CHRONIC, MULTI-GENERATIONAL EXPOSURE TO LOW DOSES OF IMIDACLOPRID: ITS EFFECTS ON CHEMICAL PRECONDITIONING AND MUTATION INDUCTION IN NICOTINIC ACETYLCHOLINE RECEPTORS IN *MYZUS PERSICAE* (HEMIPTERA: APHIDIDAE)

by

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ABSTRACT

Hormesis is a phenomenon whereby exposure to low or sublethal doses of a chemical, physical, or biological stressor stimulate or enhance biological processes in an organism. Hormesis is an evolutionarily conserved process thought to allow organisms to adapt to stress. Hormetic effects such as increased lifespan and increased reproduction induced by exposure to low dose chemical stressors have been well documented in many insect species. This is of interest in integrated pest management as insects are likely to be exposed to low dose chemical stresses from pesticides as they break down in the environment. Under the umbrella of hormesis is another phenomenon known as hormetic preconditioning, whereby exposure to low doses of stress prime organisms to better survive additional, more challenging levels of stress. This phenomenon could aid in the development of insecticide resistance. It has also been hypothesized that low levels of stress may hasten the development of pesticide resistance by increasing mutation frequencies in pests. My thesis examined low dose and hormetic priming and whether it manifests over time by exposing *Myzus persicae* to low doses of imidacloprid, over four generations. Individuals from each generation were then exposed to several, increasingly lethal concentrations of the insecticide, imidacloprid, or spirotetramat, which has a differing mode of action, to determine if and how low dose insecticide exposure primed the aphids to better survive additional, and variable chemical stresses. Insects exposed to low doses of imidacloprid were also subjected to dose response assays, which characterized the ability of exposed aphids to survive a wide range of subsequent insecticide concentrations. I also examined whether exposure to low doses of the insecticide, imidacloprid, over multiple generations, induced mutations in five subunits of the *M. persicae* nicotinic acetylcholine receptor, comprising the majority of the imidacloprid binding site. Exposure to mildly toxic concentrations of imidacloprid did not result in priming when insects were subsequently exposed to more toxic concentrations of imidacloprid and spirotetramat. Exposure to hormetic concentrations of imidacloprid did prime insects to better cope with subsequent imidacloprid stress after several generations, but not spirotetramat stress. Hormetic priming did not manifest in a transgenerational manner and only some individuals in the population subjected to hormetic concentrations of imidacloprid appeared to be adapting to the insecticide. Exposure to hormetic and mildly toxic concentrations of imidacloprid did not result in mutations in subunits of the nicotinic acetylcholine receptor. While hormesis poses a challenge to pest management, my findings suggest that the extent to which low dose exposures to chemical stress likely will not result in substantial pesticide adaptation over several generations.

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CHAPTER 1 INTRODUCTION

1.1 Preconditioning Hormesis

Biological responses to low doses of stress have been highlighted in studies on hormesis, a biphasic dose-response in which acute or chronic exposure to low doses of chemical, physical or biological stress can stimulate biological processes in an organism (Calabrese and Baldwin 2002; Calabrese and Blain 2005). Hormesis is considered to be an evolutionary strategy that allows cells or whole organisms to respond to changing environmental conditions. This has significance ecologically as adaptive responses to stress such as hormesis can allow pests to persist even when environmental conditions are unfavorable (Calabrese 2008a; Costantini 2014).

Several response models have been observed under the hormesis framework. The first is a strictly stimulatory response, in which exposure to a stressor at low doses results in an increase in biological responses, whereas at high doses inhibit biological responses. This interaction is depicted as an inverted U-shaped response. Alternatively, depending on the endpoint examined, hormesis has also been represented as a decrease in dysfunction at low doses of stress and an increase in dysfunction at high doses of stress. This interaction is depicted as a J-shaped response (Figure 1.1 a, b) (Calabrese et al. 2007; Calabrese 2008a).

The concept of hormesis and stimulatory responses to stress was established in 1854 by cellular pathologist, Rudolf Virchow, and perpetuated by Hugo Schultz in the 1880's (Henschler 2006). It has been observed in many organisms, including insects exposed to low doses of pesticides where increases in endpoints such as fecundity, and

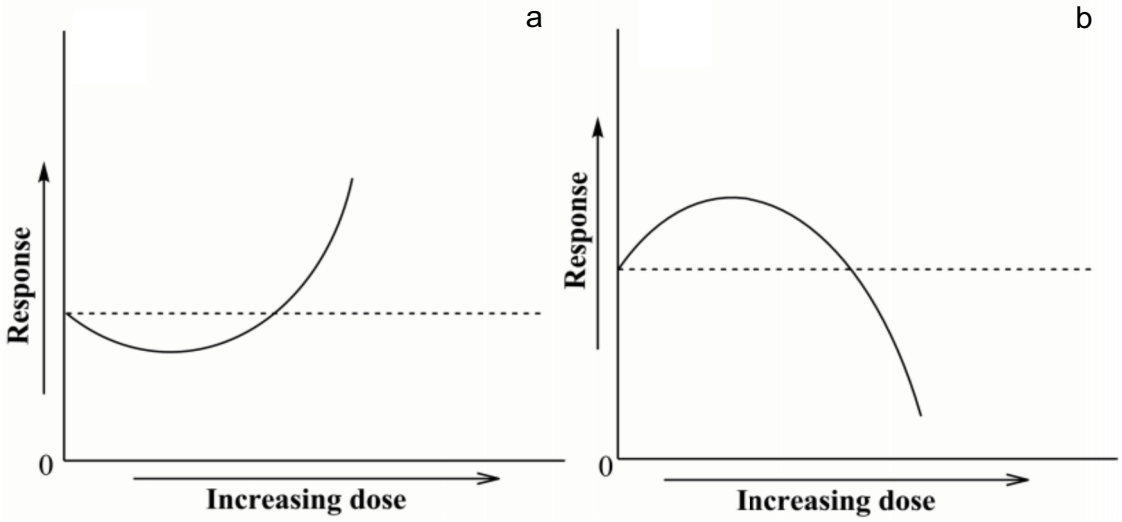

Figure 1.1: Dose response relationships (a) the J-shaped model depicting a decrease in dysfunction at low doses of stress and an increase in dysfunction at high doses of stress; (b) the inverted U-shaped model depicting an increase in response at low doses of stress and a decrease in response at high doses of stress. The dashed line represents the control levels of response (Lushchak 2014).

longevity have been well documented (Cutler 2013). For example: exposure to low doses (below the LC_1) of chlorpyrifos stimulated development and fecundity in *Plutella xylostella* (L.), the diamond back moth (Deng et al. 2016). Exposure to low doses of the pesticides: malathion, dicofol, formetanate, and fluvalinate stimulated reproduction in *Scirtothrips citri* (Moulton), the citrus thrip (Morse and Zareh 1991). Fertility and life span were increased in *Supputius cincticeps* (Stal), the predatory stink bug, after exposure to sublethal doses of the insecticide, permethrin (Zanuncio et al. 2005). The stimulatory responses are typically modest (approximately 30-60% above control values) and are thought to be the result of overcompensation in response