / --				
	Hsp70	1 / 0 / 1	2-5 fold / --				
	Hsc70	1 / 0 / 1	2-5 fold / --				
	EcRA	1 / 0 / 1	<2 fold / --				
	EcRB1	2 / 0 / 0	2-5 fold / --				
Heat, humidity	*Hsp70*	3 / 0 / 0	<2-5 fold / --	Heat tolerance	<25-50%	Fruit fly (*Drosophila simulans*)	(Bubliy et al., 2013)
				Desiccation tolerance	25-50%		
Cold, humidity	*Hsp70*	0 / 0 / 1	-- / --	Heat tolerance	<25%	Fruit fly (*Drosophila simulans*)	(Bubliy et al., 2013)
Cold	*Hsc70–2*	0 / 1 / 0	-- / 2-5 fold	Cold tolerance	25->100%	Fruit fly (*Drosophila suzukii*)	(Enriquez and Colinet, 2019)
	Hsp60C	0 / 1 / 0	-- / <2 fold				
	Hsp27	0 / 1 / 0	-- / <2 fold				
	Hsp22	1 / 0 / 0	2-5 fold / --				
	*** see extensive gene expression analysis*						
Heat	*Hsp23*	2 / 0 / 0	2-100 fold / --	Heat tolerance	<25-100%	Oriental fruit fly (*Bactrocera dorsalis*)	(Gu et al., 2019b)
	Hsp70	1 / 0 / 1	2-5 fold / --				
	Hsp90	0 / 0 / 2	-- / --				
Heat	*Hsp23*	2 / 0 / 0	2-5 fold / --	Heat tolerance	50-100%	Fruit fly (*Bactrocera correcta*)	(Gu et al., 2019b)
	Hsp70	1 / 0 / 1	5-10 fold / --				
	Hsp90	0 / 0 / 2	-- / --				
Cold	*Hsp70*	1 / 0 / 2	n/a	Cold tolerance	>100%	Bed bug *(Cimex lectularius)*	(Benoit et al., 2009)
	Hsp90	1 / 0 / 2	n/a				
Imidacloprid	Vitellin	3 / 0 / 0	<2-5 fold / --	Hatch rate	<25%	Carmine spider mite (*Tetranychus cinnabarinus*)	(Zeng and Wang, 2010)

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
				Pre-imaginal survivorship	<25%		
Acetamiprid	*Vg*	2 / 0 / 0	2-5 fold / --	Fecundity	<25%	Melon aphid (*Aphis gossypii*)	(Ullah et al., 2019)
				Intrinsic rate of increase	<25%		
				Finite rate of increase	<25%		
				Net reproductive rate	<25%		
Chlorfenapyr	GST activity	1 / 0 / 1	<2 fold / --	Fecundity	0-50%	Chinese chive maggot (*Bradysia odoriphaga*)	(Zhao et al., 2018)
	CarE activity	2 / 0 / 0	<2 fold / --	Intrinsic rate of increase	<25%		
	0-demethylation	1 / 0 / 1	<2 fold / --	Net reproductive rate	25-50%		
				Finite rate of increase	<25%		
				Gross reproductive rate	25-50%		
Chlorpyrifos	GST activity	1 / 0 / 0	<2 fold / --	Fecundity	<25%	Diamondback moth (*Plutells zylostella*)	(Deng et al., 2016)
	AChE activity	2 / 0 / 0	<2 fold / --	Development	<25%		
Deltamethrin, 2-4 D, Carbendazim	CarE activity	2 / 0 / 0	<2 fold / --	Fecundity	>100%	Brown planthopper (*Nilaparvata lugens*)	(Nanthakumar et al., 2012)
				Honeydue production	>100%		
Gossypol	*CYP9A15*	0 / 1 / 0	n/a	Larval weight gain	<25%	Cotton bollworm (*Helicoverpa armigera*)	(Celorio-Mancera et al., 2011)
	CYP6AB9	0 / 1 / 0	n/a				
	CYP6AB10	0 / 1 / 0	n/a				
	CYP6AE11	0 / 1 / 0	n/a				
	CYP6AE12	0 / 1 / 0	n/a				

	Molecular/Biochemical Response				Phenotypic Response		
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	UGT40D1	0 / 1 / 0	n/a				
	UGT33T1	0 / 1 / 0	n/a				
	UGT41B1	0 / 1 / 0	n/a				
	GST8	1 / 0 / 0	n/a				
	GST24	1 / 0 / 0	n/a				
	GST2	0 / 1 / 0	n/a				
	CEx011	0 / 1 / 0	n/a				
	CEx005	0 / 1 / 0	n/a				
	CCE001h	0 / 1 / 0	n/a				
	CCE015a	0 / 1 / 0	n/a				
	CEx011d	0 / 1 / 0	n/a				
	CEx011a	0 / 1 / 0	n/a				
	CCE016d	0 / 1 / 0	n/a				
Imidacloprid	*JH III*	3 / 0 / 0	<2 fold / --	Fecundity	25-50%	Green peach aphid (*Myzus persicae*)	(Yu et al., 2010)
Imidacloprid	SOD activity	0 / 2 / 0	-- / 2-5 fold	Longevity	25-50%	Bird cherry-oat aphid (*Rhopalosiphum padi*)	(Li et al., 2018)
	CAT activity	2 / 0 / 0	2-10 fold / --	Oviposition period	<25%		
	AChE activity	1 / 0 / 1	2-5 fold / --				
	P450 activity	0 / 0 / 2	-- / --				
	POD activity	0 / 0 / 2	-- / --				
λ-cyhalothrin	*Vg*	2 / 2 / 1	2-10 fold / <2-5 fold	Fecundity	25-50%	Oriental armyworm (*Mythimna separata*)	(Li et al., 2019)
	VgR	2 / 1 / 2	2-5 fold / 2-5 fold	Net reproductive rate	>100%		
				Intrinsic rate of population increase	<25%		
Imidacloprid	*CYP6CY3*	1 / 0 / 2	2-5 fold / --	Fecundity	50-100%	Green peach aphid (*Myzus persicae*)	(Rix et al., 2016)
	E4	2 / 0 / 1	<2-5 fold / --	Intrinsic rate of population increase	25-50%		
	Hsp60	1 / 1 / 1	2-5 fold / 2-5 fold	Food/water stress tolerance	<25%		

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
Imidacloprid	*Hsp60*	0 / 6 / 2	-- / <2-5 fold	Fecundity	50->100%	Green peach aphid (*Myzus persicae*)	(Ayyanath et al., 2013; Ayyanath et al., 2014)
	FPPS1	0 / 6 / 2	-- / <2-5 fold				
	OSD	4 / 4 / 0	<2-5 fold / <2-5 fold				
	TOL	6 / 2 / 0	<2-5 fold / <2 fold				
	ANT	3 / 3 / 2	<2-5 fold / <2-5 fold				
Precocene	*Hsp60*	1 / 4 / 1	2-5 fold / <2-10 fold	Fecundity	50-100%	Green peach aphid (*Myzus persicae*)	(Ayyanath et al., 2015)
	FPPS1	0 / 3 / 3	-- / <2-10 fold				
	OSD	3 / 2 / 1	2-5 fold / 5-100 fold				
	TOL	0 / 1 / 5	-- / 10-100 fold				
	ANT	1 / 0 / 5	2-5 fold / --				
	JHIII	0 / 0 / 2	-- / --				
Deltamethrin	*E4/FE4*	0 / 2 / 0	-- / <2 fold	Longevity	<25%	Green peach aphid (*Myzus persicae*)	(Sial et al., 2018)
λ-cyhalothrin	*E4/FE4*	1 / 0 / 1	2-5 fold / --	Fecundity	<25%	Green peach aphid (*Myzus persicae*)	(Sial et al., 2018)
Imidacloprid	*CYP6CY3*	1 / 0 / 1	2-5 fold / --	Fecundity	<25%	Green peach aphid (*Myzus persicae*)	(Sial et al., 2018)
				Gross reproductive rate	<25%		
				Longevity	<25%		
Acetamiprid	*CYP6CY3*	1 / 0 / 1	<2 fold / --	Fecundity	<25%	Green peach aphid (*Myzus persicae*)	(Sial et al., 2018)
				Gross reproductive rate	<25%		
				Longevity	<25%		
Rutin	*Tsc1*	2 / 0 / 0	<2-5 fold / --	Longevity	0-50%	Fruit fly (*Drosophila melanogaster*)	(Chattopadhyay et al., 2017)

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	Tsc2	2 / 0 / 0	<2-5 fold / --	Larval development	0-50%		
	FOXO	1 / 0 / 1	2-5 fold / --	Heat tolerance	25-50%		
	TOR	0 / 2 / 0	-- / 2-5 fold	Cold tolerance	<25-50%		
	Atg1	2 / 0 / 0	2-5 fold / --	Starvation tolerance	25-50%		
	Atg5	2 / 0 / 0	2-5 fold / --	Oxidative stress tolerance	<25 %		
	Atg7	2 / 0 / 0	2-5 fold / --				
	Sir2	0 / 0 / 2	-- / --				
	MnSOD	1 / 0 / 1	<2 fold / --				
	CAT	2 / 0 / 0	<2 fold / --				
	Thor	2 / 0 / 0	2-5 fold / --				
Imidacloprid	*JHAMT*	1 / 0 / 0	2-5 fold / --	Fecundity	50-100%	Plant bug (*Cyrtorhinus lividipennis*)	(Zhu et al., 2020)
	JHE	1 / 0 / 0	2-5 fold / --				
	JH titre	2 / 0 / 0	<2 fold / --				
Imidacloprid	*Vg*	1 / 0 / 0	<2 fold / --	Fecundity	50-100%	Plant bug (*Cyrtorhinus lividipennis*)	(Lu et al., 2017b)
	SPATA13	1 / 0 / 0	<2 fold / --	Population growth	50-100%		
Triazophos	*Vg*	1 / 0 / 0	<2 fold / --	Fecundity	50-100%	Plant bug (*Cyrtorhinus lividipennis*)	(Lu et al., 2017b)
	SPATA13	1 / 0 / 0	<2 fold / --	Population growth	50-100%		
Deltamethrin	*Vg*	1 / 0 / 0	<2 fold / --	Fecundity	50-100%	Plant bug (*Cyrtorhinus lividipennis*)	(Lu et al., 2017b)
	SPATA13	1 / 0 / 0	<2 fold / --	Population growth	50-100%		
Triazophos	*Hsp70*	1 / 0 / 0	2-5 fold / --	Heat tolerance	50-100%	Brown planthopper (*Nilaparvata lugens*)	(Ge et al., 2013)
	Argk	1 / 0 / 0	2-5 fold / --				

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
Dichloroacetic acid	GST activity	1 / 0 / 2	<2 fold / --	Longevity	<25%	Fruit fly (*Drosophila melanogaster*)	(Pandey et al., 2014)
	GSH activity	0 / 1 / 2	-- / <2 fold	Survival	<25%		
	CAT activity	1 / 0 / 2	<2 fold / --				
	SOD activity	1 / 0 / 2	<2 fold / --				
	Hsp22	2 / 0 / 1	2-5 fold / --				
	Hsp27	2 / 0 / 1	2-5 fold / --				
	Hsp70	2 / 0 / 1	2-5 fold / --				
	Caspase activity	0 / 0 / 3	-- / --				
	Hsp23	0 / 0 / 3	-- / --				
	Hsp26	0 / 0 / 3	-- / --				
	Hsp27	0 / 0 / 3	-- / --				
	Hsp60	0 / 0 / 3	-- / --				
	Hsp83	0 / 0 / 3	-- / --				
Glyphosate	*CYP6N11*	1 / 0 / 0	<2 fold / --	Imidacloprid tolerance	50-100%	Yellow fever mosquito (*Aedes aegypti*)	(Riaz et al., 2009)
	CYP6N12	1 / 0 / 0	<2 fold / --	Permethrin tolerance	50-100%		
	CYP6Z6	1 / 0 / 0	<2 fold / --	Propoxur tolerance	<25%		
	CYP6AG7	1 / 0 / 0	<2 fold / --				
	CYP325AA1	1 / 0 / 0	2-5 fold / --				
	GSTe4	1 / 0 / 0	<2 fold / --				
	GSTe7	1 / 0 / 0	<2 fold / --				
	GSTi1	1 / 0 / 0	2-5 fold / --				
	GPx	1 / 0 / 0	<2 fold / --				
	EST activity	1 / 0 / 0	<2 fold / --				
	GST Activity	0 / 0 / 1	-- / --				
	P450 activity	0 / 0 / 1	-- / --				
	CYP4G36	0 / 0 / 1	-- / --				
	CYP6Z8	0 / 0 / 1	-- / --				
	CYP6CC1	0 / 0 / 1	-- / --				
	CYP9M5	0 / 0 / 1	-- / --				
	GSTs1-2	0 / 0 / 1	-- / --				
	CCEae1o	0 / 0 / 1	-- / --				
	CCEae2o	0 / 0 / 1	-- / --				
	CCEae3o	0 / 0 / 1	-- / --				
	SOD	0 / 0 / 1	-- / --				

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	PXDN	0 / 0 / 1	-- / --				
	POD	0 / 0 / 1	-- / --				
	TPx2	0 / 0 / 1	-- / --				
	AKR	0 / 0 / 1	-- / --				
Benzo[a]pyrene	*CYP6Z6*	1 / 0 / 0	2-5 fold / --	Imidacloprid tolerance	>100%	Yellow fever mosquito (*Aedes aegypti*)	(Riaz et al., 2009)
	CYP6Z8	1 / 0 / 0	2-5 fold / --	Permethrin tolerance	50-100%		
	CYP9M5	1 / 0 / 0	2-5 fold / --	Propoxur tolerance	25-50%		
	P450 activity	1 / 0 / 0	2-5 fold / --				
	GST activity	1 / 0 / 0	<2 fold / --				
	GSTi1	1 / 0 / 0	2-5 fold / --				
	GSTs1-2	1 / 0 / 0	<2 fold / --				
	SOD	1 / 0 / 0	<2 fold / --				
	AKR	1 / 0 / 0	2-5 fold / --				
	EST activity	0 / 0 / 1	-- / --				
	CYP4G36	0 / 0 / 1	-- / --				
	CYP6N11	0 / 0 / 1	-- / --				
	CYP6N12	0 / 0 / 1	-- / --				
	CYP6AG7	0 / 0 / 1	-- / --				
	CYP6CC1	0 / 0 / 1	-- / --				
	CYP325AA1	0 / 0 / 1	-- / --				
	GSTe4	0 / 0 / 1	-- / --				
	GSTe7	0 / 0 / 1	-- / --				
	CCEae1o	0 / 0 / 1	-- / --				
	CCEae2o	0 / 0 / 1	-- / --				
	CCEae3o	0 / 0 / 1	-- / --				
	GPx	0 / 0 / 1	-- / --				
	PXDN	0 / 0 / 1	-- / --				
	POD	0 / 0 / 1	-- / --				
	TPx2	0 / 0 / 1	-- / --				
Copper	*CYP6B50*	2 / 0 / 0	2-10 fold / --	Xanthotoxin tolerance	25-100%	Tobacco cutworm (*Spodoptera litura*)	(Lu et al., 2019)
	SOD activity	2 / 0 / 0	<2 fold / --				
	POD activity	2 / 0 / 0	<2 fold / --				
	CAT activity	0 / 0 / 2	-- / --				
	CYP6B47	0 / 0 / 2	-- / --				

	Molecular/Biochemical Response				Phenotypic Response		
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	CYP6B48	0 / 0 / 2	-- / --				
	CYP6B58	0 / 0 / 2	-- / --				
Permethrin	*CYP9M9*	1 / 0 / 0	2-5 fold / --	Permethrin tolerance	<25%	Yellow fever mosquito (*Aedes aegypti*)	(Poupardin et al., 2008)
	CYP9M8	1 / 0 / 0	<2 fold / --	Temephos tolerance	<25%		
	CYP314A1	1 / 0 / 0	2-5 fold / --				
	P450 activity	1 / 0 / 0	<2 fold / --				
	GST activity	0 / 0 / 1	-- / --				
	EST activity	0 / 0 / 1	-- / --				
	CYP6M11	0 / 0 / 1	-- / --				
	CYP6Z8	0 / 0 / 1	-- / --				
	CYP6Z9	0 / 0 / 1	-- / --				
	CYP6M6	0 / 0 / 1	-- / --				
	CYP6AL1	0 / 0 / 1	-- / --				
	CYP9J15	0 / 0 / 1	-- / --				
	CYP6N12	0 / 0 / 1	-- / --				
	CYP12F8	0 / 0 / 1	-- / --				
	CCEjhe1F	0 / 0 / 1	-- / --				
Atrazine	*CYP9M9*	1 / 0 / 0	<2 fold / --	Permethrin tolerance	<25%	Yellow fever mosquito (*Aedes aegypti*)	(Poupardin et al., 2008)
	CYP9M8	1 / 0 / 0	2-5 fold / --	Temephos tolerance	<25%		
	CYP6AL1	1 / 0 / 0	<2 fold / --				
	CCEjhe1F	1 / 0 / 0	2-5 fold / --				
	GST activity	0 / 0 / 1	-- / --				
	P450 activity	0 / 0 / 1	-- / --				
	EST activity	0 / 0 / 1	-- / --				
	CYP6M11	0 / 0 / 1	-- / --				
	CYP6Z8	0 / 0 / 1	-- / --				
	CYP6Z9	0 / 0 / 1	-- / --				
	CYP6M6	0 / 0 / 1	-- / --				
	CYP9J15	0 / 0 / 1	-- / --				
	CYP6N12	0 / 0 / 1	-- / --				
	CYP12F8	0 / 0 / 1	-- / --				
	CYP314A1	0 / 0 / 1	-- / --				

	Molecular/Biochemical Response				Phenotypic Response		
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
Fluoranthene	*CYP6Z8*	1 / 0 / 0	<2 fold / --	Permethrin tolerance	25-50%	Yellow fever mosquito (*Aedes aegypti*)	(Poupardin et al., 2008)
	CYP6Z9	1 / 0 / 0	<2 fold / --	Temephos tolerance	<25%		
	CYP6M6	1 / 0 / 0	<2 fold / --				
	CYP6N12	1 / 0 / 0	2-5 fold / --				
	CYP12F8	1 / 0 / 0	<2 fold / --				
	P450 activity	1 / 0 / 0	<2 fold / --				
	GST activity	1 / 0 / 0	<2 fold / --				
	EST activity	0 / 0 / 1	-- / --				
	CYP9M9	0 / 0 / 1	-- / --				
	CYP9M8	0 / 0 / 1	-- / --				
	CYP6M11	0 / 0 / 1	-- / --				
	CYP314A1	0 / 0 / 1	-- / --				
	CYP6AL1	0 / 0 / 1	-- / --				
	CYP9J15	0 / 0 / 1	-- / --				
	CCEjhe1F	0 / 0 / 1	-- / --				
Copper	*CYP6M11*	1 / 0 / 0	2-5 fold / --	Permethrin tolerance	<25%	Yellow fever mosquito (*Aedes aegypti*)	(Poupardin et al., 2008)
	CYP9J15	1 / 0 / 0	<2 fold / --	Temephos tolerance	<25%		
	GST activity	0 / 1 / 0	-- / <2 fold				
	P450 activity	0 / 0 / 1	-- / --				
	EST activity	0 / 0 / 1	-- / --				
	CYP9M9	0 / 0 / 1	-- / --				
	CYP9M8	0 / 0 / 1	-- / --				
	CYP6Z8	0 / 0 / 1	-- / --				
	CYP6Z9	0 / 0 / 1	-- / --				
	CYP6M6	0 / 0 / 1	-- / --				
	CYP314A1	0 / 0 / 1	-- / --				
	CYP6AL1	0 / 0 / 1	-- / --				
	CYP6N12	0 / 0 / 1	-- / --				
	CYP12F8	0 / 0 / 1	-- / --				
	CCEjhe1F	0 / 0 / 1	-- / --				
Thiacloprid	*CYP315A1*	1 / 0 / 3	<2 fold / --	Thiacloprid tolerance	>100%	European honey bee (*Apis mellifera*)	(Alptekin et al., 2016)

	Molecular/Biochemical Response				Phenotypic Response		
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	cytb5	1 / 0 / 3	2-5 fold / --				
	CYP9Q1	0 / 1 / 3	-- / <2 fold				
	CCE11	0 / 1 / 3	-- / <2 fold				
Quercetin	*CYP6B6*	1 / 0 / 0	2-5 fold / --	λ-cyhalothrin tolerance	>100%	Cotton bollworm (*Helicoverpa armigera*)	(Chen et al., 2018a)
	CYP6B8	1 / 0 / 0	5-10 fold / --				
	CYP321A1	1 / 0 / 0	10-100 fold / --				
	O-deethylase activity	1 / 0 / 0	<2 fold / --				
Boric acid	GST activity	1 / 0 / 3	2-5 fold / --	Longevity	25-50%	Greater wax moth (*Galleria mellonella*)	(Hyrsl et al., 2007)
	GPx activity	2 / 1 / 1	<2-5 fold / <2 fold				
	SOD activity	3 / 0 / 1	<2-5 fold / --				
	CAT activity	2 / 2 / 0	<2-5 fold / 2-5 fold				
Thiamethoxam	*CYP6CZ1*	1 / 0 / 0	2-5 fold / --	Fecundity	<25%	Melon aphid (*Aphis gossypii*)	(Ullah et al., 2020)
	CYP6DD1	1 / 0 / 0	2-5 fold / --	Longevity	<25%		
	CYP6CY9	1 / 0 / 0	2-5 fold / --				
	CYP6CY14	1 / 0 / 0	5-10 fold / --				
	CYP6DC1	1 / 0 / 0	2-5 fold / --				
	Vg	1 / 0 / 0	2-5 fold / --				
	EcR	1 / 0 / 0	2-5 fold / --				
	CYP6CY5	0 / 0 / 1	-- / --				
Trifluralin	*CYP6B48*	4 / 0 / 0	10-100 fold	λ-cyhalothrin tolerance	50-100%	Tobacco cutworm (*Spodoptera litura*)	(Liu et al., 2019a)
	CYP9A40	0 / 2 / 4	-- / <2 fold	Bifenthrin tolerance	50-100%		
	CYP321B1	2 / 0 / 4	5-10 fold / --	Phoxim tolerance	50-100%		
	CarE	3 / 0 / 3	<2-5 fold / --				
	Ace1	3 / 0 / 3	2-5 fold / --				
	Ace2	2 / 0 / 4	2-5 fold / --				
	GSTe2	2 / 0 / 4	2-5 fold / --				
	GSTe3	2 / 0 / 4	2-5 fold / --				

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
MCPA-Na	*CYP6B48*	4 / 0 / 2	5-100 fold / --	λ-cyhalothrin tolerance	50-100%	Tobacco cutworm (*Spodoptera litura*)	(Liu et al., 2019a)
	CYP9A40	0 / 2 / 5	-- / <2 fold	Bifenthrin tolerance	50-100%		
	CYP321B1	1 / 0 / 5	5-10 fold / --	Phoxim tolerance	50-100%		
	CarE	3/ 0 / 3	2-5 fold / --				
	Ace1	3 / 0 / 3	2-5 fold / --				
	Ace2	1 / 0 / 5	2-5 fold / --				
	GSTe2	2 / 0 / 4	2-5 fold / --				
	GSTe3	2 / 0 / 4	2-5 fold / --				
Anoxia, irradiation	CuSOD activity	2 / 0 / 2	<2 fold / --	Adult emergence	0-100%	Caribbean fruit fly (*Anastrepha suspensa*)	(Lopez-Martinez and Hahn, 2012)
	MnSOD activity	3 / 0 / 1	2-10 Fold / --	Radation tolerance	<25%		
	GPx activity	4 / 0 / 0	<2 fold / --	Mating performance	>100%		
				Increased female sex ratio	<25%		
Hyperbaric normoxia	*GS*	1/ 0 / 1	<2 fold / --	Longevity	<25%	Fruit fly (*Drosophila melanogaster*)	(Yu et al., 2016)
	CAT	2 / 0 / 0	<2-5 fold / --	Heat tolerance	<25%		
	MnSOD	2 / 0 / 0	2-5 fold / --	Starvation tolerance	<25-50%		
	Jafrac I	2 / 0 / 0	2-5 fold / --	Oxidative stress tolerance	<25%		
	Hsp70	1 / 0 / 1	<2 fold / --				
	mth	0 / 0 / 1	-- / --				
	Sir2	0 / 0 / 1	-- / --				
	4E-BP	0 / 0 / 1	-- / --				
	FOXO	0 / 0 / 1	-- / --				
	Tor	0 / 0 / 1	-- / --				
	Hsp22	0 / 0 / 1	-- / --				
	Cht6	2 / 0 / 0	2-5 fold / --				
	Cht7	2 / 0 / 0	<2 fold / --				
	kkv	2 / 0 / 0	<2 fold / --				

	Molecular/Biochemical Response				Phenotypic Response		
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
Radiation	*Hsp70Aa*	0 / 1 / 9	-- / 2-5 fold	longevity	<25 %	Fruit fly (*Drosophila melanogaster*)	(Zhikrevetskaya et al., 2015)
	CYP4E2	1 / 0 / 9	2-5 fold / --				
	CYP6A20	0 / 1 / 9	-- / <2 fold				
	Clk	0 / 0 / 10	-- / --				
	Fer3	0 / 0 / 10	-- / --				
	FOXO	0 / 0 / 10	-- / --				
	GSTe3	0 / 0 / 10	-- / --				
	Hpo	0 / 0 / 10	-- / --				
	Hus 1-like	0 / 0 / 10	-- / --				
	DJNK	0 / 0 / 10	-- / --				
	Keap1	0 / 0 / 10	-- / --				
	mei-9	0 / 0 / 10	-- / --				
	mei-41	0 / 0 / 10	-- / --				
	PCNA	0 / 0 / 10	-- / --				
	mus 309	0 / 0 / 10	-- / --				
	p53	0 / 0 / 10	-- / --				
	per	0 / 0 / 10	-- / --				
	RAD54	0 / 0 / 10	-- / --				
	SOD	0 / 0 / 10	-- / --				
	spn-B	0 / 1 / 9	-- / 5-10 fold				
	tefu	0 / 0 / 10	-- / --				
	wrinkled	0 / 0 / 10	-- / --				
	Brca2	0 / 0 / 10	-- / --				
Hypoxia	*Hsp70*	0 / 5 / 0	-- / <2 fold	Cold tolerance	<25 %	False codling moth (*Thaumatotibia leucotreta*)	(Boardman et al., 2015)
Moist hypoxia	*Hsp70*	0 / 5 / 0	-- / <2 fold	Cold tolerance	<25 %	False codling moth (*Thaumatotibia leucotreta*)	(Boardman et al., 2015)
Hypoxia	*Hsp90*	1 / 4 / 0	<2 fold / 5-100 fold	Cold tolerance	>100%	Migratory locust (*Locusta migratoria*)	(Cui et al., 2014)
	Hsp70	1 / 3 / 1	<2 fold / <2 fold-100 fold				
	Hsp40	0 / 4 / 1	-- / <2 -100 fold				

	Molecular/Biochemical Response				Phenotypic Response		
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
	Hsp20.5	1 / 3 / 1	2-5 fold / <2-100 fold				
	Hsp20.6	0 / 3 / 2	-- / <2 -10 fold				
	Hsp20.7	1 / 2 / 2	2-5 fold / 5-100 fold				
CO2 narcosis	*GSTD*	0 / 3 / 0	-- / <2-5 fold	Oocyte size	25-50%	Bumble bee (*Bombus impatiens*)	(Amsalem and Grozinger, 2017)
	PRX1	0 / 3 / 0	-- / <2-5 fold				
	TPX1	0 / 2 / 1	-- / 2-5 fold				
	PHGP	0 / 2 / 1	-- / 2-5 fold				
	SOD1	0 / 2 / 1	-- / 2-5 fold				
	GSTs	0 / 0 / 3	-- / --				
Crowding	*InR*	1 / 0 / 1	2-5 fold / --	Longevity	25-50%	Fruit fly (*Drosophila melanogaster*)	(Lushchak et al., 2019)
	Sir2	1 / 0 / 1	2-5 fold / --				
	TOR	1 / 0 / 1	2-5 fold / --				
	FOXO	1 / 0 / 1	2-5 fold / --				
	Hsp70	1 / 0 / 1	2-5 fold / --				
Crowding	*CAT*	1 / 0 / 0	<2 fold / --	Heat tolerance	25-50%	Fruit fly (*Drosophila melanogaster*)	(Youn et al., 2018)
	SOD	0 / 0 / 1	-- / --	Cold tolerance	>100%		
	GS	0 / 0 / 1	-- / --				
	Hsp67	0 / 0 / 1	-- / --				
	Hsp70	0 / 0 / 1	-- / --				
	Hsp83	0 / 0 / 1	-- / --				
	Hsp22	0 / 0 / 1	-- / --				
	Hsc70	1 / 0 / 0	<2 fold / --				
	Hsp40	1 / 0 / 0	<2 fold / --				
	Hsp23	0 / 1 / 0	-- / <2 fold				
	Hsp27	0 / 1 / 0	-- / <2 fold				
	Hsp26	0 / 1 / 0	-- / <2 fold				
	stv	1 / 0 / 0	<2 fold / --				
	Hsp60	0 / 1 / 0	-- / <2 fold				
	Hsp68	0 / 1 / 0	-- / <2 fold				

	Molecular/Biochemical Response			Phenotypic Response			
Stressor	**Gene/Protein**	**Frequency (Up / Down / No Change)**	**Fold Change Range (Up / Down)**	**Response**	**% Increase Range**	**Insect**	**Reference**
Dietary restriction	*Vg-1*	0 / 1 / 0	-- / 2-5 fold	Fertility	25-50%	Oriental fruit fly (*Bactrocera dorsalis*)	(Chen et al., 2017b)
	Vg-2	0 / 1 / 0	-- / 2-5 fold	Longevity	50-100%		
	Lsp-2	0 / 1 / 0	-- / 10-100 fold	Starvation tolerance	>100%		
				Desiccation tolerance	25-50%		
				Heat resistance	<25%		
Crowding	*Hsp70*	1 / 0 / 2	n/a	Longevity	25-50%	Fruit fly (*Drosophila melanogaster*)	(Sorensen and Loeschcke, 2001)
				Heat tolerance	50-100%		
Dietary restriction	CAT activity	0 / 1 / 0	-- / <2 fold	Starvation tolerance	>100%	Oriental fruit fly (*Bactrocera dorsalis*)	(Chen et al., 2017a)
	SOD activity	0 / 1 / 0	-- / <2 fold	Desiccation tolerance	<25%		
	GST activity	0 / 1 / 0	-- / 5-10 fold				
	POD activity	0 / 1 / 0	-- / 2-5 fold				
	Vg-1	0 / 1 / 0	-- / >100 fold				
	Vg-2	0 / 1 / 0	-- / >100 fold				
Starvation	JH titre	2 / 0 / 0	<2-fold-5 fold	Starvation tolerance	<25%	European honey bee (*Apis mellifera*)	(Wang et al., 2016b)
	TRY1	0 / 1 / 1	-- / <2 fold				
	ILP1	0 / 0 / 1	-- / --				
	ILP2	0 / 0 / 1	-- / --				
	InR1	0 / 0 / 1	-- / --				
	InR2	0 / 0 / 1	-- / --				
	AKH	0 / 0 / 1	-- / --				
	AKHR	0 / 0 / 1	-- / --				
	Vg	0 / 0 / 1	-- / --				

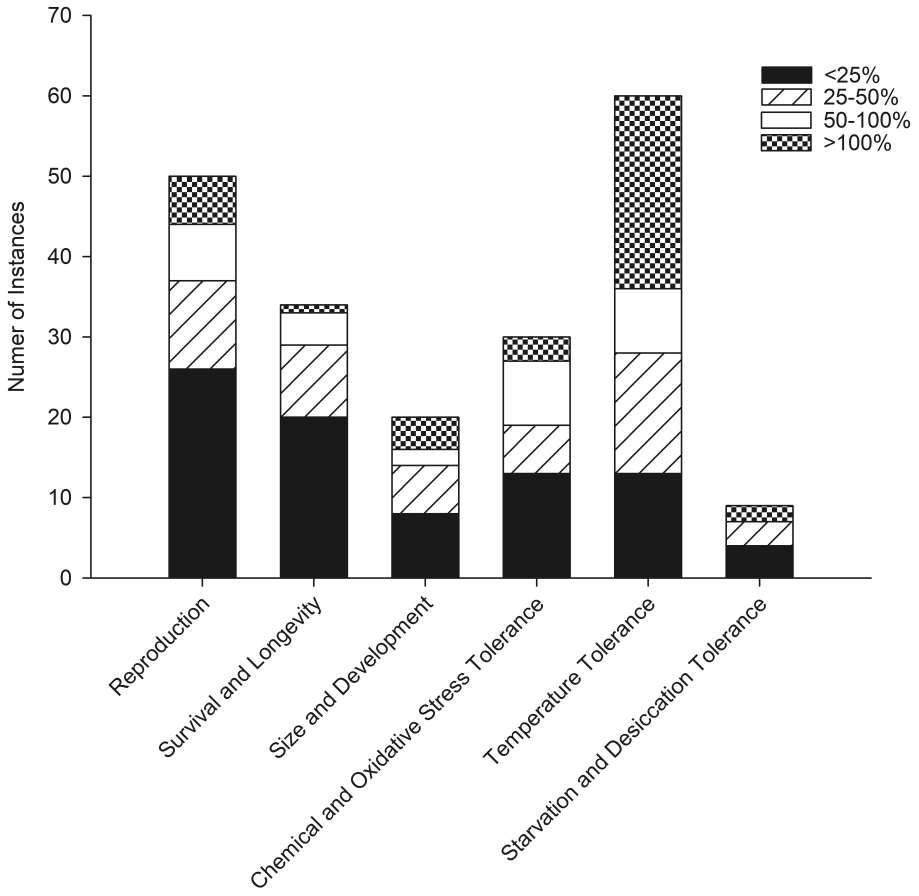

Figure 4.1: Summary of frequencies and magnitudes of stimulated phenotypic effects observed following exposure to various stressors. Response magnitudes are expressed as percentage increases above controls.

Molecular and/or biochemical responses could be broadly grouped into those related to detoxification, including cytochrome P450, glutathione-s-transferase, and esterase gene expression and activity; antioxidation, including catalase, superoxide dismutase, and peroxidase gene expression and activity; chaperones, which included heat shock protein gene expression; growth, development, and reproduction, including vitellin and vitellogenin gene expression and regulation, juvenile hormone gene expression and regulation, and ecdysone gene expression and regulation; and, genes involved in insulin/insulin-like and target of rapamycin signalling (IIS/TOR) (Figure 4.2a-d). Except

in the case of heat shock protein gene expression, where the frequency of up vs. down regulation was relatively similar, gene expression or enzyme activity mostly increased when accompanying phenotypic hormetic stimulation. The vast majority of molecular and biochemical responses, whether increasing or decreasing relative to controls, were <2-fold or 5-10-fold. Cytochrome P450s and heat shock proteins were the only genes where 10-100-fold changes in expression were observed (Figure 4.2a-d). During phenotypic hormetic responses, increased expression of these genes was most often reported, with greater than 50 instances of cytochrome P450s being upregulated, and over 70 instances of heat shock proteins being upregulated, although there were also 50 instances of heat shock protein down-regulation (Figure 4.2a,c). Only vitellogenin genes had levels of expression that differed from controls by more than 100-fold during hormesis in insects, where >100-fold down-regulation was observed (Figure 4.2d).

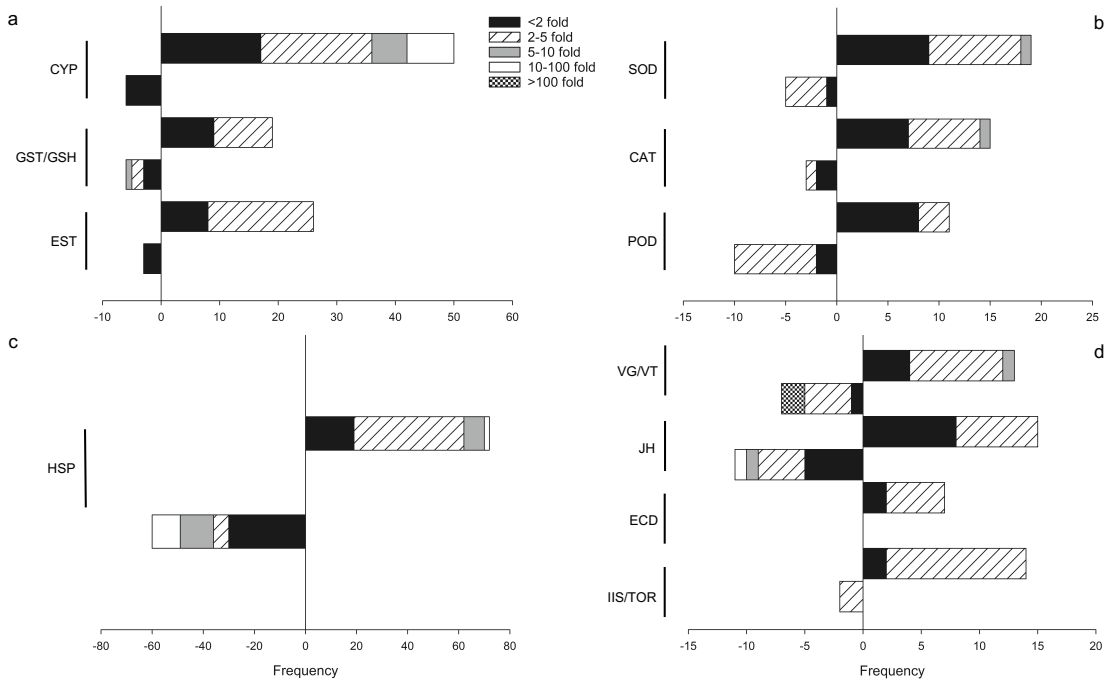

Figure 4.2: Frequencies of increases and decreases in the main molecular and/or biochemical responses associated with phenotypic stimulation following stress. These were grouped into those related to a) detoxification, including cytochrome P450 (CYP), glutathione-s-transferase (GST), and esterase (EST) gene expression and activity; b) antioxidation, including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) gene expression and activity; c) chaperones, which included heat shock protein (HSP) gene expression; d) growth, development and reproduction, including vitellin (VT) and vitellogenin (VG) gene expression and regulation, juvenile hormone (JH) gene expression and regulation, and ecdysone (ECD) gene expression and regulation, and genes involved in insulin/insulin-like and target of rapamycin signalling (IIS/TOR).

4.4 Discussion

Hormetic responses are phenotypic adaptations to mild amounts of stress that challenge homeostasis (Calabrese et al., 2010). The modest nature of hormetic stimulation is important as efficient resource allocation would only result in moderate stimulatory responses (Calabrese, 2013a). Maximum hormetic responses are shown to be on average 30-60% increased above controls, and 80% of maximum responses to be less than twice the control (Calabrese and Blain, 2005; Calabrese and Blain, 2011; Calabrese, 2013a). My analysis showed that hormetic responses in insects are usually 25-50% increased

above controls, but for reproduction and longevity half of responses were <25% increased above controls. It is possible some studies with a limited range of treatments did not capture the entire hormetic dose-response, and thus 'missed' the maximum stimulatory response, although traits that are energetically costly, like reproduction, require high amounts of resources and may have limited response magnitudes (Calabrese and Mattson, 2011). Breakdown in the regulatory mechanisms involved in resource allocation that may naturally occur with aging may in some instances account for greater amounts of hormetic stimulation (Calabrese, 2008b). Several studies showing stimulatory responses >100% increased above controls involved exposure of immature insects to mild levels of chemical (Riaz et al., 2009), anoxia and radiation (Lopez-Martinez and Hahn, 2012), or heat stress (Sorensen et al., 2007).

Stimulatory effects generally occur at doses or levels of stress below the NOAEL (Calabrese, 2013a), but stimulatory effects in response to chemical stress in insects can be found in dose ranges outside the NOAEL (Cutler, 2013), For example, longevity or fecundity were stimulated in *M. persicae* and the plant bug, *Cyrtorhinus lividipennis* Reuter, exposed at LD_{20} doses of insecticide (Lu et al., 2017b; Sial et al., 2018; Zhu et al., 2020). Further study is needed to determine the factors and mechanisms that govern some of the variability in levels of stress that elicit hormesis in insects.

4.4.1 Molecular and Biochemical Responses Associated with Hormesis

Hormetic responses may have an obvious direct relationship to the stressor, such as heat stress conditioning an organism to withstand higher levels of heat stress. However, a range of stressor types may elicit similar effects on the same physiological or life-history traits (Costantini et al., 2010). For example, chemical and heat stress can stimulate

longevity and fecundity (Sial et al., 2018; Zhou et al., 2020). A single stressor may also stimulate multiple physiological or life-history traits at the same time or impair some traits while stimulating others. In the following sections, I summarize the most common molecular responses from my analysis that coincided with hormesis in insects, focusing on the functional aspects of these responses and their connections to the stressors and stimulatory effects.

Heat shock proteins

Stress induced changes in the expression of heat shock proteins corresponding to increased stress tolerance or longevity were most frequently reported. This mainly occurred following temperature stress, but also following crowding, hypoxia, and insecticide exposure in some instances. The primary role of heat shock proteins is maintaining protein homeostasis by preventing and repairing protein misfolding and aggregation following stress, as well as mediating protein autophagy (Akerfelt et al., 2010; Tower, 2011). The latter is crucial for stress resistance and longevity, and is largely mediated by Hsp70, Hsc70, Hsp90, and Hsp40 proteins (Tower, 2011). Differential expression of more than one of the genes encoding these proteins can occur (Benoit et al., 2009; Cui et al., 2014; Shen et al., 2014; Kim et al., 2017; Youn et al., 2018). Increased *Hsp70* expression was often associated with increased longevity and temperature stress tolerance in my analysis and has been well studied for its role in aging and stress resistance in *Drosophila* (Feder et al., 1996; Khazaeli et al., 1997; Tatar et al., 1997; Bettencourt et al., 2008). Although the role of Hsp70 in hormetic stimulation of longevity and stress tolerance has been questioned given *Hsp70* gene expression may return to baseline levels shortly after stress (Sorensen et al., 2003; Sarup et al., 2014), *Hsp70*

expression can be impacted long after an initial stress (Sorensen et al., 2007). A number of studies showing increased lifespan or stress tolerance reported increased levels of *Hsp70* expression later in life or after cessation of the mild stress (Hercus et al., 2003; Sorensen et al., 2007; Ge et al., 2013; Sarup et al., 2014; Lushchak et al., 2019). Early life exposures and induction of repair processes may play a key role in these responses. Accumulation of damaged and denatured proteins following early life stress could result in stronger induction of *Hsp70* gene expression later in life, to extended lifespan or precondition insects for subsequent stress (Kristensen et al., 2003). On the other hand, *Hsp70* induction from mild stress in early life may also lessen deleterious effects that would occur during aging, thereby extending lifespan (Sorensen et al., 2003).

Hsp90 expression was also associated with enhanced tolerance to temperature stress. In addition to mitigating the effects of stress on protein integrity, Hsp90 interacts with a diverse range of proteins including those involved in cell communication and signalling, control of growth, and gene expression and regulation (Rutherford et al., 2007). In insects, Hsp90 has roles in reproduction, longevity, and stress tolerance. Whereas increased *Hsp90* expression and fecundity in insects coincides with selection for thermal tolerance (Gu et al., 2019a), reduced thermotolerance, longevity, fecundity, embryogenesis, and eclosion coincide with decreased expression of *Hsp90* (Chen and Wagner, 2012; Will et al., 2017; Farahani et al., 2020). Increased expression of *Hsp90* corresponded to heat stress and increased heat tolerance in my analysis (Shen et al., 2014; Kim et al., 2017) but not reproduction, which was reduced in one instance (Shen et al., 2014). Few papers on hormesis have examined Hsp90 expression outside of its role in thermotolerance, yet Hsp90 could also influence hormetic responses given its additional

roles regulating phenotypic expression under stressful conditions (Jarosz and Lindquist, 2010; Chen and Wagner, 2012).

Detoxification

Expression of detoxification genes and activity was most frequently associated with hormetic stimulation of chemical stress tolerance and growth, longevity, and reproductive endpoints. The detoxification system is responsible for the breakdown of xenobiotics, protecting insects from oxidative stress that can result from chemical exposure, but also maintains roles in antioxidant defense and redox balance (Veith and Moorthy, 2018; Zhang et al., 2018). Common enzymes like cytochrome P450s and esterases are respectively responsible for oxidation and hydrolysis of xenobiotics, and glutathione-s-transferases break down xenobiotics through conjugation with glutathione and peroxidase activity (Feyereisen, 1999; Hayes et al., 2005; Schuler, 2011; Hatfield et al., 2016; Pavlidi et al., 2018). Unsurprisingly, there is a strong relationship between chemical exposure and expression of detoxification enzymes, and enhanced detoxification and tolerance to insecticides or other chemicals (Enayati et al., 2005; Bass and Field, 2011; Feyereisen, 2012; Montella et al., 2012). A single transcription factor may induce expression of genes involved in multiple phases of detoxification (Misra et al., 2011), and expression of multiple genes involved in metabolism of different chemicals can result in cross tolerance. As an example from my analysis showing hormetic stimulation accompanied by induction of detoxification genes, exposure to contaminants increased yellow fever mosquito, *Aedes aegypti* (L.), tolerance to insecticides (Poupardin et al., 2008; Riaz et al., 2009), which was associated with increases in multiple cytochrome P450s and glutathione-s-transferase genes important in insecticide metabolism/resistance

in mosquitoes (Marcombe et al., 2012; Strode et al., 2012; Riaz et al., 2013). Similar expression of cytochrome P450 genes important in insecticide resistance was observed in *S. litura* where stimulated increases in insecticide and xanthotoxin tolerance was observed following herbicide and xenobiotic exposure (Liu et al., 2019a; Lu et al., 2019).

The roles of detoxification genes outside of chemical detoxification are not entirely established in insects, but there is evidence they may play roles in reproduction, longevity, and growth and development. A number of studies in my survey linked increased carboxylesterase and acetylcholinesterase activity, and *E4/FE4* gene expression to hormetic increases in reproduction, growth, and also longevity (Nanthakumar et al., 2012; Deng et al., 2016; Rix et al., 2016; Li et al., 2018; Sial et al., 2018; Zhao et al., 2018). Functional analysis of carboxylesterase and acetylcholinesterase enzymes has demonstrated their importance in these processes. For example, silencing of acetylcholinesterase genes in red flour beetle, *Tribolium castaneum* (Herbst), and cotton bollworm, *Helicoverpa armigera* (Hübner), resulted in delayed or inhibited growth and development, and dramatic reduction in fecundity and fertility, and increased mortality (Kumar et al., 2009; Lu et al., 2012). Overexpression of carboxylesterase enzymes in reproductive tracts and seminal fluid enhances sperm storage and utilization in male *Drosophila spp.*; fecundity is also increased in *Drosophil spp.* females following copulation, possibly due to enhanced detoxification of background xenobiotic stress (Mikhailov and Torrado, 1999). Such effects could account for the association between increased detoxification and longevity or reproductive hormesis observed in other studies in my survey (Deng et al., 2016; Rix et al., 2016; Sial et al., 2018; Zhao et al., 2018).

More recent research has identified cytochrome P450 genes involved in ecdysteroid biosynthesis in insects, known as Halloween genes (Feyereisen, 2012). Ecdysteroids are responsible for mediating growth and development of juvenile insects, and oogenesis and initiation of vitellogenesis in females (Belles and Piulachs, 2015). Halloween genes have also been shown to be induced by stress and involved in detoxification. For example, expression of *CYP302A1* (*disembodied*), *CYP306A1* (*spook*), *CYP307A1* (*phantom*) and *CYP314A1* (*shade*) was induced in rice stem borer, *Chilo suppressalis* (Walker), and domestic silk moth, *Bombyx mori* (L.), following exposure to chlorantraniliprole and phoxim (Li et al., 2015; Meng et al., 2020). Overexpression of these genes, in addition to *CYP315A1* (*shadow*) were observed in thiamethoxam resistant *A. gossypii* (Wu et al., 2018). *Disembodied*, *shade*, and *shadow* are also thought to be involved in reproduction, given their expression in the ovaries of some insects, and negative effects on yolk protein expression, reproductive development, and egg production following gene silencing (Wu et al., 2018; Peng et al., 2019). This demonstrates the role of Halloween genes in both detoxification and reproduction. While these genes were not studied with respect to reproductive hormesis in the studies I examined, *CYP314A1* and *CYP315A1* expression was increased in *A. aegypti* and European honey bee, *Apis mellifera* L.*,* respectively, in response to permethrin or thiacloprid, priming the insects for subsequent higher exposures (Poupardin et al., 2008; Alptekin et al., 2016). Thus, Halloween genes may be involved in chemical or insecticide preconditioning, but their role in reproductive hormesis should not be ruled out, given the strong association between sublethal insecticide exposure and stimulated reproduction.

Antioxidation, Growth, Development, and Reproduction

Antioxidants have long been proposed as arbiters of the hormetic response given their role in mediating both stress – chemical, temperature, dehydration, radiation, anoxia/hypoxia, etc. – and numerous life history traits including growth, development, longevity, and reproduction, and survival. Stimulation of these sorts of life history traits following mild stress is a hallmark of hormesis in insects (Cutler, 2013; Berry and Lopez-Martinez, 2020) and is frequently associated with increased antioxidant activity or gene expression (Hyrsl et al., 2007; Riaz et al., 2009; Lopez-Martinez and Hahn, 2012; Ge et al., 2013; Yu et al., 2016; Chattopadhyay et al., 2017; Matsumura et al., 2017; Li et al., 2018; Youn et al., 2018; Lu et al., 2019).

Reactive oxygen species are produced during normal metabolism but may also be produced at higher rates under stress, signalling the production of cellular protection and repair mechanisms (Berry and Lopez-Martinez, 2020). Antioxidants are responsible for protecting cells from cytotoxicity and mutagenesis caused by excessive build-up of ROS in cells following stress (Dalton et al., 1999; Kodrik et al., 2015). This is predominantly carried out by superoxide dismutases, which catalyse the dismutation of the superoxide anion into oxygen and hydrogen peroxide; catalases and peroxidases which breakdown hydrogen peroxide to water and oxygen (Costantini, 2014a). Preconditioning *A. suspensa* with mild anoxia stress increased superoxide dismutase and glutathione peroxidase activity, resulting in reduced oxidative damage and concomitantly improved radiation tolerance, fly emergence, and male sexual performance when under combined effects of anoxia and radiation (Lopez-Martinez and Hahn, 2012).

The ability to buffer oxidative stress enhances longevity and reproduction. Reactive oxygen species scavenging by catalase enzymes reversed age related decreases in fecundity in the sandfly, *Lutzomyia longipalpis* (Lutz and Neiva), (Diaz-Albiter et al., 2011). Extended lifespan in *D. melanogaster* selected for longevity was observed with increased antioxidant gene expression and activity (Arking et al., 2000). Genes in the IIS/TOR signalling pathway are thought to be involved in the regulation of the oxidative stress response and its relationship to longevity, reproduction, and stress resistance. Expression of FOXO transcription factors is directly affected by oxidative stress and their expression has been associated with increased longevity through regulation of genes in the IIS/TOR pathway (Kops et al., 2002; Kapahi et al., 2004; Storz, 2011). Hormetic increases in longevity and stress tolerance following crowding or rutin exposure in *D. melanogaster* were associated with increased *FOXO* along with differential expression of several genes in the IIS/TOR signalling pathway known to be involved in longevity extension, *Tsc1*, *Tsc2, Atg1, Atg5, Atg7* (Chattopadhyay et al., 2017; Lushchak et al., 2019). FOXO indirectly inhibits TOR through activation of Tsc1/Tsc2 and genes involved in autophagy, which results in increased lifespan and oxidative stress resistance (Hay, 2011). Recent work has suggested that the Nrf2-Keap1-ARE signalling may also be involved in hormetic effects on lifespan or survival, and oxidative stress resistance. Under low levels of stress, Nrf2 activates ARE-mediated antioxidant responses, enhancing survival and oxidative stress resistance (Calabrese and Kozumbo, 2021b; Calabrese and Kozumbo, 2021a). In insects, the Nrf2 ortholog, CncC, has largely been involved in regulating detoxification, but recent work has shown a connection between increased FOXO, Nrf2, and Keap1, and antioxidant enzyme gene expression in response

to insecticide exposure (Lu et al., 2021); detoxification genes and antioxidants are frequently expressed in tandem. Further research is needed to confirm, but this could potentially be a mechanism for insecticide-induced hormesis and associated increases in detoxification and antioxidant responses following insecticide exposure.

In some insects, FOXO influences reproduction by regulating synthesis of vitellogenin, an egg yolk protein responsible for ovarian maturation, in response to insulin signalling (Roy et al., 2018). FOXO can suppress expression of juvenile hormone degradation genes (Wu et al., 2021). Juvenile hormone regulates synthesis of vitellogenin and vitellin (vitellogenin after uptake by the ovary) (Corona et al., 2007). Moreover, TOR positively influences juvenile hormone biosynthesis and vitellogenesis (Wu et al., 2021). Low doses of pesticide or heat exposure can increase juvenile hormone and related gene expression or titres (Yu et al., 2010; Ayyanath et al., 2014; Ayyanath et al., 2015; Zhou et al., 2020; Zhu et al., 2020), or vitellogenin gene expression or content (Zeng and Wang, 2010; Lu et al., 2017b; Li et al., 2019; Ullah et al., 2020; Zhou et al., 2020), resulting in increased reproduction. However, in a number of instances vitellogenin genes were highly downregulated or variably regulated in response to stress where hormetic increases in reproductive endpoints or longevity were observed (Chen et al., 2017b; Li et al., 2019; Zhou et al., 2020), suggesting that additional factors are involved in stress induced reproductive stimulation and increased longevity. This was also true in Ayyanath et al. (2014, 2015) where patterns in differential expression of the juvenile hormone regulator gene *FPPS I*, although associated with chemical induced reproductive hormesis, were similarly observed in response to doses not inducing hormesis. This again suggesting additional factors are involved in reproductive hormesis.

4.5 Conclusions

Studies on hormesis in insects concomitant with examination of gene expression overall reveal consistent similarities between phenotypic and molecular/biochemical responses. The evidence herein shows there are key molecular and biochemical responses that are consistently linked to the stimulatory and hormetic responses to stress in insects. Coordination of the expression of chaperones, and genes or proteins associated with detoxification and antioxidation were most prominently observed demonstrating that protection from oxidative stress and DNA and protein damage are implicit in hormetic responses. The stress response itself may also trigger signalling pathways that play direct or indirect roles in the regulation of commonly observed biological traits stimulated during hormesis. The observed commonalities among hormetic responses suggest there may be a universal mechanism for hormesis however the wide array of stressors and magnitudes that induce hormesis and organisms that display hormesis in response to low doses of stress suggest that there are organismal and stress specific responses. In my assessment, studies examining expression of multiple genes, or expression over multiple time points demonstrated varying patterns of gene expression suggesting hormesis manifests from a coordination of stress response elements.

The consistency and ubiquitous nature of hormesis has spurned desire to understand its underlying molecular basis. This work has served to bring together the clear and present evidence of the robust patterns in hormesis and its associated molecular and biochemical responses. Future work should focus on examining regulatory mechanisms of stress responses, taking into account the diversity of insect species, biology, and development.

CHAPTER 5 TRANSCRIPTOME ANALYSIS OF PODISUS MACULIVENTRIS EXPOSED TO HORMETIC AND INHIBITORY CONCENTRATIONS OF IMIDACLOPRID

This chapter is formatted as a manuscript in preparation for submission to peer review.

5.1 Introduction

Hormesis is now recognized as being fundamentally representative of the dose-response. In contrast to traditional models of the dose-response where low doses have minimal to no effect on an organism, the hormetic dose-response is characterized by stimulation at low doses and inhibition at high doses (Calabrese, 2014; Calabrese, 2016a; Calabrese and Agathokleous, 2022). Hormesis is widespread, occurring across the taxonomic spectrum and is not strictly limited to exposure to chemical toxins, occurring in response to many forms of stress including heat, nutrition, radiation, exercise, and oxidative stress (Mattson, 2008). Hormesis also has consistent and predictable quantitative features, in most cases occurring at levels of stress below the NOAEL, with stimulatory effects being mild, ranging between 30-60% increased above control levels (Calabrese, 2013a). The ubiquitous nature of hormesis would suggest evolutionary conservation. Hormesis is an adaptive response to stress. Organisms are continuously exposed to stress in ever changing environments, and without the ability to adapt, extinction would be inevitable. Therefore, hormesis is likely fundamental to natural selection and thus evolution (Zhang et al., 2008; Mattson, 2010).

When an organism is under stress, defensive and protective cellular responses are triggered. At intermediate and high levels of stress, defensive and protective cellular responses begin to exceed the capacity of the cell to defend and protect itself and damage may begin to accumulate, resulting in deleterious or inhibitory effects in an organism or

even death (Wiegant et al., 2012; Rossnerova et al., 2020). At low levels of stress, these defensive and protective processes are carried out in slight excess of what was needed to overcome the adverse effects of the stress, resulting in the stimulatory responses and adaptation to stress (Rossnerova et al., 2020). Cellular regulatory networks are complex, involving multiple genes, proteins, metabolic reactions, and interactions between proteins and DNA. It is through small, coordinated changes in gene expression that cellular stress responses are regulated at low doses, resulting in adaptive homeostasis, without cell detriment, which may be key to the hormetic response (Zhang et al., 2008; Calabrese and Mattson, 2017).

In Chapter 4 (Rix and Cutler, 2022), I systematically analyzed the literature studying molecular and biochemical responses associated with hormesis. This review showed that while commonalities exist in these responses (i.e., changes in the expression of genes and proteins involved in DNA repair, detoxification and protection against oxidative stress), the responses were dynamic. Examination of multiple genes, timepoints, or low-dose concentrations showed varying patterns of gene expression, suggesting hormesis manifests by means of a coordinated stress response. However, most studies on hormesis have focused only on small subsets of targeted genes. While this has provided valuable insights into the hormetic response, it is limited in its scope. By utilizing next generation sequencing technologies, we can examine whole transcriptomes, which can provide more detailed insights into the dynamics of stress responses and hormesis.

Therefore, in this chapter I used RNA-seq to conduct a full transcriptome analysis with the objective of examining gene expression in *P. maculiventris* exposed to a

reproductively hormetic concentration of imidacloprid compared with a control and inhibitory/deleterious concentration to provide more detailed insights into the manifestation of reproductive hormesis in this insect. Given that hormesis is thought to manifest from coordinated expression of cellular stress and repair and protective processes, and that reproductive hormesis was induced in *P. maculiventris* exposed to a low-dose concentration of imidacloprid insecticide, I expected to see increased expression of transcripts involved in general stress and repair, detoxification, and growth, development, and reproduction. I also hypothesized that given hormesis occurs in response to low doses and manifests as moderate stimulation of biological processes, I would see fewer expressed genes involved in general stress and repair, and detoxification compared with an inhibitory concentration of imidacloprid. I therefore expected to see more genes involved in general stress and repair, and detoxification in insects exposed to a high-dose (inhibitory) concentration of imidacloprid. Given insects exposed at this concentration had reduced survival and inhibited reproduction I also expected to see decreased expression of transcripts involved in growth, development, and reproduction.

5.2 Methods

5.2.1 Insect Rearing

Insects were reared as previously described in Rix and Cutler (2020). Briefly, a *P. maculiventris* colony was established from eggs obtained from the Agriculture and Agri-Food Canada Research and Development Centre (London, ON, Canada), and reared in the Entomology Laboratory at Dalhousie University Faculty of Agriculture (Truro, NS, Canada). Newly hatched nymphs from eggs (100-200) were provided with a fresh cabbage leaf (*B. oleracea*) in 10 cm diameter Petri plates lined with filter paper (Fisher

Scientific, Ottawa, ON, Canada) until they reached their 2nd instar and were large enough to be transferred to transparent plastic containers (Dart Canada, Scarborough, ON, Canada). These were lined with filter paper, and insects were provided, as needed, with fresh cabbage leaves and several *T. molitor* larvae (PetValue, Truro, NS, Canada) as food and water. Late 3rd instars were transferred to larger, ventilated plastic containers and reared to adulthood. Nymphs (3rd to 5th instar) and adults were reared separately in ventilated containers on 2–3-week-old whole cabbage plants and provided with *T. molitor* larvae twice a week as needed. Eggs laid by adults were collected and the rearing cycle continued as above. Eggs, larvae, and adults were all reared in a growth chamber at 23 ± 0.3 ºC, 55 ± 5.0% RH, and 16:8 hours (L:D).

5.2.2 Insecticide Treatment and Sample Selection

Adult female *P. maculiventris* were randomly collected from among my previously conducted experiments (Chapter 2) (Rix and Cutler, 2020). Briefly, insects were treated topically, using a Potter spray tower (Burkard Scientific Ltd, Uxbridge, United Kingdom), with a range of sublethal and field relevant concentrations of imidacloprid (Admire 240 SC; 240 g/L AI; Bayer CropScience Canada, Calgary AB, Canada). Insects were collected 24 hours following exposure, consistent with research showing effects occurring at the molecular level 24 hours following insecticide exposure (Cabrera-Brandt et al., 2014; Liu et al., 2016; Reid et al., 2018; Xiong et al., 2019). Insects were placed into sterile cryogenic vials (VWR, Randor, PA, USA), flash frozen in liquid nitrogen, and placed at -80 ºC until RNA extraction. In my previously conducted experiments (Chapter 2) (Rix and Cutler, 2020), I observed that insects treated with 0.5 mg/L of imidacloprid had stimulated fecundity, and that treatment with 100.0 mg/L of imidacloprid reduced

survival and inhibited fecundity across the timespan of my experiments. Therefore, *P. maculiventris* treated with 0.5 mg/L and 100 mg/L, in addition to water treated controls, were sampled for further molecular analysis to assess the patterns of gene expression in insects treated with hormetic and inhibitory levels of imidacloprid.

5.2.3 RNA Extraction and Sequencing

Insects were removed from -80 ºC and placed into 15 mL sterile centrifuge tubes with 1.5 mL of Tri reagent (1.4 M guanidine isocyanate, 0.1 M sodium acetate, 38% phenol, 5% sterile glycerol) and homogenized with a TH electric homogenizer (OMNI International, Kennesaw, GA, USA). Chloroform (200 µL) was added to the homogenate and shaken for 15 seconds, then incubated on the benchtop at room temperature for 3 min. Samples were then centrifuged at 12,000 *g* for 15 minutes at 4 ºC. The aqueous phase (600 µL) was mixed with equal parts 70% ethanol and RNA was extracted using an RNeasy® Universal Kit (Qiagen, Toronto, ON, Canada), according to manufacturer's instructions. An on-column DNase I-digestion step was conducted using an RNase-Free DNase Set (Qiagen, Toronto, ON, Canada) to remove any contaminating DNA. RNA was eluted in 30 µL of RNase-free water and quantified via spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific, Ottawa, ON, Canada). Quality was assessed on an Agilent Bioanalyzer 2100 (model G2939A, Agilent, Santa Clara, CA, USA) using an Agilent RNA 6000 Nano chip (Agilent, Santa Clara, CA, USA) following manufacturer's instructions. RNA samples (9 samples per concentration randomly collected across 3 experimental batches/blocks) were sent to the Génome Québec Innovation Centre (McGill University, Montreal, QC, Canada) for sequencing. Sequencing libraries were

prepared with an NEBNext® mRNA Library Prep kit (Illumina) and Paired End 100 sequencing was performed on an Illumina NovaSeq 6000 at a 25M read depth.

5.2.4 Bioinformatics

Raw sequence reads were uploaded to the main Galaxy web platform and analyzed on the Galaxy server (usegalaxy.org) (Jalili et al., 2021). Read quality was assessed with FastQC (Andrew, n.d.) (Galaxy Version 0.72+galaxy1), and adaptor sequences trimmed with Trim Galore! (Krueger, 2015) (Galaxy Version 0.6.3). A subsample of 70% of the reads taken from each sample set of sequences was used to construct the transcriptome (seqtk_sample; Galaxy Version 1.3.2). The transcriptome was assembled de novo using the Trinity suite (Grabherr et al., 2011) (Galaxy Version Galaxy Version 2.9.1+galaxy1) with a *k-mer* of 30. Higher *k-mer*s produce a more contiguous assembly, with fewer errors, better mapping efficiency, and smaller size (Durai and Schulz, 2016; Manekar and Sathe, 2018; Raghavan et al., 2022). Reads were aligned to the transcriptome using HiSAT2 (Kim et al., 2015) (Galaxy Version 2.2.1+galaxy0). Count files were generated with StringTie (Pertea et al., 2015) (Galaxy Version 2.1.1) and merged with StringTie Merge (Pertea et al., 2015) (Galaxy Version 2.1.1). Read counts were normalized with featureCounts (Liao et al., 2014) (Galaxy Version 2.0.1+galaxy1), and unwanted variation was removed using RUVr in the RUVseq package (Risso et al., 2014; Peixoto et al., 2015) (Galaxy Version 1.26.0+galaxy0). DESeq2 was used to identify differentially expressed transcripts between treatments, using the Benjamini-Hochberg procedure to control for false discovery rate (FDR) (Love et al., 2014) (Galaxy Version 2.11.40.6+galaxy2). I considered transcripts to be significantly differentially expressed if the adjusted (adj) p-value ≤ 0.01 and a fold change (FC) >1 ($\log_2 FC > 0$).

Differentially expressed transcripts were uploaded into OmicsBox (BioBam Bioinformatics, 2019) and functional analysis conducted with the Blast2Go suite (Gotz et al., 2008). Transcripts were annotated using Blastx (Camacho et al., 2009) against the GenBank protein nr database, and functional terms assigned with Gene Ontology (GO) (Ashburner et al., 2000; Carbon et al., 2021). Gene Ontology enrichment analysis was also conducted in OmicsBox using all differentially expressed transcripts (adj p-value ≤ 0.01) to identify biological processes that were overrepresented in response to treatments. Gene Ontology terms were considered to be over-represented if the p-value from the enrichment analysis was ≤ 0.05. Gene Ontology term reduction was further conducted in OmicsBox to remove redundant GO terms. Enzyme names were assigned with KEGG (Kanehisa and Goto, 2000; Kanehisa, 2019) and domain information assigned with Interpro (Quevillon et al., 2005; Blum et al., 2021).

5.3 Results and Discussion

5.3.1 Transcriptome Summary

The *de novo* assembly of the *P. maculiventris* transcriptome yielded 34,725 transcripts, with maximum and minimum transcript lengths of 14,174 bp and 181 bp, and an N50 of 1550 bp. Of these, 19,909 transcripts returned blast hits from the nr database. Given *P. maculiventris* is not a model organism, it would be expected that novel transcripts would constitute a large portion of the transcriptome. Transcriptome annotations in addition to RNA sample quality and quantity, and read quality assessments for RNA-seq libraries for each sample can be found in the supplementary material (Table S1; see Appendix 2). Of the transcripts returning blast hits, 9,183 transcripts were functionally annotated to GO terms in at least 1 of the 3 major categories: molecular function (MF), cellular component

(CC), and biological process (BP). Analysis identified 2224 unique GO categories represented in the transcriptome, 860 attributed to molecular function, 332 attributed to cellular components, and 1032 attributed to biological processes. The top 30 GO terms from each category are listed in the supplementary material (Figure S1; see Appendix 3).

5.3.2 RNA-seq Analysis and Differential Expression

RNA-seq has become the major method of choice for gene expression profiling. Historically, gene expression profiling was conducted with DNA microarray-based methods. Microarrays, although having high level performance, were subject to background noise and bias, which was shown to affect reproducibility (Hrdlickova et al., 2017; Coenye, 2021). As such, researchers found it necessary to validate microarrays with qRT-PCR. RNA-seq is not subject to the same issues as microarrays, although there remains some debate as to the necessity of qRT-PCR validation (Hrdlickova et al., 2017; Coenye, 2021). However, recent investigation into the subject of qRT-PCR validation has demonstrated that RNA-seq is robust and highly correlated with qRT-PCR results across multiple workflows, with approximately 85% of genes showing concordance between RNA-seq and qRT-PCR (Everaert et al., 2017). Those genes with non-concordance were mainly only marginally non-concordant, and maintained the same direction (increased/up or decreased/down) of expression (Everaert et al., 2017). Validation with qRT-PCR can provide added value to an RNA-seq analysis, however, given the proven robust nature of RNA-seq, current research is moving away from the use of qRT-PCR validation. Many recently published RNA-seq papers using a variety of organisms including insects, and a variety of RNA-seq assembly and analysis methods that have not conducted qRT-PCR validation see (Des Marteaux et al., 2017; Chen et al., 2018b; Liu et al., 2018; Camargo

et al., 2020; Harrington et al., 2020; Lawrie et al., 2020; Ramond et al., 2020; Amaro et al., 2021; Khan et al., 2021; Kim et al., 2021; Lebenzon et al., 2021; Niemisto et al., 2021). Due to constraints and limitations associated with a major building fire, followed by the Covid-19 pandemic, it was not possible to conduct qRT-PCR validation, however the proven robustness of RNA-seq demonstrates that it alone provides accurate and valuable insights into the nature of gene expression. Furthermore, of the multiple software programs available for the identification of differentially expressed genes, DEseq2 (used in my analysis) performs among the highest for accuracy in correctly detecting differentially expressed genes when compared with qRT-PCR (Costa-Silva et al., 2017). The current knowledge of the accuracy of RNA-seq and the recent literature precedent demonstrates my work fits within the current norm for RNA-seq analysis.

5.3.3 Differential Expression Patterns

As expected for a non-model organism, a large portion of transcripts having significant differential expression, including some of the most highly differentiated transcripts in both hormetic and deleterious imidacloprid treatments, did not annotate to genes with known function in the nr database, demonstrating that there are many genes involved in the response to imidacloprid that remain to be identified. Future work dedicated to determining the functions of novel genes in *P. maculiventris* is likely to provide crucial insights on the distinct response to insecticide exposure and stress, potentially providing a blueprint for the stress response and hormesis of *P. maculiventris* and other related species.

Following annotation of differentially expressed transcripts, a selection of biologically relevant transcripts characteristic of general stress response during

insecticide exposure or hormesis were manually categorized according to involvement in DNA damage response, metabolism (lipid, carbohydrate, and energy metabolism), detoxification, and growth, development and reproduction. The full lists of differentially expressed transcripts and top 15 GO terms for both increased and decreased expression can be found in the supplementary material (Table S1; see Appendix 2, Figure S2-4; See Appendix 3).

5.3.3.1 Differential Expression in Response to a Hormetic Concentration of Imidacloprid

Hormesis is typically induced under low to moderate levels of stress. When the quantity and duration of a stressor is below the capacity of the defensive machinery, the stressor only activates defensive and protective processes that remain active to repair the existing damage and protect the organism against future stress (Rossnerova et al., 2020). Therefore, the stress response is mounted in slight excess of what is needed to re-establish homeostasis and repair damage. This is thought to result in the modest stimulation of life history traits and biological processes that we call hormesis. Efficient resource allocation under low levels of stress would result in only modest responses (Calabrese, 2013a). I would therefore expect to see a more tempered molecular response at low, hormetic levels of stress than at high levels of stress. Indeed, I observed far fewer differentially expressed transcripts in *P. maculiventris* exposed to a reproductively hormetic concentration of imidacloprid, than in insects treated with an inhibitory/deleterious concentration of imidacloprid. I observed 363 transcripts having FC >1 increased expression and 205 having FC >1 decreased expression (Log_2 fold change > 0; adj p-value ≤ 0.01) (Fig 5.1a, Table S1; see Appendix 2).

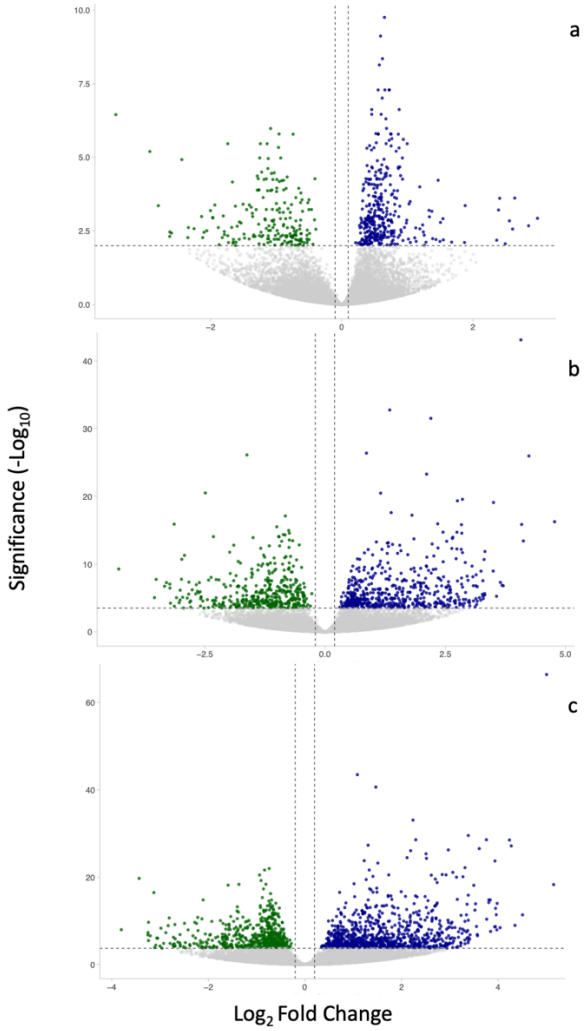

Figure 5.1: Volcano plots showing distribution of significantly differentially expressed transcripts (Log_2 fold change > 0; adj p-value ≤ 0.01) a) hormetic vs control, b) high vs control, c) high vs hormetic. Transcripts with adj p-values ≤ 0.01 and a Log_2 fold change > 0 are indicated in blue (increased expression) and green (decreased expression).

DNA Damage Response

Transcripts with increased expression were largely involved in the DNA damage response including autophagy and apoptosis, and DNA repair. These processes are integral to maintenance of cellular homeostasis under stress, preventing damage from occurring, and repairing or eliminating damaged cells. Exposure to imidacloprid, like many chemical pesticides and other stressors, can result in damage to DNA and

activation of damage response and repair systems (Ge et al., 2015; Wang et al., 2016a; Wei et al., 2020). These processes are also crucial to inducing hormesis (Wiegant et al., 2012; Agathokleous et al., 2018; Bhattacharya and Rattan, 2018; Mägdefrau et al., 2018; Berry and Lopez-Martinez, 2020). Damage to cells sufficient to elicit a response is necessary to induce hormesis, wherein the response is over-compensatory, resulting in enhancement of biological processes such as survival, longevity, and reproduction (Calabrese and Agathokleous, 2022).

The transcript with the highest increase in expression in insects exposed to a hormetic concentration of imidacloprid was myb-like protein X (Table 5.1). Myb proteins are transcription factors found in all eukaryotes that also function in cell cycle regulation and regulation of apoptosis, and cell proliferation in response to stress and DNA damage (Oh and Reddy, 1999; Chen et al., 2017c). They have thus been shown to play an important role in stress tolerance (Ambawat et al., 2013). Organisms are exposed to genotoxic stressors daily, and low doses of genotoxic stressors are beneficial for a variety of cellular processes through the induction of DNA repair (Mägdefrau et al., 2018). In insects treated with the hormetic concentration of imidacloprid, I also observed increased expression of transcripts involved in DNA repair processes. Histone-lysine N-methyltransferase SETMAR-like (SETMAR) was significantly increased and is involved in mediating DNA double-strand break repair by recruiting DNA repair proteins at damaged sites (Tellier, 2021). Increased expression of *SETMAR* has been observed in insects exposed to sublethal temperature stress (Lebenzon et al., 2021; Vatanparast et al., 2021). I also observed an increase in DNA ligase 1 expression (Table 5.1). DNA ligase 1 contributes to genome stability by catalyzing the ligation of DNA strands following DNA

repair (Tang et al., 2020). Its expression has been increased in response to sublethal imidacloprid exposure in bees (Chen et al., 2021), and was associated with increased fecundity in rock ant queens, *Temnothorax rugatulus* (Emery), subject to physical stress (Negroni et al., 2021).

Table 5.1: Differential expression of select biologically relevant transcripts associated with the DNA damage response in *Podisus maculiventris* treated with a hormetic concentration (0.5 mg/L) of imidacloprid compared with a control.

Sequence Description	**Transcript ID**	**Fold Change**
autophagy protein 5	DN16252_c0_g1_i1	1.34
autophagy-related protein 16-1	DN676_c0_g1_i2	1.85
autophagy-related protein 16-1 isoform X2	DN676_c0_g1_i1	1.74
autophagy-related protein 16-1-like	DN676_c0_g2_i1	1.49
BRCA1-associated protein	DN15740_c0_g1_i1	1.35
cellular tumor antigen p53	DN170_c1_g2_i5	2.01
DNA excision repair protein ERCC-1	DN15677_c0_g1_i1	1.51
DNA ligase 1	DN207_c0_g1_i7	6.09
DNA repair protein RAD50	DN6986_c0_g1_i1	1.30
DNA repair protein RAD51 homolog 1	DN13262_c0_g1_i1	1.71
dnaJ homolog subfamily C member 24-like	DN9257_c0_g1_i1	1.57
dnaJ homolog subfamily C member 28	DN4083_c0_g1_i1	-1.56
heat shock 70 kDa protein 14-like	DN2222_c0_g1_i1	1.32
histone-lysine N-methyltransferase SETMAR-like	DN1692_c0_g1_i8	5.25
mitogen-activated protein kinase kinase kinase 7-like	DN3467_c0_g5_i1	2.77
myb-like protein X	DN1901_c0_g1_i3	7.21
transcription elongation factor 1 homolog	DN929_c0_g1_i1	2.89

Other notable transcripts involved in DNA repair included increased expression of transcription elongation factor 1 (ELOF1) homolog (Table 5.1) and DNA excision repair protein ERCC-1 (ERCC-1) (Table 5.1), both involved in nucleotide excision repair following stress induced damage (Mägdefrau et al., 2018; Geijer et al., 2021), DNA repair protein RAD50 (RAD50) DNA repair protein RAD51 homolog 1 (RAD51) and a BRCA1-associated protein (Table 5.1), all involved in DNA double strand break repair, and associated with molecular response pathways involved in hormetic responses (Bhattacharya and Rattan, 2018; Mägdefrau et al., 2018). Increased levels of expression of DNA repair genes have also been shown to be involved in maintaining high reproduction and longevity in some insects (Lucas et al., 2016).

Chaperones are also crucial to the DNA damage response and are well known for their role in hormesis (Calabrese et al., 2009; Calabrese et al., 2012). Heat shock proteins, molecular chaperones that repair and eliminate damaged proteins, preventing their aggregation in the cell, are activated by many forms of stress including chemical and oxidative stress, osmotic and nutritional stress (Rattan et al., 2004; Calabrese et al., 2009; Calabrese et al., 2012; King and MacRae, 2015). Insects treated with a hormetic concentration had increased expression of two heat shock protein transcripts, including an Hsp70 (heat shock 70 kDa protein 14-like), and an Hsp40 family member (dnaJ homolog subfamily C member 24-like). Decreased expression of an additional Hsp40 transcript (dnaJ homolog subfamily C member 28) was also observed (Table 5.1). Hsp40s act as cochaperones with major heat shock proteins (King and MacRae, 2015), regulating the activity of Hsp70s by stabilizing their interactions with protein substrates (Qiu et al., 2006). Proper function of Hsp70 plays an important role in insect fecundity and life

history, by protecting insects from the negative effects of stress (Sorensen et al., 2003). For example, silencing of Hsp70 gene expression reduces fecundity in the alligator weed flea beetle, *Agasicles hygrophila* Selman and Vogt, under stress (Jin et al., 2020). Increased thermotolerance and increased fecundity were associated with increased expression of Hsp70 in rice leaf folder larvae, *Cnaphalocrocis medinalis* (Guenee) (Gu et al., 2019a).

One important regulator of DNA damage responses is the transcription factor p53 (cellular tumor antigen p53), which had increased expression in my insects exposed to a reproductively hormetic concentration of imidacloprid (Table 5.1). p53 regulates cellular homeostasis by either inducing transient cell cycle arrest, where the cell is repaired, promoting cell survival, stress resistance, and longevity (Suzuki et al., 2021), or inducing permanent cell cycle arrest, preventing accumulation of damaged cells through senescence, or apoptosis (Suzuki et al., 2021), which has been shown to be important for development, fecundity, and fertility (Suh et al., 2006; Hu, 2009; Vousden and Prives, 2009; Hu and Feng, 2019). Induction of apoptosis in nurse cells in the insect ovary is essential to providing nutrients to developing oocytes, allowing for their maturation (Mpakou et al., 2011). p53 is highly expressed in insect nurse cells (Jin et al., 2000), and increased apoptosis of nurse cells has been shown to increase fecundity, and can occur following stress (Guo et al., 2018; Zhang et al., 2021). Apoptosis induced by p53 under stress can also protect genome stability in oocytes and eliminate damaged oocytes (Suh et al., 2006; Hu and Feng, 2019). In *D. melanogaster*, p53 plays a role in the development of ovary and oocytes (Hu, 2009). *D. melanogaster* with p53 null mutations have degenerative ovarioles (which produce oocytes), demonstrating the importance of p53 for

the maintenance of fecundity (Lee et al., 2003). Increased p53 gene expression was associated with increased fecundity of Asian citrus psyllid, *Diaphorina citri* Kuwayama, exposed to toxic plant metabolites when surviving on flush shoots of *Murraya paniculate* (L.) (Guo et al., 2021). Addition of a carotenoid, fucoxanthin, to a *D. melanogaster* diet regime stimulated fecundity by 30-48% during the first week of fucoxanthin intake, and increased the expression of genes involved in the DNA damage response, including p53 (Lashmanova et al., 2015). The mitogen-activated protein (MAP) kinase pathway may also play a role, as the MAP kinase pathway is involved in regulating p53 mediated apoptosis (Gen, 2004; Brown and Benchimol, 2006) and associated increased fecundity (Guo et al., 2021). I observed increased expression of mitogen-activated protein kinase kinase kinase 7 (MAP3K7), also known as TAK-1 protein that activates p53 in response to stress (Table 5.1) (Gen, 2004; Shin and Min, 2016; Zonneville et al., 2020). Increased expression of *MAP3K7* was also associated with insecticidal Vip3 protein induced apoptosis in fall armyworm, *Spodoptera frugiperda* (J.E. Smith), larvae (Jin et al., 2022).

A major question surrounding hormesis is the regulation of trade-offs (Jager et al., 2013). In insects, trade-offs are often observed between reproduction and longevity or survival (Flatt, 2011), and these may occur trans-generationally (Ayyanath et al., 2013). Activation of Nrf2 may play a key role in hormetic stimulation of longevity and survival under low doses of stress (Calabrese and Kozumbo, 2021a), and occurs under p53 suppression (Chen et al., 2012; Calabrese and Kozumbo, 2021b; Calabrese and Kozumbo, 2021a). Therefore, regulation of p53 may play a key role in moderating the hormetic response and trade-offs between reproduction and longevity and survival. p53 also plays a role in regulating autophagy (Suzuki et al., 2021), which is involved in

survival and longevity, and reproduction, including under stress (Malagoli et al., 2010; Agathokleous et al., 2018; Maruzs et al., 2019; Weng and Shiao, 2020; Suzuki et al., 2021). I also found increased expression of transcripts involved in autophagy including autophagy-related protein 16-1, and autophagy protein 5 (Table 5.1). My previous work (Chapter 2) (Rix and Cutler, 2020) demonstrated that *P. maculiventris* survival was not impaired when adults were exposed to a hormetic concentration of imidacloprid. It is thus possible that the regulation of p53 by MAP3K7/TAK-1 was associated with imidacloprid induced reproductive hormesis in *P. maculiventris*. Given Nrf2 may be a key moderator of the hormetic response (Calabrese and Kozumbo, 2021a), the p53-Nrf2 regulatory axis should be further studied and considered as a mechanism behind hormetic responses.

Detoxification

I expected to see significant differences in the expression of many genes involved in detoxification in insects treated with a hormetic concentration of imidacloprid compared with control. Hormesis in response to insecticides in insects is often associated with differential expression, mainly increased expression, of detoxification genes (Chapter 4) (Rix and Cutler, 2022). However, I found only a small number of differentially expressed transcripts involved in detoxification in insects exposed to a hormetic concentration of imidacloprid. I saw increased expression of a glutathione-s-transferase transcript and decreased expression of UDP-glucuronosyltransferase 2B9, and esterase FE4-like (Table 5.2). While I expected to see more increased expression of detoxification genes, dynamic expression of detoxification genes during and following the course of hormetic insecticide exposure has been shown across numerous studies (Celorio-Mancera et al., 2011; Rix et al., 2016; Liu et al., 2019a; Rix and Cutler, 2022), and is dependent upon the

extent of the exposure, and recovery time. I collected insects 24 hours following the initial imidacloprid exposure, and therefore the insects may have moved out of an initial detoxification phase and into a repair/recovery phase, given transcripts involved in DNA repair are largely increased observed in insects treated with a hormetic concentration of insecticide.

Table 5.2: Differential expression of select biologically relevant transcripts associated with detoxification in *Podisus maculiventris* treated with a hormetic concentration (0.5 mg/L) of imidacloprid compared with a control.

Sequence Description	Transcript ID	Fold Change
esterase FE4-like	DN1366_c0_g1_i2	-4.17
glutathione S-transferase-like	DN1156_c0_g1_i1	1.51
UDP-glucuronosyltransferase 2B9	DN2091_c0_g1_i1	-2.02

Metabolism

The minimal detoxification response may explain why I observed reduced expression of some genes involved in metabolic processes under a hormetic exposure, as detoxification is energy intensive (Berenbaum and Zangerl, 1994; Castañeda et al., 2009). Previous work in the model organism, *Caenorhabditis elegans* (Maupas), has shown metabolic processes dominate expression patterns during initial exposure to an organophosphorus insecticide, and expression of DNA repair mechanisms dominate during recovery (Lewis et al., 2013). I observed decreased expression of transcripts involved in lipid, carbohydrate, and energy metabolism. I observed decreased expression of transcripts such as long-chain-fatty-acid--CoA ligase 5, very long-chain specific acyl-CoA dehydrogenase (mitochondrial), probable medium-chain specific acyl-CoA dehydrogenase (mitochondrial), hydroxyacyl-coenzyme A dehydrogenase, mitochondrial-like, and pancreatic triacylglycerol lipase-like (Table 5.3) which are

involved in the degradation of lipids for energy production (McAndrew et al., 2008; He et al., 2011; Klett et al., 2017; Lebenzon et al., 2021). I also saw decreased expression of a transcript encoding a maltase A2-like protein (Table 5.3). Maltase enzymes catalyze the hydrolysis of maltose into glucose, which can then be used to generate energy through glycolysis (Turner and Turner, 1980). I did however, observe increased expression of an isocitrate dehydrogenase [NADP] cytoplasmic-like transcript and a 2-acylglycerol O-acyltransferase 1-like transcript, which are involved in lipid biosynthesis (Koh et al., 2004; Majerowicz et al., 2017), suggesting the hormetically treated insects are reducing the breakdown of energy sources and moving into storing energy, which could be used for other processes such as reproduction. Further, I observed decreased expression of multiple transcripts encoding enzymes in the tricarboxylic acid (TCA) cycle, which work in tandem to breakdown and release stored energy in acetyl-CoA derived from the breakdown of carbohydrates, lipids, and some proteins (Fernie et al., 2004). Further decreased were transcripts encoding proteins that make up the components of the electron transport chain (ETC; mitochondrial respiratory system) complexes (Table 5.3). This included NADH dehydrogenase transcripts (ETC complex I), succinate dehydrogenase transcripts (ETC complex II), cytochrome b-c1 transcripts (ETC complex III), and cytochrome c oxidase transcripts (ETC complex IV) which use electrons donated from NADH and $FADH_2$ generated by the TCA cycle to drive the production of ATP, the main source of cellular energy, in complex V (ATP synthase); expression of ATP synthase transcripts was also decreased (Fernie et al., 2004; Meng et al., 2019a; Nolfi-Donegan et al., 2020). The decreased expression of genes involved in lipid and carbohydrate catabolism, and decreased expression of genes involved in the TCA cycle and ETC

suggests the insects are slowing down energy production. Decreased expression of genes in the ETC has also been observed in other insects exposed to sublethal doses of insecticides, such as *P. xylostella*, exposed to chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad, where preconditioning hormesis was observed (Gao et al., 2018). Decreased expression of genes involved in carbohydrate metabolism such as glycolysis and the ETC were also observed in *C. suppressalis* exposed to sublethal chlorantraniliprole (Meng et al., 2019b), and in the aquatic insect, *Chironomus dilutes* (Fabricius), exposed to sublethal levels of imidacloprid (Wei et al., 2020). Genes involved in the TCA cycle and ETC were both decreased in the mosquito, *Anopheles coluzzii* Coetzee and Wilkerson, exposed to low doses of deltamethrin, and similar to what I observed in *P.* maculiventris, this was also coordinated with increased expression of genes involved with DNA repair, in particular p53, which was consistently increased throughout the insecticide recovery period (Ingham et al., 2021). It has been proposed that reduced energy metabolism associated with sublethal insecticide exposure may be beneficial, allowing the insect to survive the stress (Gao et al., 2018). This could indicate that energy metabolism is tightly regulated at low levels of insecticide-induced stress, resulting in recovery of energy loss and the regulation of energy balance, and preserving energy so as not to induce resource trade-offs. Efficient use of energy resources is likely an important factor in survival to insecticide stress and could therefore be an important factor in hormesis.

It is also thought that reduced energy metabolism under low doses of insecticide stress is a compensatory mechanism to reduce mitochondrial ROS production due to exogenous ROS production from insecticide exposure (Ingham et al., 2021). If energy

metabolism is reduced as a compensatory mechanism under low levels of insecticide-induced stress, but at a hormetic level the insecticide is detoxified quickly, a reduction of energy metabolism with a corresponding increase in DNA repair or other aspects of the DNA damage response could be the mechanism by why which protective and repair processes are over compensatory, in slight excess of what is needed to repair and recover from the stress. This could be one of the ways in which insecticide-induced hormesis manifests.

Table 5.3: Differential expression of select biologically relevant transcripts associated with metabolism in *Podisus maculiventris* treated with a hormetic concentration (0.5 mg/L) of imidacloprid compared with a control.

Sequence Description	**Transcript ID**	**Fold Change**
Lipid Metabolism		
Long-chain-fatty-acid--CoA ligase 5	DN5849_c0_g1_i1	-2.24
pancreatic triacylglycerol lipase-like	DN2094_c0_g1_i1	-2.08
probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial	DN1905_c0_g1_i1	-1.93
very long-chain specific acyl-CoA dehydrogenase, mitochondrial	DN6266_c0_g1_i2	-2.36
hydroxyacyl-coenzyme A dehydrogenase, mitochondrial-like	DN1017_c0_g1_i2	-2.39
isocitrate dehydrogenase [NADP] cytoplasmic-like	DN1790_c0_g1_i1	1.38
2-acylglycerol O-acyltransferase 1-like	DN14015_c0_g1_i1	1.29
Carbohydrate Metabolism		
maltase A2-like isoform X2	DN4146_c2_g1_i1	-1.78
Energy Metabolism		
TCA Cycle		

Sequence Description	Transcript ID	Fold Change
Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	DN6972_c0_g1_i1	-2.19
isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	DN16209_c0_g1_i1	-2.57
malate dehydrogenase, mitochondrial	DN3834_c0_g1_i1	-1.94
probable citrate synthase 2, mitochondrial	DN3115_c0_g1_i1	-1.83
fumarate hydratase, mitochondrial-like isoform X1	DN2026_c0_g1_i1	-1.69
probable isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial isoform X1	DN14083_c0_g1_i1	-1.98
succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	DN16409_c0_g1_i1	-2.10
succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	DN1332_c0_g1_i2	-1.76
probable aconitate hydratase, mitochondrial	DN5873_c0_g1_i1	-2.44
dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial-like	DN16218_c0_g1_i1	-2.12
<u>Electron Transport Chain</u>		
cytochrome b-c1 complex subunit 2, mitochondrial	DN3415_c0_g1_i1	-1.93
cytochrome b-c1 complex subunit 7-like	DN2084_c0_g1_i2	-2.27
cytochrome b-c1 complex subunit Rieske, mitochondrial	DN8165_c0_g1_i1	-1.93
cytochrome c oxidase subunit 4 isoform 1, mitochondrial-like	DN2698_c0_g2_i1	-1.65
cytochrome c oxidase subunit 5A, mitochondrial	DN15596_c0_g1_i1	-1.85

Sequence Description	Transcript ID	Fold Change
cytochrome c oxidase subunit 5B, mitochondrial-like	DN10367_c0_g1_i2	-2.18
cytochrome c oxidase subunit NDUFA4	DN1602_c0_g1_i1	-1.87
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	DN15893_c0_g1_i1	-1.55
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	DN8570_c0_g1_i1	-1.64
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	DN6168_c0_g1_i1	-1.63
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	DN10786_c0_g1_i1	-1.89
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	DN10039_c0_g1_i1	-1.5
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	DN1321_c0_g1_i1	-1.68
NADH dehydrogenase [ubiquinone] 1 subunit C2	DN3512_c0_g1_i1	-1.69
NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial	DN249_c0_g3_i1	-2.16
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	DN3398_c0_g1_i1	-1.61
NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	DN6921_c0_g1_i1	-1.56
NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	DN7624_c0_g1_i1	-1.83
NADH-quinone oxidoreductase subunit B 2-like	DN15960_c0_g1_i1	-1.85
NADH-ubiquinone oxidoreductase 49 kDa subunit	DN6071_c0_g1_i1	-2.38

Sequence Description	Transcript ID	Fold Change
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	DN5255_c0_g1_i2	-2.09
succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial	DN3031_c0_g1_i1	-1.70
succinate dehydrogenase cytochrome b560 subunit, mitochondrial-like	DN466_c0_g1_i1	-1.98
ATP synthase lipid-binding protein, mitochondrial	DN6344_c0_g1_i1	-1.62
ATP synthase subunit alpha, mitochondrial	DN16402_c0_g1_i1	-1.95
ATP synthase subunit b, mitochondrial	DN10949_c0_g2_i1	-1.62
ATP synthase subunit beta, mitochondrial	DN1800_c0_g1_i1	-1.99
ATP synthase subunit gamma, mitochondrial	DN16545_c0_g1_i1	-1.57

Growth, Development, and Reproduction

I expected to see significant increases in genes directly involved in reproduction associated with a reproductively hormetic concentration of imidacloprid. Interestingly, in insects treated with a reproductively hormetic concentration of imidacloprid, I observed decreased expression of two transcripts encoding vitellogenin, a yolk protein involved in oocyte maturation in insects (Roy et al., 2018). I did, however, observe an increase in expression of the vitellogenin receptor, which is responsible for the uptake of vitellogenin in developing oocytes (Table 5.4) (Wu et al., 2021). This could suggest that insects had an increased uptake of vitellogenin into the ovaries, and further vitellogenin production was not needed, thus vitellogenin gene expression was decreased while the vitellogenin receptor was increased. Increased vitellogenin gene expression, however, is often associated with enhanced reproduction (Huang et al., 2016; Lu et al., 2017b; Ullah et al.,

2019), although this may not always be the case. For example, expression of *Vg-1*, *Vg-2* and the vitellogenin receptor, *VgR*, were significantly reduced in *B. dorsalis* for 7 days following exposure to a low dose of cyantraniliprole that also increased fecundity (Li et al., 2022). Acute heat stress that increased fecundity in the melon fly, *Bactrocera cucurbitae* (Coquillett), resulted in reduced expression of *Vg-1*, *Vg-2*, but also increased expression of *Vg-3* and *VgR* (Zhou et al., 2020), similar to insects in my experiments. In the Oriental armyworm, *Mythimna separata* (Walker), exposure to lambda-cyhalothrin increased fecundity, net reproductive rate, and rate of population increase. This was accompanied by dynamic changes (up and down) in *Vg* and *VgR* expression over the oviposition period, while control expression of these genes remained stable over time (Li et al., 2019). Thus, my findings regarding the expression of vitellogenin related genes in insects treated with a hormetic imidacloprid concentration are consistent with reports from others. It is possible that dynamic expression of vitellogenin related genes over time, following stress, plays a role in reproductive hormesis, however, more research is needed to understand how changes in the expression patterns of vitellogenin related genes impact reproductive hormesis.

In addition, I did observe increased expression of an E78 transcript (ecdysone-induced protein 78C isoform X2), known to be involved in oogenesis and proper egg formation (Ables et al., 2015), and a juvenile hormone acid O-methyltransferase (JHAMT) transcript (Table 5.4), involved in the regulation of juvenile hormone synthesis, which is important for regulating vitellogenin synthesis and uptake into the developing oocytes (Roy et al., 2018; Wu et al., 2021). Increased expression of *JHAMT* was also observed in *C. lividipennis*, following imidacloprid exposure that induced

reproductive hormesis (Zhu et al., 2020). Silencing of JHAMT removed the hormetic effect suggesting JHAMT played a key role in imidacloprid induced reproductive hormesis in *C. lividipennis* (Zhu et al., 2020), and thus may also have played a role in reproductive hormesis observed in *P. maculiventris*.

Table 5.4: Differential expression of select biologically relevant transcripts associated with growth, development, and reproduction in *Podisus maculiventris* treated with a hormetic concentration (0.5 mg/L) of imidacloprid compared with control.

Sequence Description*	**Transcript ID**	**Fold Change**
ecdysone-induced protein 78C isoform X2	DN9595_c0_g1_i1	1.36
juvenile hormone acid O-methyltransferase-like	DN5075_c0_g1_i9	1.65
vitellogenin	DN1006_c5_g3_i2	-6.96
	DN685_c0_g1_i1	-5.06
vitellogenin receptor	DN3123_c0_g1_i1	1.64

* Additional transcript IDs following a sequence description are gene variants.

5.3.3.2 Differential Expression in Response to an Inhibitory Concentration of Imidacloprid

As previously hypothesized, I observed far more differentially expressed transcripts when insects were exposed to an inhibitory concentration (100.0 mg/L) of imidacloprid compared with control and hormetic (0.5 mg/L) concentrations. Compared with control, insects treated with an inhibitory concentration of imidacloprid had 1245 transcripts with FC >1 increased expression, and 965 with FC >1 decreased expression (Log_2 fold change > 0; adj p-value ≤ 0.01) (Fig 5.2, Table S1; see Appendix 2). When compared with a hormetic concentration, insects treated with an inhibitory concentration had 1942 transcripts with FC >1 increased expression, and 1339 with FC >1 decreased expression (Log_2 fold change > 0; adj p-value ≤ 0.01) (Fig 5.2, Table S1; see Appendix 2).

DNA Damage Response

Transcripts related to the DNA damage response were also increased in insects treated with the inhibitory concentration of imidacloprid (100.0 mg/L) (Table 5.5, Table 5.6). Increased expression of transcripts involved in autophagy and apoptosis were observed. While essential for normal cellular maintenance and maintenance of homeostasis during stress, high levels of stress can cause excessive levels of autophagy and apoptosis, which can be deleterious (Shintani and Klionsky, 2004; Elmore, 2007). The BCL-2 family of proteins are important regulators of autophagy and apoptosis (Singh et al., 2019). I observed a significant increase in the expression of a transcript encoding a BCL-2 protein, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) in insects exposed to the inhibitory concentration of imidacloprid compared with control and hormetic concentration treatments (Table 5.5, Table 5.6). Under stress, BNIP3 proteins localize to the mitochondria where they increase production of ROS through release and subsequent increase of cytochrome c (also increased in insects treated with an inhibitory concentration of imidacloprid), resulting in cell damage and inducing cell death, whether apoptotic, autophagic, or necrotic (Burton and Gibson, 2009). This is consistent with changes I observed in the expression (mainly increased) of a number of transcripts encoding antioxidants, including peroxiredoxin transcripts, thioredoxin transcripts, and superoxide dismutase transcripts (Table S1; see Appendix 2) (Lee et al., 2013; Perkins et al., 2015; Garcia-Caparros et al., 2020) in insects exposed to an inhibitory concentration compared with either control or hormetic concentrations. It is also consistent with observed expression changes in a number of autophagy-related protein transcripts. I observed a high number of transcripts encoding cathepsins compared with control and

hormetic concentrations (Table S1; see Appendix 2). Among their many cellular functions, cathepsins are involved in autophagy. High levels of autophagy are commonly observed in dying cells (Yu et al., 2004; Debnath et al., 2005). This is further consistent with observed increases in death-associated protein kinase related-like, death ligand signal enhancer-like isoform X1, and death-inducer obliterator 1 isoform X1 transcripts in insects treated with an inhibitory concentration compared with a control or a hormetic concentration (Table 5.5, Table 5.6). These are all involved in autophagy, apoptosis and cell death (Fulda et al., 2010; Green and Llambi, 2015; Singh et al., 2016). The involvement of BNIP3 in mediating autophagy, apoptosis, and cell death could be associated with the negative effects on reproduction and survival in insects treated with the inhibitory concentration of imidacloprid. When exposed to plant defenses in resistant rice plants, *BNIP3* gene expression increased in *N. lugens*, and was associated with reduced survival, weight gain, and honey dew secretions (Zhang et al., 2019). Similarly, *H. armigera* showed increased *BNIP3* and *cytochrome c* expression when exposed to an anthraquinone from an endophyte derivative of the *Acremonium vitellinum* Gams fungus having insecticidal activity (Yuan et al., 2020).

Table 5.5: Differential expression of select biologically relevant transcripts associated with the DNA damage response in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with a control.

Sequence Description*	**Transcript ID**	**Fold Change**
BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	DN4558_c0_g2_i1	2.50
death-associated protein kinase related-like	DN336_c2_g1_i1	2.27
death-inducer obliterator 1 isoform X1	DN10532_c0_g1_i1	2.56
Heat shock 70 kDa protein	DN330_c0_g1_i2	7.34
heat shock 70 kDa protein 12A isoform X1	DN6949_c0_g1_i2	2.00

Sequence Description*	Transcript ID	Fold Change
heat shock 70 kDa protein cognate 5	DN4491_c0_g1_i1	1.48
heat shock protein 83	DN516_c0_g1_i3	-1.84
dnaJ homolog subfamily C member 13 isoform X2	DN5784_c0_g1_i1	1.77
dnaJ homolog subfamily C member 2	DN1793_c1_g1_i1	-1.47
dnaJ homolog subfamily C member 21	DN2200_c0_g1_i1	-1.65
major heat shock 70 kDa protein Ba-like	DN330_c0_g1_i22	4.65
	DN330_c0_g1_i5	5.76
myb-like protein X	DN1901_c0_g1_i9	-1.83
	DN1901_c0_g1_i4	-1.81
nuclear factor NF-kappa-B p105 subunit isoform X1	DN14429_c0_g2_i1	1.81
DNA repair and recombination protein RAD54-like	DN16198_c0_g1_i1	1.68
DNA repair and recombination protein RAD54B-like	DN13602_c0_g1_i1	-1.73
ras GTPase-activating protein-binding protein 2	DN16417_c0_g1_i1	1.60
ras-like GTP-binding protein Rho1 isoform X2	DN15957_c0_g1_i1	1.31
	DN4831_c0_g1_i1	1.55
ras-related GTP-binding protein C	DN2457_c0_g1_i1	2.80
ras-related protein Rab-14	DN3111_c0_g1_i1	1.55
ras-related protein Rab-18-B-like	DN1595_c0_g1_i2	1.83
ras-related protein Rab-39B	DN6317_c0_g1_i1	1.64
ras-related protein Rab-44-like isoform X4	DN570_c0_g1_i3	2.81
ras-related protein Rab-4B	DN16295_c0_g1_i1	1.88
ras-related protein Rab-5B	DN2107_c2_g1_i1	1.27
ras-related protein Rab-8A isoform X2	DN7400_c0_g1_i1	1.31
ras-related protein rab7	DN2661_c0_g1_i1	1.57
ras-related protein Rap-2a	DN4778_c0_g1_i1	1.31

Sequence Description*	Transcript ID	Fold Change
ras-related protein Rap1	DN15895_c0_g1_i1	1.25
rho GTPase-activating protein 20-like isoform X3	DN808_c0_g1_i2	2.61
rho GTPase-activating protein 21 isoform X6	DN5148_c0_g1_i1	-1.37
rho GTPase-activating protein 27-like isoform X1	DN4631_c0_g1_i1	1.46
rho GTPase-activating protein 44-like isoform X1	DN2923_c0_g1_i2	-4.48

* Additional transcript IDs following a sequence description are gene variants.

Yuan et al. (2020) additionally observed increased expression of *FOXO* (forkhead box protein O), and genes in the Ras superfamily (Rho and Rab families) associated with the deleterious effects of the anthraquinone derivative. This was also true of insects in my study that were treated with an inhibitory concentration of imidacloprid compared with either a control or hormetic concentration (Table 5.5, Table 5.6). Both FOXO and proteins in the Ras superfamily play complex roles in cell cycle regulation and growth, autophagy, apoptosis, metabolism, and the oxidative stress response (Furukawa-Hibi et al., 2005; Hay, 2011; Goitre et al., 2014). Increased *FOXO* expression under low levels of stress has been associated with hormetic increases in longevity and stress resistance in *D. melanogaster* through regulation of the IIS/TOR signalling pathway and autophagy (Chattopadhyay et al., 2017; Lushchak et al., 2019). Under higher levels of stress, however, increased *FOXO* expression has been associated with deleterious effects. For example, Cui et al. (2020), who observed that LC_{30} and LC_{50} concentrations of benzothiazole that reduced growth, fecundity, and fertility in *T. castaneum* also had

increased *FOXO* expression, consistent with observed inhibition of fecundity in insects in my experiments.

Table 5.6: Differential expression of select biologically relevant transcripts associated with the DNA damage response in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with a hormetic (0.5 mg/L) concentration of imidacloprid.

Sequence Description*	**Transcript ID**	**Fold Change**
BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	DN4558_c0_g2_i1	3.45
death-associated protein kinase related-like	DN336_c2_g1_i1	2.58
death ligand signal enhancer-like isoform X1	DN5248_c0_g2_i1	1.53
DNA excision repair protein ERCC-1	DN15677_c0_g1_i1	-1.48
DNA repair and recombination protein RAD54-like	DN16198_c0_g1_i1	1.30
DNA repair and recombination protein RAD54-like isoform X1	DN3313_c0_g1_i1	-2.15
DNA repair and recombination protein RAD54B-like	DN13602_c0_g1_i1	-2.47
DNA repair protein RAD50	DN6986_c0_g1_i1	-1.39
DNA repair protein RAD51 homolog 1	DN13262_c0_g1_i1	-1.49
DNA repair protein RAD51 homolog 4	DN10104_c0_g1_i1	-1.95
dnaJ homolog subfamily C member 13 isoform X2	DN5784_c0_g1_i1	1.79
dnaJ homolog subfamily C member 2	DN1793_c1_g1_i1	-1.52
dnaJ homolog subfamily C member 21	DN2200_c0_g1_i1	-1.72
dnaJ homolog subfamily C member 28	DN4083_c0_g1_i1	1.57
dnaJ homolog subfamily C member 9	DN1293_c0_g1_i1	-1.46
forkhead box protein O isoform X1	DN16455_c0_g1_i1	2.95
Heat shock 70 kDa protein	DN330_c0_g1_i2	5.96
heat shock 70 kDa protein 12A isoform X1	DN6949_c0_g1_i2	2.47
heat shock 70 kDa protein 14-like	DN2222_c0_g1_i1	-1.36

Sequence Description*	Transcript ID	Fold Change
heat shock 70 kDa protein cognate 5	DN4491_c0_g1_i1	1.65
heat shock protein 83	DN516_c0_g1_i3	-1.91
major heat shock 70 kDa protein Ba-like	DN330_c0_g1_i5	10.69
myb-like protein X	DN1901_c0_g1_i3	-8.65
	DN1901_c0_g1_i4	-1.85
nuclear factor NF-kappa-B p105 subunit isoform X1	DN14429_c0_g2_i1	3.06
ras-like GTP-binding protein Rho1	DN15723_c0_g1_i1	2.96
ras-like GTP-binding protein Rho1 isoform X2	DN4831_c0_g1_i1	1.68
	DN15957_c0_g1_i1	1.80
ras-like GTP-binding protein RhoL isoform X2	DN3920_c0_g1_i1	2.47
ras-related protein Rab-10	DN3700_c0_g1_i1	1.34
ras-related protein Rab-18-B-like	DN1595_c0_g1_i2	2.04
ras-related protein Rab-1A	DN1172_c0_g1_i1	1.42
ras-related protein Rab-21	DN4067_c0_g1_i1	-1.23
Ras-related protein Rab-30	DN6876_c0_g1_i1	1.47
ras-related protein Rab-39B	DN6317_c0_g1_i1	2.36
ras-related protein Rab-44-like isoform X4	DN570_c0_g1_i3	1.89
ras-related protein rab7	DN2661_c0_g1_i1	1.86
ras-related protein Rap1	DN15895_c0_g1_i1	1.27
rho GTPase-activating protein 20-like isoform X3	DN808_c0_g1_i2	2.13
rho GTPase-activating protein 21 isoform X6	DN5148_c0_g1_i1	-1.21
rho GTPase-activating protein 27-like isoform X1	DN4631_c0_g1_i1	1.29
rho GTPase-activating protein 44-like isoform X1	DN2923_c0_g1_i2	-4.36

* Additional transcript IDs following a sequence description are gene variants.

Imidacloprid and other neonicotinoids at high concentrations are well known to cause excessive oxidative stress (Ge et al., 2015; Wang et al., 2016a; Qi et al., 2018; Wei et al., 2020). I observed increased expression of nuclear factor NF-kappa-B p105 subunit isoform X1 transcript in insects treated with an inhibitory concentration compared with control and hormetic concentrations (Table 5.5, Table 5.6). Expression of members of the nuclear factor kappa beta (NFκB) family are indicative of high levels of oxidative stress and are involved in associated pathologies (Calabrese et al., 2012). Excessive oxidative stress leads to genotoxic effects (DNA damage) in insects and other organisms (Ge et al., 2015; Wang et al., 2016a; Qi et al., 2018; Wei et al., 2020), to which germline cells are highly susceptible (Metcalfe and Alonso-Alvarez, 2010), thereby negatively affecting reproduction.

In insects treated with an inhibitory concentration of imidacloprid, I interestingly observed decreased expression in a number of transcripts involved in DNA repair that were increased in insects treated with a reproductively hormetic concentration of imidacloprid. I observed decreased expression of DNA excision repair protein ERCC-1 when insects were treated with an inhibitory concentration of imidacloprid compared with a hormetic concentration, and decreased expression of myb-like protein X when compared with both a control and hormetic concentration (Table 5.5, Table 5.6). In addition, I observed decreased expression in a number of transcripts encoding RAD proteins (although one was increased) when compared with both control and hormetic concentrations (Table 5.5, Table 5.6). The decreased expression of some transcripts involved in DNA-repair could also have played a role in the deleterious effects on reproduction or survival observed after exposure to a high concentration of imidacloprid.

In *D. melanogaster*, mutations in myb during oogenesis reduced embryonic viability, and myb mutants showed impaired development and survival under stress. This demonstrates the essential function of myb in stress mitigation, the maintenance of survival and reproduction under stress (Katzen and Bishop, 1996). Similarly, work in mice models showed that Rad54 was required for normal germ cell development under stress (Messiaen et al., 2013). Therefore, this could indicate that some aspects of DNA repair are regulated differently at high doses than they are at low doses, with cell death dominating, having negative effects on reproduction and survival.

I observed a greater number of heat shock proteins with increased expression in insects treated with high concentrations of imidacloprid. I observed increased expression in several Hsp70 transcripts in insects treated with the inhibitory concentration of imidacloprid when compared with control and hormetic concentrations, and I also observed increased or decreased expression of several members of the Hsp40 family, dnaJ homolog subfamily C members 2, 13, 21 (inhibitory vs control), and dnaJ homolog subfamily C members 2, 9, 13, 21, 28 (inhibitory vs hormetic) (Table 5.5, Table 5.6). Differential expression of genes encoding Hsp70s and Hsp40s (both up and down) have been observed in response to pesticide exposure in insects (Park et al., 2010; Lencioni et al., 2016; Yang et al., 2016a; Lu et al., 2017a). While Hsp70 has been implicated in hormesis, overexpression of Hsp70 is energy intensive and may revert resources that would otherwise be used for reproduction (Sorensen et al., 2003; Huang et al., 2007). I also observed a decrease in the expression of Hsp83 (the insect homolog of Hsp90) in insects treated with the inhibitory concentration of imidacloprid compared with both control and hormetic concentrations. Hsp90 mediates biological fitness under stressful

conditions by regulating physiological responses to environmental conditions (Will et al., 2017). Decreased expression of *Hsp90* is associated with reductions in stress tolerance, growth, longevity, fecundity, and fertility in insects (Chen and Wagner, 2012; Will et al., 2017; Farahani et al., 2020).

Detoxification

Under the inhibitory imidacloprid exposure, I observed significant increases in the expression of multiple classes of detoxification genes when compared with control and hormetic concentrations of imidacloprid. This included UDP-glucuronosyltransferases which catalyze the addition of sugars to lipophilic molecules, a critical role in the elimination of exogenous chemicals (Meech et al., 2019); cytochrome P450s, which largely act as monooxygenases, catalyzing the reduction of chemical substrates (Feyereisen, 2012); glutathione-s-transferases, which metabolize xenobiotics through conjugation with glutathione (Hayes et al., 2005), and FE4 esterases, which metabolize xenobiotics by hydrolyzing carboxylesters into alcohol and carboxylic acid (Hatfield et al., 2016) (Table 5.7, Table 5.8). I also observed increased expression of ABC transporters in insects treated with the inhibitory concentration of imidacloprid compared with the hormetic treatment, but not when compared with control (Table 5.7, Table 5.8). High doses of neonicotinoids have been shown to rapidly induce the expression of a wide variety of detoxification genes (Liang et al., 2015; Yang et al., 2016b). The classes of detoxification genes I observed have all been shown to be induced by neonicotinoids and involved in their metabolism (Johnson et al., 2012; Yang et al., 2016b; Kaplanoglu et al., 2017; Chen et al., 2019; He et al., 2019; Liu et al., 2019b; Tian et al., 2019; Xia et al., 2019). The significant increase of multiple transcripts across different classes of

detoxification genes suggests that 24 hours following exposure to the inhibitory imidacloprid concentration, insects were still working to detoxify the imidacloprid.

Table 5.7: Differential expression of select biologically relevant transcripts associated with detoxification in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with a control.

Sequence Description*	Transcript ID	Fold Change
cytochrome P450 6a2-like	DN14341_c0_g1_i1	1.98
	DN4255_c0_g1_i1	2.05
	DN11223_c0_g1_i1	2.15
	DN10431_c0_g2_i1	6.60
cytochrome P450 6B1	DN9396_c0_g1_i1	4.10
esterase FE4	DN12154_c0_g2_i1	2.85
esterase FE4-like	DN5485_c1_g4_i1	4.15
glutathione S-transferase	DN5214_c0_g1_i1	-3.52
glutathione S-transferase D8	DN3177_c0_g1_i1	2.49
glutathione S-transferase theta-1-like	DN1290_c0_g1_i1	1.34
glutathione S-transferase-like	DN3897_c0_g1_i1	1.76
	DN10645_c0_g1_i1	2.80
	DN6717_c0_g1_i1	3.31
probable cytochrome P450 6a14	DN7550_c0_g1_i1	2.91
probable cytochrome P450 6d5	DN7887_c0_g1_i3	4.52
	DN870_c0_g2_i2	4.63
putative cytochrome P450 6a14	DN3717_c0_g1_i1	2.66
UDP-glucuronosyltransferase 2B9	DN2091_c0_g1_i1	3.04
UDP-glucuronosyltransferase 2C1	DN75_c0_g1_i1	2.29
	DN5988_c0_g2_i1	2.48
	DN6192_c0_g1_i1	2.75
	DN75_c0_g1_i2	5.09
UDP-glycosyltransferase UGT5-like	DN1775_c0_g1_i1	1.52

* Additional transcript IDs following a sequence description are gene variants

Table 5.8: Differential expression of select biologically relevant transcripts associated with detoxification in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with an hormetic (0.5 mg/L) concentration of imidacloprid.

Sequence Description*	**Transcript ID**	**Fold Change**
ATP-binding cassette sub-family A member 3-like isoform X3	DN11022_c0_g2_i1	2.82
	DN11022_c0_g4_i1	4.79
ATP-binding cassette sub-family B member 7, mitochondrial isoform X2	DN13192_c0_g1_i1	2.29
ATP-binding cassette sub-family F member 1	DN12499_c1_g1_i2	-1.63
ATP-binding cassette sub-family G member 1	DN11385_c0_g1_i1	2.57
ATP-binding cassette sub-family G member 4	DN176_c1_g1_i7	3.41
cytochrome P450 306a1 isoform X1	DN8825_c0_g1_i1	-3.04
cytochrome P450 6a2-like	DN11223_c0_g1_i1	1.96
	DN14341_c0_g2_i1	2.11
	DN13600_c0_g2_i1	2.40
	DN4255_c0_g1_i1	3.14
	DN738_c0_g1_i2	4.19
	DN10431_c0_g2_i1	8.90
cytochrome P450 6B1	DN9396_c0_g1_i1	2.72
cytochrome P450 6j1-like isoform X1	DN9280_c0_g1_i1	3.59
esterase FE4	DN3945_c0_g1_i1	2.03
	DN12154_c0_g2_i1	3.12
	DN10579_c0_g1_i1	3.20
	DN12154_c0_g1_i1	5.67
esterase FE4-like	DN1366_c0_g1_i2	7.08
	DN5485_c1_g4_i1	7.80
glutathione S-transferase D8	DN3177_c0_g1_i1	2.81
glutathione S-transferase-like	DN1156_c0_g1_i1	-1.61
	DN9408_c0_g1_i1	1.98
	DN3897_c0_g1_i1	2.274

Sequence Description*	Transcript ID	Fold Change
	DN6717_c0_g1_i1	3.06
	DN10645_c0_g1_i1	4.11
probable cytochrome P450 6a13 isoform X2	DN9669_c0_g1_i1	2.58
probable cytochrome P450 6a14	DN7550_c0_g1_i1	4.93
probable cytochrome P450 6d5	DN870_c0_g1_i1	1.85
	DN6282_c0_g1_i1	1.92
	DN10327_c0_g2_i1	2.81
	DN7887_c0_g1_i3	3.45
	DN7887_c0_g1_i1	4.35
UDP-glucuronosyltransferase 1-9	DN590_c1_g1_i6	3.48
UDP-glucuronosyltransferase 2B13-like	DN2368_c0_g2_i1	3.29
UDP-glucuronosyltransferase 2B16-like isoform X2	DN4075_c0_g1_i2	2.52
UDP-glucuronosyltransferase 2B9	DN2091_c0_g1_i1	6.13
UDP-glucuronosyltransferase 2C1	DN75_c0_g1_i1	2.39
	DN9178_c0_g1_i1	2.78
	DN5988_c0_g2_i1	2.93
	DN6192_c0_g1_i1	3.55
	DN5988_c0_g1_i1	3.66
	DN75_c0_g1_i2	8.34

* Additional transcript IDs following a sequence description are gene variants.

Metabolism

As previously discussed, detoxification is energy intensive. In contrast to insects exposed to a hormetic concentration, expression of genes involved in metabolic processes were significantly increased in my insects exposed to an inhibitory concentration of imidacloprid compared with control and hormetic concentrations. For example, I observed significant increases in multiple lipophorin receptor transcripts, lipid storage

droplets surface-binding protein transcripts, and a patatin-like phospholipase domain-containing protein 3 transcript compared with both control and hormetic concentrations of imidacloprid (Table 5.9, Table 5.10). These are involved in the transport of lipids, and regulation of lipid metabolism and homeostasis (Ryan and Van Der Horst, 2000; Kienesberger et al., 2009). I observed significant increases in lipid catabolic transcripts that were decreased in hormetic insects, including long-chain-fatty-acid--CoA ligase 5, very long-chain specific acyl-CoA dehydrogenase (mitochondrial), probable medium-chain specific acyl-CoA dehydrogenase (mitochondrial), and pancreatic triacylglycerol lipase-like, in addition to increased expression of lipase-like 3 transcripts when insects were treated with an inhibitory concentration of imidacloprid compared with control or hormetic concentrations (Table 5.9, Table 5.10).

Table 5.9: Differential expression of select biologically relevant transcripts associated with metabolism in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with a control.

Sequence Description*	**Transcript ID**	**Fold Change**
Lipid Metabolism		
lipase 3-like	DN10427_c0_g1_i1	2.95
	DN1442_c0_g1_i1	4.47
lipid storage droplets surface-binding protein 2	DN14206_c0_g1_i1	1.85
lipophorin receptor	DN644_c2_g1_i1	1.24
	DN2627_c0_g1_i1	2.59
Carbohydrate Metabolism		
trehalase-like isoform X2	DN3424_c1_g2_i1	3.64
facilitated trehalose transporter Tret1	DN16448_c0_g1_i1	2.34
facilitated trehalose transporter Tret1-2 homolog	DN724_c0_g1_i1	-2.31
facilitated trehalose transporter Tret1-like	DN4273_c0_g3_i1	1.70

Sequence Description*	Transcript ID	Fold Change
	DN3248_c0_g1_i1	5.07
	DN2827_c0_g2_i1	5.26
maltase A1-like	DN599_c0_g1_i26	2.46
maltase A3 isoform X2	DN1215_c1_g2_i5	3.56
	DN1215_c1_g2_i4	5.52
maltase A3-like	DN599_c0_g1_i21	6.04
	DN599_c0_g1_i23	6.96
Energy Metabolism		
Glycolysis		
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-like	DN2769_c0_g1_i1	2.01
enolase-like isoform X2	DN8089_c0_g1_i1	1.35
fructose-bisphosphate aldolase	DN9163_c0_g1_i1	1.76
glyceraldehyde-3-phosphate dehydrogenase 2	DN5003_c0_g1_i1	1.64
hexokinase type 2 isoform X3	DN7567_c0_g1_i4	2.43
phosphoglycerate mutase 1	DN8578_c0_g1_i1	1.41
TCA Cycle		
malate dehydrogenase, cytoplasmic	DN1055_c2_g1_i1	1.44
probable pyruvate dehydrogenase E1 component subunit alpha, mitochondrial	DN3872_c0_g1_i1	2.67
Electron Transport Chain		
cytochrome c oxidase assembly protein COX15 homolog	DN1379_c0_g1_i2	1.40
cytochrome c oxidase subunit 6B2-like	DN15972_c0_g1_i1	2.52
cytochrome c oxidase subunit 7A, mitochondrial-like	DN8813_c0_g1_i1	1.36
cytochrome B5	DN15964_c0_g1_i1	1.52

Sequence Description*	Transcript ID	Fold Change
ATP synthase F0 subunit 6	DN5980_c0_g1_i2	-2.36
ATP synthase F0 subunit 6	DN5980_c0_g1_i1	-1.99

* Additional transcript IDs following a sequence description are gene variants.

Carbohydrate metabolism was also increased. I observed mainly increased expression of transcripts involved in trehalose transport, and the breakdown of trehalose, trehalase-like isoform X2 (Table 5.9, Table 5.10). Trehaolse is the major source of sugar for energy production in insects and important for dealing with stress (Shukla et al., 2015). In contrast to insects exposed to a hormetic concentration, exposure to an inhibitory concentration increased expression of maltase transcripts, and additional transcripts involved in glycolysis (Table 5.9, Table 5.10). Transcripts involved in the TCA cycle and components of the electron transport chain were also increased when compared with control and hormetic concentrations (Table 5.9, Table 5.10). Although decreased expression of two ATP synthase transcripts was observed when compared with control, increased expression of several V-type proton ATPase transcripts indicate ATP was being produced and used (Pamarthy et al., 2018) (Table 5.9, Table S1; see Appendix 2). The increase in expression of transcripts involved in lipid and carbohydrate metabolism are an indication of the mobilization of energy reserves, and are consistent with increased resource and energy demands in *P. maculiventris* that would be expected for repair and detoxification following exposure to a much higher, inhibitory concentration of imidacloprid. Increases in lipid and carbohydrate metabolism in the aphid endoparasitoid, *Aphidius gifuensis* Ashmead, were reported following exposure to an LC_{20} of imidacloprid (Kang et al., 2018).

Table 5.10: Differential expression of select biologically relevant transcripts associated with metabolism in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with a hormetic (0.5 mg/L) concentration of imidacloprid.

Sequence Description*	**Transcript ID**	**Fold Change**
Lipid Metabolism		
lipase 3-like	DN8787_c0_g1_i1	2.53
	DN6821_c3_g1_i1	3.53
	DN10427_c0_g1_i1	4.21
	DN1009_c0_g1_i2	5.86
	DN1442_c0_g1_i1	6.10
Long-chain-fatty-acid--CoA ligase 5	DN5849_c0_g1_i1	2.83
pancreatic triacylglycerol lipase-like	DN2094_c0_g1_i1	2.29
probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial	DN1905_c0_g1_i1	1.97
	DN5832_c0_g1_i3	2.54
very long-chain specific acyl-CoA dehydrogenase, mitochondrial	DN6266_c0_g1_i2	2.22
isocitrate dehydrogenase [NADP] cytoplasmic	DN5349_c0_g1_i1	-1.33
lipid storage droplets surface-binding protein 1-like	DN760_c0_g1_i2	2.99
lipid storage droplets surface-binding protein 2	DN14206_c0_g1_i1	2.20
2-acylglycerol O-acyltransferase 1-like	DN758_c0_g1_i1	1.40
lipophorin receptor	DN2627_c0_g1_i1	2.53
Carbohydrate Metabolism		
maltase A1-like	DN599_c0_g1_i26	3.85
maltase A2-like isoform X2	DN4146_c2_g1_i1	1.77
maltase A3 isoform X2	DN1215_c1_g2_i5	4.75
	DN1215_c1_g2_i4	10.34
maltase A3-like	DN599_c0_g1_i21	5.11

Sequence Description*	Transcript ID	Fold Change
	DN599_c0_g1_i23	6.45
trehalase-like isoform X2	DN3424_c1_g2_i1	5.57
facilitated trehalose transporter Tret1	DN3846_c0_g1_i1	2.09
	DN16448_c0_g1_i1	2.74
facilitated trehalose transporter Tret1-2 homolog	DN724_c0_g1_i1	-2.54
facilitated trehalose transporter Tret1-2 homolog isoform X1	DN14397_c0_g1_i2	1.57
facilitated trehalose transporter Tret1-2 homolog isoform X3	DN4751_c0_g2_i1	2.79
facilitated trehalose transporter Tret1-like	DN4273_c0_g3_i1	1.84
	DN1633_c0_g1_i1	2.23
	DN5727_c0_g1_i1	3.14
	DN8651_c0_g2_i1	4.91
	DN2827_c0_g2_i1	6.83
	DN3248_c0_g1_i1	7.82
facilitated trehalose transporter Tret1-like isoform X2	DN3555_c0_g2_i1	1.52
Energy Metabolism		
Glycolysis		
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-like	DN2769_c0_g1_i1	2.40
enolase-like isoform X2	DN8089_c0_g1_i1	1.58
fructose-bisphosphate aldolase	DN9163_c0_g1_i1	1.69
glyceraldehyde-3-phosphate dehydrogenase 2	DN5003_c0_g1_i1	1.61
hexokinase type 2 isoform X3	DN7567_c0_g1_i2	2.90
hexokinase-2-like isoform X1	DN3984_c0_g1_i1	1.56

Sequence Description*	Transcript ID	Fold Change
phosphoglycerate mutase 1	DN8578_c0_g1_i1	1.54
probable citrate synthase 2, mitochondrial	DN3115_c0_g1_i1	2.01
<u>TCA Cycle</u>		
Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	DN6972_c0_g1_i1	1.98
malate dehydrogenase, cytoplasmic	DN1055_c2_g1_i1	1.68
malate dehydrogenase, mitochondrial	DN3834_c0_g1_i1	2.45
probable isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial isoform X1	DN14083_c0_g1_i1	2.09
probable pyruvate dehydrogenase E1 component subunit alpha, mitochondrial	DN3872_c0_g1_i1	2.55
probable pyruvate dehydrogenase E1 component subunit alpha, mitochondrial isoform X2	DN5691_c0_g2_i1	1.77
succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	DN16409_c0_g1_i1	2.05
succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	DN1332_c0_g1_i2	1.94
probable aconitate hydratase, mitochondrial	DN5873_c0_g1_i1	2.65
dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial-like	DN16218_c0_g1_i1	2.40
<u>Electron Transport Chain</u>		
ATP synthase lipid-binding protein, mitochondrial	DN6344_c0_g1_i1	1.59
	DN5130_c0_g1_i1	3.03

Sequence Description*	Transcript ID	Fold Change
ATP synthase subunit alpha, mitochondrial	DN16402_c0_g1_i1	1.97
ATP synthase subunit b, mitochondrial	DN10949_c0_g2_i1	1.76
ATP synthase subunit beta, mitochondrial	DN1800_c0_g1_i1	2.22
ATP synthase subunit d, mitochondrial	DN6891_c0_g1_i1	1.53
ATP synthase subunit delta, mitochondrial	DN3552_c0_g1_i1	1.84
ATP synthase subunit gamma, mitochondrial	DN16545_c0_g1_i1	2.04
ATP synthase subunit O, mitochondrial	DN4325_c0_g1_i1	1.69
cytochrome b-c1 complex subunit 2, mitochondrial	DN3415_c0_g1_i1	2.40
cytochrome b-c1 complex subunit 6, mitochondrial	DN16028_c0_g1_i1	1.59
cytochrome b-c1 complex subunit 7-like	DN2084_c0_g1_i2	2.23
	DN2084_c0_g1_i1	2.37
cytochrome b-c1 complex subunit 8	DN10327_c0_g1_i1	1.86
cytochrome b-c1 complex subunit 9	DN3884_c0_g1_i1	1.90
cytochrome b-c1 complex subunit Rieske, mitochondrial	DN8165_c0_g1_i1	2.63
cytochrome B5	DN15964_c0_g1_i1	1.51
cytochrome c oxidase assembly protein COX11, mitochondrial	DN10251_c0_g1_i1	1.48
cytochrome c oxidase assembly protein COX15 homolog	DN1379_c0_g1_i2	1.40
cytochrome c oxidase subunit 4 isoform 1, mitochondrial-like	DN2698_c0_g2_i1	1.86

Sequence Description*	Transcript ID	Fold Change
cytochrome c oxidase subunit 5A, mitochondrial	DN15596_c0_g1_i1	2.11
cytochrome c oxidase subunit 5A, mitochondrial-like	DN15905_c0_g1_i1	-2.87
cytochrome c oxidase subunit 5B, mitochondrial-like	DN10367_c0_g1_i2	2.74
cytochrome c oxidase subunit 6A, mitochondrial	DN4893_c0_g1_i1	1.7
cytochrome c oxidase subunit 6B1	DN1977_c0_g2_i2	1.80
cytochrome c oxidase subunit 6B2-like	DN15972_c0_g1_i1	2.92
cytochrome c oxidase subunit 6C-1	DN1120_c0_g1_i1	1.89
	DN1120_c0_g1_i2	2.27
cytochrome c oxidase subunit 7A, mitochondrial-like	DN8813_c0_g1_i1	1.41
cytochrome c oxidase subunit II	DN15345_c0_g1_i3	-1.86
cytochrome c oxidase subunit III	DN3719_c0_g1_i1	-2.18
cytochrome c oxidase subunit NDUFA4	DN1602_c0_g1_i1	2.59
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	DN15893_c0_g1_i1	1.62
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	DN16277_c0_g1_i1	2.01
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	DN10630_c0_g2_i1	1.72
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	DN16478_c0_g1_i1	1.74
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7-like	DN2757_c0_g1_i1	1.55

Sequence Description*	Transcript ID	Fold Change
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	DN6168_c0_g1_i1	1.65
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	DN10786_c0_g1_i1	1.59
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial	DN4867_c0_g1_i1	1.50
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 2, mitochondrial-like	DN1313_c0_g1_i1	3.16
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	DN10039_c0_g1_i1	1.68
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	DN15703_c0_g1_i1	1.56
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	DN1321_c0_g1_i1	1.65
NADH dehydrogenase [ubiquinone] 1 subunit C2	DN3512_c0_g1_i1	1.80
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	DN16408_c0_g1_i1	1.96
NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	DN10180_c0_g1_i1	1.77
NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial	DN249_c0_g3_i1	2.08
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	DN3398_c0_g1_i1	1.72

Sequence Description*	Transcript ID	Fold Change
NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	DN6921_c0_g1_i1	1.78
NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	DN16587_c0_g1_i1	1.56
NADH dehydrogenase subunit 5	DN6512_c0_g1_i1	-4.28
NADH-quinone oxidoreductase subunit B 2-like	DN15960_c0_g1_i1	1.80
NADH-ubiquinone oxidoreductase 49 kDa subunit	DN6071_c0_g1_i1	2.32
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	DN5255_c0_g1_i2	2.16
NADH-ubiquinone oxidoreductase subunit 8	DN1376_c0_g1_i1	1.70
probable NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	DN4485_c0_g1_i1	1.78
succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial	DN3031_c0_g1_i1	1.77
succinate dehydrogenase cytochrome b560 subunit, mitochondrial-like	DN466_c0_g1_i1	2.19

* Additional transcript IDs following a sequence description are gene variants

Similarly, *A. mellifera* exposed to nicotine also had significant overexpression of genes involved in carbohydrate, lipid, and energy metabolism (oxidative phosphorylation, TCA cycle, and electron transport), accompanied by a dynamic detoxification response (du Rand et al., 2017). My results suggest that detoxification and remediation of stress associated with higher levels of imidacloprid exposure have increased energetic costs. Energy used for reproduction and coping with DNA damage may have shunted resources

that would have been used for reproduction or maintenance, contributing to decreases in reproduction and survival in insects treated with the inhibitory concentration of imidacloprid (100.0 mg/L).

Growth, Development, and Reproduction

In insects treated with the inhibitory concentration of imidacloprid compared with control, I observed decreased expression in several transcripts encoding vitellogenin, but increased expression of vitellogenin-like protein 1 (Table 5.11). While no decreased expression of vitellogenin transcripts was observed between the inhibitory and hormetic treatments, the expression of vitellogenin-like protein 1 was increased, while juvenile hormone acid O-methyltransferase and ecdysone-induced protein 78C isoform X2 were decreased (Table 5.12). I also observed increased expression in a number of other genes involved in reproduction and growth and development, including insulin receptor substrate 1 isoform X3 (inhibitory vs control and hormetic), and ecdysone-induced protein 74EF (inhibitory vs control), as well as increased and decreased expression of insulin-like growth factor-binding protein complex acid labile subunit (inhibitory vs control or hormetic) (Table 5.11, Table 5.12) (Xu et al., 2015; Ables et al., 2016). It is possible these proteins may be acting in a stress response capacity, and not a reproductive capacity. For example, the ecdysone-induced protein 74EF also has a role in regulating autophagy under stress and is upregulated in response to dehydration in the Antarctic midge, *Belgica antarctica* Jacobs (Teets and Denlinger, 2013). Insulin-like growth factor acid labile subunits are involved in carbohydrate and lipid metabolism, and activation of insulin receptor substrate signalling pathways is responsible for increases in energy metabolism (De La Monte et al., 1999; Arquier et al., 2008), all of which were induced in

P. maculiventris following an inhibitory imidacloprid stress. Furthermore, overexpression of insulin receptor substrates is involved in mitigating the effects oxidative stress induced autophagic cell death (Chan et al., 2012).

Table 5.11: Differential expression of select biologically relevant transcripts associated with growth, development, and reproduction in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with a control.

Sequence Description*	**Transcript ID**	**Fold Change**
ecdysone-induced protein 74EF isoform X1	DN7477_c0_g2_i2	3.16
insulin receptor substrate 1 isoform X3	DN9854_c0_g2_i1	2.51
	DN9854_c0_g5_i1	3.57
	DN9854_c0_g4_i1	4.17
insulin-like growth factor-binding protein complex acid labile subunit	DN3665_c0_g2_i1	-1.37
Vitellogenin	DN1006_c5_g3_i2	-19.54
	DN626_c0_g1_i19	-9.27
	DN626_c0_g1_i9	-7.68
	DN1103_c0_g1_i2	-6.08
	DN626_c0_g1_i14	-4.91
	DN685_c0_g1_i1	-4.61
vitellogenin 2	DN12815_c0_g1_i1	-8.19
vitellogenin-like protein 1	DN10984_c0_g4_i1	4.53

* Additional transcript IDs following a sequence description are gene variants.

Given that differential expression of transcripts involved in reproduction were observed following exposure to both low-dose hormetic and high-dose inhibitory concentrations of imidacloprid, it is clear that insecticide exposure has an effect on the expression of these genes. As discussed above, dynamic expression of genes involved in reproductive processes following exposure to insecticides is consistent with findings in

the literature, and further suggests that genes involved in reproductive development in insects have alternative functions. This requires further exploration.

Table 5.12: Differential expression of select biologically relevant transcripts associated with growth, development, and reproduction in *Podisus maculiventris* treated with an inhibitory concentration (100.0 mg/L) of imidacloprid compared with a hormetic (0.5 mg/L) concentration of imidacloprid.

Sequence Description*	**Transcript ID**	**Fold Change**
ecdysone-induced protein 78C isoform X2	DN9595_c0_g1_i1	-1.44
insulin receptor substrate 1 isoform X3	DN9854_c0_g2_i1	3.88
	DN9854_c0_g5_i1	4.37
	DN9854_c0_g4_i1	4.75
insulin-like growth factor-binding protein complex acid labile subunit	DN3665_c0_g2_i1	-1.64
	DN7549_c0_g2_i1	2.04
juvenile hormone acid O-methyltransferase	DN2672_c0_g2_i2	-2.56
juvenile hormone acid O-methyltransferase-like	DN5075_c0_g1_i9	-1.63
vitellogenin-like protein 1	DN10984_c0_g3_i1	2.51

* Additional transcript IDs following a sequence description are gene variants.

5.4 Conclusions

Under high levels of stress, insects mobilize energy and devote these resources to processes that will help the organism to survive the stressor. Consequently, this diverts energy away from processes that are not imminent (such as reproduction and growth), sacrificing these functions in favour of immediate survival to the stress. This can result in detrimental effects to other life history traits (McNamara and Buchanan, 2005). This was reflected in insects exposed to and inhibitory concentration of imidacloprid (100.0 mg/L) which inhibited reproduction and reduced long term survival (Chapter 2) (Rix and Cutler, 2020), where increases in the expression of transcripts involved in detoxification,

metabolic processes, and the DNA damage response were observed. The type and magnitude of the response was indicative of the intensity of the stress induced by this concentration of imidacloprid. On the other hand, when insects were exposed to a reproductively hormetic concentration (0.5 mg/L) of imidacloprid there was increased expression of transcripts involved in the DNA damage response, and reduced expression of transcripts involved in lipid, carbohydrate, and energy metabolism, and some detoxification transcripts, suggesting treated insects may have moved from an initial detoxification phase, into a recovery/repair phase. Stress recovery and repair processes, also referred to as cellular quality control, have been implicated in the hormetic response (Zhang et al., 2008; Wiegant et al., 2012). At high doses, endpoints measured are indicative of cellular damage whereas, at low doses the endpoints are indicative of a reparative and adaptive response (Calabrese et al., 2012). When increased in slight excess of what is needed to repair and recover from stress, these processes are thought to result in hormetic effects. As discussed above, this could occur due to a compensatory reduction in energy metabolism during detoxification, that is coincident with increased DNA damage and repair processes, reducing the amount of ROS produced, and DNA damage and repair processes are thus in slight excess of what is needed to repair remaining damage, but only if the stressor is low enough.

One regulatory mechanism that could be key to the hormetic response is the p53-Nrf2 axis. The Nrf2 transcription factor has recently been proposed as a potential mediator of hormesis, as its increase in expression under low levels of stress has been shown to enhance survival and longevity (Calabrese and Kozumbo, 2021a). p53 regulates Nrf2, and its expression has been shown to be involved in reproduction (Suh et al., 2006;

Hu and Feng, 2019). Exposure to an hormetic concentration of imidacloprid that increased fecundity without a subsequent reduction in survival also resulted in increased expression of a p53 transcript. It is possible that p53 plays a key role in reproductive hormesis, and its regulation of Nrf2 could mediate trade-offs with longevity and survival under stress.

It is important to recognize that molecular responses to stress are dynamic, with effects being influenced by the type of stress, extent of the exposure (concentration and duration), sex of the individual, and time. Conclusions that can be drawn from molecular studies on stress responses are thus limited by these types of factors. Ayyanath et al. (2014) demonstrated that even small changes in concentrations in the hormetic zone could have different effects on gene expression, and that there were also generational impacts on gene expression in the hormetic zone. Thus, even similar stimulatory phenotypic effects can present with different molecular responses. Cui et al. (2014) and (Matsumura et al., 2017) showed that gene expression under hormetic levels of stress can even vary across hours, during and after stress is removed. I collected insects at a single time point, 24 hours following exposure to imidacloprid, thus differing patterns of gene expression would likely have been evident across different sampling times. Therefore, the molecular mechanisms of hormesis may not be identifiable by examining a specific complement of genes, but rather by establishing a molecular stress response profile over time. Future studies on hormesis would benefit from examining full complements of gene expression over time, which may provide more insights into the expression dynamics. However, gene expression studies are often limited by costs, time, and other factors such as sampling population sizes, thus it is not always feasible to examine full complements

of gene expression across many concentrations, time points, or generations. Thus, examining transcriptional regulators of the stress response identified by smaller transcriptome studies may be a cost-effective method of studying hormetic mechanisms at the molecular level. My work has also added to the increasing body of work showing dynamic molecular responses to hormetic and inhibitory levels of stress consisting of increased and decreased expression of DNA damage responses, detoxification, antioxidation, energy metabolism, and reproduction, growth, and development.

CHAPTER 6 DISCUSSION

Toxicological studies with insects and other organisms have traditionally focused mainly on lethal and deleterious effects of toxins at high doses. This was largely rooted in the assumption of a toxicological threshold (NOAEL) below which no measurable consequential effects occur, and above which effects begin to occur (Calabrese, 2005). The study of sublethal effects of toxic substances, namely pesticides, on insects had likewise focused mainly on the inhibitory effects to such biological endpoints as survival, longevity, fecundity, and behavior (Cutler, 2013). Consequently, pesticide toxicology largely ignored potential stimulatory effects at low doses. The study of hormesis has brought to light the importance of examining stimulatory effects stemming from mild chemical stress, in addition to deleterious effects. Comprehensive analyses of toxicological studies have shown that hormesis is a widespread occurrence, having been observed in microbes, plants, and animals (vertebrate and invertebrate), particularly insects (Calabrese and Blain, 2011; Cutler, 2013; Cutler and Guedes, 2017). In insects, exposure to pesticides, among other stressors, has resulted in stimulatory effects on reproduction, growth, longevity, survival, and increases in stress tolerance (Cutler, 2013; Cutler and Guedes, 2017).

The impacts of these types of stimulatory effects on insects in agroecosystems are not inconsequential. In agroecosystems, insects are exposed to wide ranging concentrations of pesticides. While growers try to evenly apply pesticides on their fields, deposition of variable amounts on plants is inevitable. The size and depth of the plant canopy can result in uneven distribution of pesticides on plant foliage. Hot, dry weather may result in volatilization of pesticides, and even light wind can result in spray drift that

affects even distribution of pesticides. Environmental degradation of pesticides over time may also expose insects to varying pesticide amounts. Insects are thus likely to encounter concentrations within ranges that could be stimulatory rather than inhibitory (Cutler, 2013). Pest resurgence and secondary pest outbreaks are a frequent occurrence following pesticide application. Although often attributed to pesticide induced reductions in natural enemies, there is some evidence this may occur because of pesticide induced stimulation of reproduction and population growth, having been observed in brown plant hopper (Chelliah and Heinrichs, 1980), and southern red mite (Cordeiro et al., 2013). Furthermore, hormesis may result in transgenerational effects and preconditioning which could increase tolerance or resistance to pesticides or other stressors over time (Poupardin et al., 2008; Riaz et al., 2009; Ayyanath et al., 2013; Gong et al., 2015; Rix et al., 2016). On the other hand, if hormesis can be induced in beneficial insects, such as natural enemies, there could be benefit to agriculture. The occurrence of natural enemies in agroecosystems would naturally expose them to the same kinds of low doses as pests, and could result in stimulation of reproduction, survival and longevity, or predatory behavior, which could enhance biological control (Cutler et al., 2022). Furthermore, strong, long lived, robust natural enemy populations may enhance the success of rearing and release of natural enemies introduced in augmentative biological control programs (Cutler et al., 2022). This formed the basis for the first half of my thesis, examining stimulatory phenotypic effects induced by low-dose concentrations of imidacloprid insecticide on *P. maculiventris*.

The first objective of my thesis was to examine whether the insecticide imidacloprid could induce stimulatory effects on life history traits: time to oviposition,

fecundity, fertility, and survival. I examined this in *P. maculiventris* exposed to a range of imidacloprid concentrations as nymphs, and in those exposed as adults across two generations (Chapter 2) (Rix and Cutler, 2020). A key determinant of adult phenotypes, importantly fitness, is early life stress. High levels of stress can be costly. Increased allocation of resources to combat a stressor can divert resources away from cellular maintenance, resulting in the build-up of cellular damage. If not immediately fatal, this can result in reduced survival, lifespan, and even reproduction over time as additional damage incurred over the organism's lifespan compounds (McNamara and Buchanan, 2005). This was clearly observed when *P. maculiventris* nymphs were treated with higher concentrations of imidacloprid that reduced fecundity, fertility, and survival (Chapter 2) (Rix and Cutler, 2020). On the other hand, low doses of stress experienced in early life can be protective. Exposures that increase protective responses such as DNA and protein repair, or antioxidant capacity may delay inhibitory effects that normally occur with aging (Sorensen et al., 2003). Early life stress can also prime stress responses to enable the organism to more efficiently cope with stress later in life (Kristensen et al., 2003), and stimulate life history traits in later life. I observed stimulated fecundity when insects were treated with concentrations of imidacloprid below the LC1 (0.015 mg/L and 1.5 mg/L) as nymphs. At the higher hormetic concentration (1.5 mg/L), however, stimulated reproduction was accompanied by reduced fertility and survival (Chapter 2) (Rix and Cutler, 2020). Some work has suggested that early life stress can cause trade-offs to occur if the stressor is not again experienced in adulthood (Costantini et al., 2014), as was the case with insects experiencing an acute exposure as nymphs, but not as adults (Chapter 2) (Rix and Cutler, 2020). Other work suggests that trade-offs associated with stimulatory

effects are inevitable due to the inherent reallocation of resources during the stress response (Jager et al., 2013), although it has been shown that stimulatory effects can occur without trade-offs (Pereira et al., 2009; Ayyanath et al., 2013).

I also observed stimulated fecundity in insects treated as adults, and in offspring of treated adults that were not subjected to imidacloprid exposure. Reproductive hormesis is one of the most commonly observed stimulatory effects induced by exposure to pesticides (Cutler, 2013; Cutler and Guedes, 2017), and has been consistently shown in stink bugs exposed to low doses of multiple insecticides (Zanuncio et al., 2003; Guedes et al., 2009; Pereira et al., 2009; Zanuncio et al., 2011; Zanuncio et al., 2013; Santos et al., 2016). Reproductive hormesis can also be transgenerational (Ayyanath et al., 2013; Margus et al., 2019), though this is energy intensive (Costantini, 2014b). I did not observe trade-offs in adults treated with mild amounts of imidacloprid in the first generation, but their offspring had reduced fertility (Chapter 2) (Rix and Cutler, 2020). Stress of this sort can have significant effects on offspring phenotypes and quality. To ensure reproductive success, oviparous females may invest energy in egg production or egg quality. Investing energy in the number of eggs may increase the potential of producing more offspring (Koch and Meunier, 2014).

On the other hand, investing in egg quality may result in increased egg development and hatching, or better-quality offspring. This trade-off was shown in European earwig, *Forficula auricularia* L. (Koch and Meunier, 2014). *Podisus maculiventris* females subjected to insecticide stress may have invested energy in egg production rather than in egg quality. Under stress, this may have resulted in increased fecundity in parental and F1 generations but resulted in offspring with reduced fitness,

manifested as reduced fertility of F1 progeny (Chapter 2) (Rix and Cutler, 2020). My work also demonstrated that parental exposure to imidacloprid did not prime offspring for secondary exposure given F1 insects of treated parents that were also subsequently treated with imidacloprid as adults had reduced fertility and no stimulation of fecundity (Chapter 2) (Rix and Cutler, 2020). In contrast to other work showing positive transgenerational effects (Rix and Cutler, 2018; Sial et al., 2018; Margus et al., 2019), I did observe that reduced fertility did not have an overall negative impact on the population. Increased fecundity compensated for the decreased fertility and the number of offspring was higher compared to the control (Chapter 2) (Rix and Cutler, 2020). This is significant, as it shows pesticide exposures can increase population size of *P. maculiventris*.

Successful predators, in addition to having robust populations, must maintain their predatory abilities such as searching for, locating, and subduing prey. If population sizes are increased but insects are unable to maintain their predatory abilities, the significance of hormesis for biological control may be compromised. This formed the basis for Chapter 3 (Rix and Cutler, 2021), where I examined predation, and commonly observed behaviors in adult male and female *P. maculiventris* exposed to a series of sublethal concentrations of imidacloprid. This was examined in two settings, a simple setting in a plastic container arena, and a complex plant arena.

Imidacloprid is an agonist of the nicotinic acetylcholine receptor (nAChR), mimicking acetylcholine, a major excitatory neurotransmitter (Matsuda et al., 2001; Picciotto et al., 2012). Nicotinic acetylcholine receptors are highly concentrated in sensory and motor neurons in insects and important for governing movement and visual

and olfactory processing (Gauthier, 2010). I therefore expected that certain mild concentrations of imidacloprid would stimulate aspects of locomotory or searching behavior such as walking-antennating, antennating, and probing in my insects, and that this might result in increased predation through increased interactions with prey or through increased responsiveness to prey. Locomotive performance is important for prey hunting in addition to dispersal and colonization in agricultural ecosystems (Muller, 2018). Antennae and the insect proboscis have sensory and olfactory roles, helping insects to sense and locate potential prey (Kwon et al., 2006; Maekawa et al., 2011; Elgar et al., 2018).

Generally, I observed that behavioral hormesis is a dynamic response, with stimulatory effects being associated with exposure, sex of the insect, and study arena. Specifically, I observed that a reproductively hormetic concentration (0.5 mg/L) had no inhibitory effects on behavior or predation (Chapter 3) (Rix and Cutler, 2021). Although stimulatory effects on probing behavior in males treated with a hormetic concentration (0.5 mg/L) occurred, and stimulation of antennating behavior in females exposed to a milder (0.05 mg/L) and a higher concentration (5.0 mg/L), this did not result in enhanced predation. In fact, the 5.0 mg/L treatment resulted in reduced predation. Behavior and predation were also impaired following exposure to 20.0 mg/L (Chapter 3) (Rix and Cutler, 2021). My work suggests that the stimulatory effects from imidacloprid do not necessarily increase sensory reception or recognition of prey. This was shown in a lack effect of these same treatments on frequency of location switches on plants, a metric of prey searching (Chapter 3) (Rix and Cutler, 2021). Despite a lack of stimulation of predation, stimulation of probing on plants by males could be of significance. Plant

probing by the zoophytophagous mirid, *N. tenuis*, induced the release of plant volatiles which had a repellant effect on whitefly, *Frankliniella occidentalis* Pergande, and thrip, *Bemisia tabaci* (Gennadius), pests (Bouagga et al., 2018). Furthermore, insect assemblages in agricultural ecosystems are complex, often consisting of multiple pest and predator populations. Predators may be attracted to prey through olfactory signals, but attraction can also be indirect. The release of plant volatiles induced through plant probing by the same zoophytophagous mirid, *N. tenuis*, also attracted the whitefly parasitoid, *Encarsia Formosa* Gahan (Bouagga et al., 2018). Therefore, activity of a zoophytophagous predator can have indirect effects on prey.

These experiments, and those of Chapter 2, show that insects may be exposed to a reproductively hormetic concentration of imidacloprid without detriment to essential predatory behaviors and predation. The increased population, therefore, could be of benefit in an agroecosystem, with an increased number of predators having a greater impact on pest population reduction. A number of potential scenarios exist for how the predator-prey dynamic could manifest in a hormetic context (Figure 6.1). Under a scenario of no hormesis, high concentrations of insecticide suppress both pest and predator populations, but they recover as the insecticide degrades over time, and both populations return to their respective population equilibrium (Figure 6.1a). Under a scenario where reproductive hormesis is induced in a predator, the initial high concentration suppresses both the predator and prey population, but as the insecticide degrades over time, lower doses induce reproductive hormesis in the predator population. The increased number of predators reduces the pest population below equilibrium, suppressing the pest for the period of time over which hormesis is maintained, and

eventually returns to equilibrium (Figure 6.1b). During the period of pest suppression, plant damage could be reduced. In addition, both pest and predator could be hormetically stimulated (Figure 6.1c). Under this scenario, one might expect these effects to cancel each other out, but this may not necessarily be the case. It is unlikely pests and predators would be stimulated at the exact same dose-range. Some evidence suggests that in some instances, insect predators may be more sensitive to pesticides (Croft, 1990), therefore hormesis could occur at a lower dose in predators. In other words, in some instances, hormesis could occur later in predators. As before, both pest and predator populations would decrease initially, and begin to rebound as the insecticide degraded. Further degradation would first result in a stimulation of the pest population, but no stimulation in the predator. An increase in the pest population would provide more prey for predators, which could naturally increase their population. The pest population then begins to decrease as the predator population naturally increases. Hormetic stimulation of the pest could still maintain its population above equilibrium, but as the insecticide further degrades into the predators' hormetic zone, with the additional population stimulation from hormesis, the predators could suppress a stimulated pest population (Figure 6.1c). A somewhat similar scenario was observed in a *Acyrthosiphon pisum* (pea aphid) - *Aphidius ervi* Haliday (parasitoid) experiment. Exposure to temperature just below the detrimental heat shock, resulted in aphid population increases over average/control temperature. When parasitoids were also in the system, their population rose more rapidly, suppressing the aphid population (Meisner et al., 2014). This occurred even when aphid populations were initially large prior to introduction of parasitoids. Aphid populations did rebound in the second generation in situations where aphid populations were initially low in the first

generation, consistent with typical cyclical patterns in predator-prey dynamics (Meisner et al., 2014).

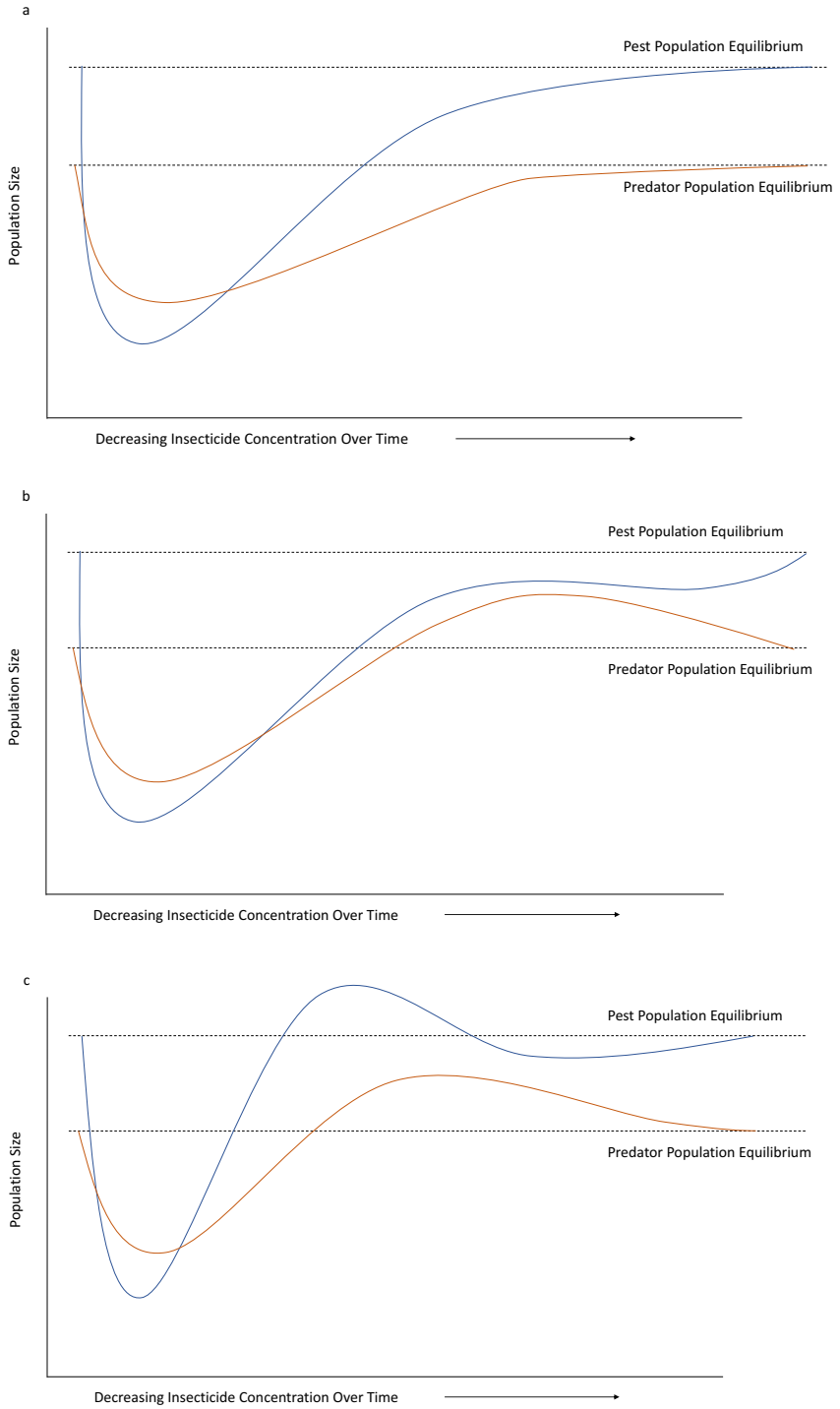

Figure 6.1: Theoretical responses of predator and pest populations when exposed to an insecticide over time a) without induction of hormesis; b) with reproductive hormesis in a predator; c) with reproductive hormesis in both predator and pest. Initial pest populations sizes are prior to insecticide treatment.

The above are some of the potential scenarios of how hormesis could affect agriculture in the context of a simple predator-prey dynamic, although other scenarios have also been proposed. For example, in a situation where a predator population is stimulated, but a pest population is inhibited, a rapid decline in the predator population would be expected following the hormetic response, due to a reduction in the pest population (Cutler et al., 2022). Predator populations would however rebound as the pest population begins to increase as the intensity of a stressor decreases over time. If the pest population is then hormetically stimulated, the predator population may increase and exceed equilibrium simply because of increased food availability with the increased pest population (Cutler et al., 2022). This could similarly be the case in a situation where a pest population was stimulated at a lower dose (later) than a predator. Although predators can be more sensitive to pesticides, pests are often smaller than predators and it is possible a pest could be stimulated at a lower dose than a predator. The scale of complexity of agroecosystems in general may reduce our ability to predict hormesis, and the overall significance of hormesis for agroecosystems. Interspecific interactions are likely to effect whether stimulatory effects have an impact. Changes in species population sizes can have impacts on the broader community, disrupting species competition and trophic level interactions. Changes in the population dynamics of crop pests may have unique effects on competitive interactions among insects. Foote et al. (2017) observed that cereal grass aphid, *Metopolophium festucae* (Theobald) populations on wheat undergoing water stress, increased 12% whereas bird cherry-oat aphid *Rhopalosiphum padi* (L.) populations were decreased compared with controls and 16% decreased compared with cereal grass aphid. However, when both aphid species were present under

stress, the opposite occurred, bird cherry-oat aphid populations doubled those of cereal grass aphid, thereby neutralizing any competition between the two under stressful conditions. Similarly, mild exposure to a pyrethroid insecticide stimulated reproduction in soybean aphid, *Aphis glycines* Matsumura, alone, but if the generalist aphid, *Aulacorthum solani* (Kaltenbach), was also present on soybean, *A. glycines* reproduction was not increased, possibly due to interspecific competition (Qu et al., 2020).

In addition, if a hormetic increase in the population of a secondary pest suppressed the activities of a primary pest, this could be of benefit to the plant. Ramirez and Eubanks (2016) showed that an increase in *A. gosypii* populations on cotton plants, reduced the population of the more damaging beet armyworm, *Spodoptera exigua* (Hübner), in part due to aphid induced plant defenses. This also impacted predator-prey interactions, as ladybeetle, *Hippodamia convergens* Guérin-Méneville predation on beet armyworm was also increased by the presence of aphids. Damage to cotton plants was subsequently reduced (Ramirez and Eubanks, 2016). These examples highlight the probability that predicted outcomes based upon hormesis studies on a single species may manifest differently in complex systems. Most studies conducted on hormesis in insects to date have been in laboratory on a single species. A need exists for future studies to employ more complex scenarios. This could help determine how predator-prey interactions in a given system would manifest where hormesis is induced in predators, prey, or both, and which are most likely to occur in a given scenario.

Given the above complexities, the use of hormesis to stimulate predator populations in controlled rearing of insects for biological control may have more realistic opportunities, although this is also not without challenges. I observed a fair amount of

variability in reproductive outputs in *P. maculiventris*, and peak population reproductive outputs under hormetic levels of stress did not occur until 30-40 days into adulthood (Chapter 2) (Rix and Cutler, 2020). Therefore, the cost-effective benefits may not be realized in a timely manner. A natural enemy with a shorter life cycle or more predictable reproductive outputs may be a better candidate for hormetic stimulation in rearing for biological control. For example, Hymenoptera parasitoids of aphids such as *A. ervi* have rapid larval development, and short pre-oviposition and oviposition periods, completing their life cycles in as low as 10-15 days (Danks, 2006). Reproductive stimulation in such a short time window would be realized quickly and could compound quickly if stimulation was transgenerational. Future studies examining hormesis in biological control agents could include a cost benefit analysis of stimulatory effects to determine whether there are indeed benefits to any hormetic effects.

On a more basic level, there exist other gaps in our knowledge that may limit our ability to make definitive predictions about hormesis and its benefits for biological control. Firstly, the dose-range of the stimulatory response, while typically within 10-fold of the NOAEL, can be as high as 1000-fold (Calabrese, 2013a). This may influence the extent and range of the stimulatory effects observed, with different effects observed across lower and higher hormetic concentration ranges. Different responses to different doses of stressor could be reflective of biological trade-offs. In my experiments, trade-offs in fertility and survival accompanied stimulated fecundity, but only at a higher hormetic imidacloprid concentration (1.5 mg/L), whereas no trade-offs were observed with stimulated fecundity at a lower concentration (0.015 mg/L) (Chapter 2) (Rix and Cutler, 2020). This suggests that trade-offs may only manifest in a significant manner at

higher hormetic concentrations. This has been previously observed in *P. turionellae*, where stimulation of longevity and fecundity occurred at the expensive of fertility at higher hormetic dose of malathion, but not a lower hormetic dose (Buyukguzel, 2006). Furthermore, typical modelling of hormetic effects demonstrates that hormesis is more often biphasic, with a single hormetic zone/dose range and peak. However, other work has shown multiple hormetic peaks (Ayyanath et al., 2013). The existence of multiple stimulatory zones could account for the anomalous stimulatory effects outside the traditional hormetic NOAEL range in some insects- LC_{50} (Li et al., 2019), and LC_{20} (Sial et al., 2018). It is clear that a range of effects can occur across the dose-response continuum relative to the amount of stress.

It will be important for future studies on insect hormesis to examine multiple species and their interactions, multiple concentrations, and generations. Studies specific to rearing biological control agents should consider the types of stressors that could be used to induce hormesis; physical stressors such as heat or hypoxia may be more appropriate methods of inducing hormesis in reared natural enemies. Studies should also consider species that if stimulated by hormesis would be most effective in biological control of pests and in terms of cost.

While many aspects of phenotypic effects of hormesis continue to be well characterized across taxa and within Insecta (Cutler, 2013; Guedes and Cutler, 2014; Cutler and Guedes, 2017), molecular responses during hormesis have not been comprehensively studied in insects. The fourth Chapter of my thesis systematically analyzed the commonly observed stimulatory responses and associated molecular and biochemical responses following exposure to low doses of stress to more clearly show the

patterns and manifestation of hormesis in insects. Stress that induced hormesis in insects included chemical stress, oxidative stress, temperature stress, dietary restriction, crowding, and radiation (Chapter 4) (Rix and Cutler, 2022). On the phenotypic level, I largely observed stimulation of endpoints associated with reproduction, longevity and survival, size and development, and increased tolerance to certain stressors including chemical and oxidative stress, temperature stress, and starvation and desiccation stress. These effects were increased 25-50% above controls (Chapter 4) (Rix and Cutler, 2022), which is consistent with the modeled 30-60% of hormetic stimulatory effects increased above controls (Calabrese and Blain, 2011; Calabrese, 2013a).

Molecular and biochemical responses associated with hormesis were associated with: detoxification, including cytochrome P450, esterase, and glutathione-s-transferase gene expression and activity; antioxidation, including superoxide dismutase, catalase, and peroxidase gene expression and activity; chaperones including heat shock protein gene expression; those involved in growth, development, and reproduction including, vitellin and vitellogenin gene expression and regulation, juvenile hormone gene expression and regulation, and ecdysone gene expression and regulation; and genes involved in insulin/insulin-like and target of rapamysin signalling (IIS/TOR) (Chapter 4) (Rix and Cutler, 2022). Patterns were observed in the types of molecular responses and associated phenotypic effects in response to hormesis. For example, heat stress frequently induced the expression of Hsp70 genes when insects were exposed to heat, and this was associated with increased longevity and/or increased heat tolerance (Chapter 4) (Rix and Cutler, 2022). Chemical stress often induced the expression of detoxification genes and/or antioxidant gene expression, frequently cytochrome P450 genes. This was often

associated with increased tolerance to other chemical exposures, but also increased fecundity or longevity/survival (Chapter 4) (Rix and Cutler, 2022). These results demonstrated that protection from oxidative stress and repair of DNA and protein damage are implicit in hormetic responses. While these patterns were observed, variation was also prevalent in coordinated molecular and biochemical responses to stress. This was demonstrated in gene expression studies over time or generations. For example: heat exposure that increased heat tolerance in *M. separata* was associated with increased *Hsp70*, and antioxidant gene expression, but this varied over time. *Hsp70* and *CAT* expression increased early into heat stress and continued to elevate for several hours following heat stress. Expression of *SOD* on the other hand, had a pulse of expression 3h following heat stress (Matsumura et al., 2017). In *M. persicae*, reproduction increased across three generations, with variable changes in gene expression in each generation: cytochrome P450 *CYP6CY3* was increased only in the first generation, remaining insignificant in the second and third generations; *E4/FE4* esterase expression was increased in the first and third generations, at different levels, 2-5 fold and <2 fold respectively; *Hsp60* expression was decreased in the second generation but increased in the third generation (Rix et al., 2016). Multi-gene expression studies have shed further light on the coordination of molecular responses. Different stressors, although having similar hormetic effects in an organism, can show different gene expression patterns. This was shown by Riaz et al. (2009) where exposure to glyphosate and benzo[a]pyrene induced different gene expression patterns, but both resulted in increased tolerance to imidacloprid, permethrin, and propoxur in *A. aegypti*. The same was also true for Liu et al. (2019) studying *S. litura* where exposure to different herbicides increased insecticide

tolerance, but induced variable gene expression patterns (Chapter 4) (Rix and Cutler, 2022).

Responses also display some specificity to individual and stressor. For example, increased longevity associated with hyperbaric normoxia in both males and females did not significantly increase expression of *Hsp70* or *GS* (*glutathione synthase)* in females as it did in males, although other genes were similarly expressed (Yu et al., 2016). Exposure to heat stress increased longevity, heat tolerance, and *Hsp70* expression in *D. melanogaster* males, while reducing heat tolerance without effect on *Hsp70* expression in females (Sorensen et al., 2007). Genes may also respond differently to stress in different species. Reviews of the data on heat shock protein gene expression show a wide variety of induction profiles in insects. For example, strong upregulation of *Hsp90* is observed in response to heat in *Rhagoletis pomonella* (Walsh), but not in *Drosophila triauraria Drosophila triauraria* Bock and Wheeler (King and MacRae, 2015). Multiple stressors can also elicit similar responses across organisms, with varying molecular effects. For example, heat stress and insecticide stress that increased fecundity were associated with increased vitellogenin and juvenile hormone in some cases, and increased antioxidant and detoxification genes and activity in others (Chapter 4) (Rix and Cutler, 2022).

Hormetic responses at the phenotypic level have molecular/biochemical underpinnings, but gene expression patterns vary, suggesting hormesis manifests as a coordination of processes in the stress response. This highlighted an important gap in the molecular hormesis literature. While studies examining the expression of a multitude of genes exist, many papers focus on a small number of select genes, which limits the ability to examine the coordination of molecular responses. Next generation sequencing

technologies enhance the ability to examine full suites of gene expression which can allow for a more detailed analysis of the dynamics of stress responses and hormesis. This formed the basis for Chapter 5 of my thesis which employed the use of RNA-seq to perform a transcriptome analysis on *P. maculiventris*, comparing gene expression in response to a reproductively hormetic concentration of imidacloprid and an inhibitory/deleterious concentration that inhibited reproduction and reduced survival (Chapter 2) (Rix and Cutler, 2020).

My transcriptome analysis provided more information on the nuances of the stress response and hormesis. Repair of DNA and protein damage was a key element of the hormetic response in *P. maculiventris* exposed to imidacloprid. Genes involved with DNA damage response had increased expression, and increased and decreased expression in a small number of detoxification genes was observed, with mainly decreased expression in genes involved in energy metabolism. In contrast, insects treated with an inhibitory concentration of imidacloprid had significant increases in expression of numerous genes involved in the above processes. This is significant. In order for hormesis to occur, an organism must sustain enough damage to elicit defensive and reparative processes, but not in such excess that the organism's resources are overwhelmed, but rather in slight excess to what is needed to repair the immediate damage and protect the organism from potential subsequent damage (Rossnerova et al., 2020). For hormesis to occur without significant detriment to an organism, the response to the stress must not overwhelm the organism's resources and repair capacity. This may be evident in the fact that 24 hours post exposure, when insects were collected, genes involved in detoxification and metabolic processes had decreased expression, while genes

involved in the response to DNA damage were increased, suggesting hormetically exposed insects moved from an energy intensive, detoxifying phase, to a repair and recovery phase (Chapter 5). In *C. elegans*, energy metabolism dominated expression patterns during initial exposure to insecticide, and expression of DNA repair mechanisms dominated during recovery (Lewis et al., 2013). Other studies have shown decreased expression of genes involved in metabolic processes, including the breakdown of carbohydrates, lipids and amino acids for energy, and energy metabolism, in insects exposed to low doses of insecticide (Gao et al., 2018; Meng et al., 2019b; Wei et al., 2020; Ingham et al., 2021). It has been suggested that reduced energy metabolism associated with sublethal insecticide exposure could be beneficial and may be a compensatory mechanism to reduce ROS production due to exogenous ROS production from insecticide exposure, thereby reducing the impact of oxidative stress, allowing the insect to survive, repair damage, and re-establish homeostasis (Gao et al., 2018; Ingham et al., 2021). This could indicate that energy metabolism is tightly regulated under low doses of stress, and that efficient use of energy resources is a factor influencing hormesis (Chapter 5). A compensatory reduction in energy metabolism or enhanced metabolic efficiency, corresponding to an increase in DNA damage response and repair processes could be one of the mechanisms by why which hormesis manifests in response to insecticide exposure.

At intermediate and high levels of stress however, defensive and reparative processes exceed resource capacities, and with accumulation of damage, organisms sustain deleterious or inhibitory effects (Wiegant et al., 2012). Indeed, *P. maculiventris* treated with 100.0 mg/L imidacloprid had reduced survival and impaired reproduction in

association with substantial increases in genes involved in energy metabolism, lipid and carbohydrate metabolism, detoxification, and response to DNA damage (Chapter 5). This suggests that 24 hours following the inhibitory imidacloprid exposure, treated *P. maculiventris* were still detoxifying imidacloprid and repairing ongoing damage. The associated increase in expression of metabolic processes suggested that ongoing detoxification and repair from stress induced damage following inhibitory levels of imidacloprid exposure have increased energetic costs which likely contributed to decreases in reproduction and survival in insects treated with 100.0 mg/L imidacloprid (Chapter 5).

A general theoretical framework for how the hormetic response manifests over time in response to insecticide or chemical stress is presented in Figure 6.2. Under insecticide or other chemical exposure, detoxification would be expected to be the primary response, therefore following exposure to the low-dose insecticide, the detoxification response is triggered. Energy stores are mobilized, and energy metabolism increases to drive detoxification (Rand et al., 2015; Gong and Diao, 2017), which is energy intensive (Berenbaum and Zangerl, 1994). The process of detoxification and increased energy metabolism leads to increases in ROS, and increased antioxidation (Rand et al., 2015; Gong and Diao, 2017). The increased generation of ROS would be expected to cause an accumulation of cellular and DNA damage, triggering the DNA damage response (Mägdefrau et al., 2018). As discussed above, at low doses of exposure, the increase in ROS possibly induces a compensatory reduction in energy metabolism (Gao et al., 2018; Ingham et al., 2021). Given the insecticide or chemical exposure is low, a sustained detoxification response is unlikely, and therefore detoxification begins to

decrease as does antioxidation. The DNA damage response remains elevated to repair remaining damage and recover the insect back to homeostasis as the organism shifts from a detoxifying phase to a repair phase, and then declines as homeostasis is re-established (Figure 6.2). In accordance with the overcompensation stimulation theory of hormesis, this would manifest in slight excess of what is needed to repair the remaining damage in expectation of additional stress (Calabrese, 2001; Rozman and Doull, 2003; Rossnerova et al., 2020). This could potentially be because of the compensatory reduction in energy metabolism. Hormesis is characterized by simultaneous stimulation of multiple cellular and molecular stress responses, and it has been proposed that each is expected to have its own quantitatively hormetic features. In other words, its own response magnitude and duration (Calabrese and Mattson, 2017). It is therefore also possible and/or likely that detoxification or antioxidation, during their activation, are also elevated in slight excess as they are consistently linked to hormesis (Chapter 4) (Berry and Lopez-Martinez, 2020; Rix and Cutler, 2022). The integration of these responses thus results in a stimulatory response in one or more biological processes. In contrast, inhibitory exposures, whether in amount, intensity, or duration, are expected to elicit stress responses in magnitudes that exceed the resource capacity of an organism. Homeostasis is either re-established in the organism (albeit with depleted energy stores and sustained residual damage), or the organism succumbs to the combined effects of the insecticide and the stress response (if the dose is high enough to be lethal) (Sancho et al., 2009; Ge et al., 2015; Johnson, 2017; Wei et al., 2020). In some instances, a stressor may be so toxic that an organism is killed early into the manifestation of the stress response, or such a stressor may damage or inhibit processes that are important for the normal functioning of the stress response

(Sancho et al., 2009; Johnson, 2017; Wei et al., 2020; Holen et al., 2022). Therefore, stress response profiles for inhibitory or deleterious levels of exposure may be variable.

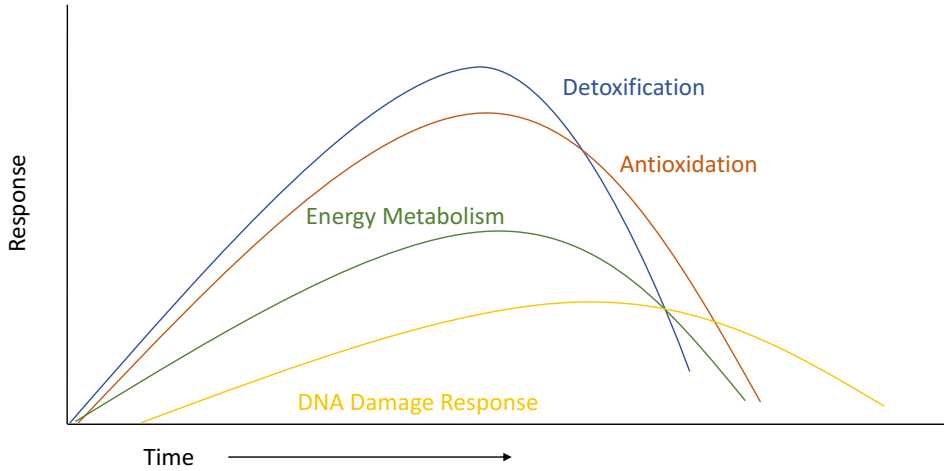

Figure 6.2: Theoretical framework for molecular stress response profiles in insects exposed to a hormetic level of insecticide.

One of the primary goals of examining hormesis at the molecular level has been to establish whether there is a common, universal pattern that explains or distinguishes hormesis. On a broader scale, it is largely considered that a stress is either harmful or beneficial depending on the frequency, duration, and intensity of the stress and the effect on metabolism and energy utilization, and cellular damage (Rattan, 2013). At the small-scale molecular level, numerous regulatory and signalling molecules within stress response pathways can be diversely expressed. Therefore, expression of specific genes associated with the stress response would be expected to be dynamic. In addition, not all processes will respond equally with time, amplitude, or kinetics (Demirovic and Rattan, 2013). The curves in Figure 6.2 are depicted as such to show that differences in magnitudes of stress response processes are likely to be expected, but may be different from what is depicted. Differences in relative magnitudes of responses could be influenced by a number of factors. For example, the overall magnitude of a response

relative to other responses would be expected to be dependent on basal expression levels of individual genes in an organism. Expression patterns can be influenced by background environment of an organism, for example the host plant of an insect (Birnbaum and Abbot, 2020), or life stage (Cao and Jiang, 2017). Individual gene expression patterns would be characterized by a combination of increased and decreased expression throughout the duration of the stress response depending on the needs of the cell, and to maintain or maximize energy efficiency (Demirovic and Rattan, 2013). Different expression patterns may be triggered by different levels of stress even within the hormetic zone, with different aspects of the stress response being regulated and expressed. Even small changes in the level of stress can cause significant differences in gene expression profiles. For example, in *M. persicae* undergoing hormesis, *OSD* expression was increased when nymphs were exposed to 0.1μg/L of imidacloprid, but decreased when exposed to 0.25 μg/L. In adults *OSD* expression was increased 4-fold at 0.1μg/L exposure, but less than 2-fold at 0.25 μg/L. Expression patterns also differed across generations (Ayyanath et al., 2014). It is unlikely that there is a single identifying molecular maker of hormesis, and that instead it is the way in which the stress response manifests at the molecular level over time that identifies hormesis. Although, there may be important transcriptional regulators that are differentially expressed at low/hormetic and high-doses. For instance, while p53 and BNIP3 are both important regulators of autophagy and apoptosis, I observed that *P. maculiventris* exposed to a hormetic concentration had increased expression of p53, but increased expression of BNIP3 when exposed to an inhibitory imidacloprid concentration. Furthermore, under the inhibitory concentration, I observed decreased expression of several transcripts involved in DNA

repair that were increased following exposure to a hormetic concentration, such as the transcriptional regulator myb-like protein X (Chapter 5).

Establishing concrete or robust molecular response profiles of hormesis may continue to present challenges, as the type of stressor, its intensity, the organism and its life stage, and even level of biological organization studied (cell, tissue, whole organism) may show differing stress response profiles. Therefore, the design of a comprehensive analysis and the conclusions drawn should take this into consideration. Large comprehensive studies of this nature are likely to be constrained by costs and limited by time constraints and may not always be feasible. It may however be more feasible to conduct small transcriptome studies, with a small number of time points to identify potential transcriptional regulators of the hormetic response and use future studies to examine this across a greater range of time points. Despite challenges, molecular studies on hormesis in insects will need to move away from relatively small-scale studies examining single phenotypic endpoints and small numbers of target genes, to more comprehensive and robustly designed studies that can provide more concrete explanations for the hormesis phenomenon at the regulatory level.

Overall, in this thesis I found that insecticide-induced reproductive hormesis in the beneficial insect predator, *P. maculiventris* may occur across different life stages and generations, with intra- and inter- generational trade-offs in survival or fertility according to treatment. Despite trade-offs I found that overall reproduction can be increased without detriment to predator behavior and predation. This added to the recent expansion of insecticide-induced hormesis work into beneficial insects in addition to pests. My thesis also sought to expand molecular knowledge of hormesis through both a systematic

analysis of the molecular hormesis literature and more specifically through a transcriptome analysis of *P. maculiventris* exposed to a hormetic concentration of insecticide. My systematic analysis showed that protection from and repair of oxidative damage is implicit in the hormetic response. My transcriptome analysis also demonstrated this to be true, specifically observing that transcripts involved in the regulation of autophagy and apoptosis, and those involved DNA repair were expressed. My work also highlighted gaps in knowledge on hormesis and in the current body of research. I have proposed the need to 'level-up' the complexity of hormesis research, in a more ecological context by examining the effects of hormesis on species interactions, and in the molecular context through establishing a molecular stress response profile in insects exposed to hormetic levels of stress. This will lay a foundation for the next stages in the study on the significance of hormesis in toxicology and pest management.

APPENDIX 1

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APPENDIX 2

Full lists of the annotated transcriptome and annotated differentially expressed transcripts, and RNA sample quality, quantity, and read quality assessments are found in the tabs of the spreadsheet labelled Appendix 2_Table S1, which can be found separate to this thesis and on Dalspace.

Tab-1 contains the full annotated transcriptome. Tabs-2-4 contain the full annotated lists of differentially expressed genes (Tab-2 hormetic vs control; Tab-3 inhibitory vs control; Tab-4 inhibitory vs hormetic). Tab-5 consists of data on RNA sample quality and quantity (quantified spectrophotometrically) and read quality assessments (following Illumina NovaSeq 6000 sequencing) for replicate insect samples treated with control, hormetic, and inhibitory concentrations of imidacloprid: the nucleic acid concentration; ratios of absorbance at 260 nm/280 nm (A260/A280) and at 260 nm/230 nm (A260/A230); phred scores; and the number of reads for each replicate sample.

APPENDIX 3

Supplementary figures S1-S4 showing top 30 GO terms for the annotated transcriptome and top 15 increased and decreased GO terms for differentially expressed genes. These can be found in the document labelled Appendix 3_Supplementary Figures, separate to this thesis and on Dalspace.

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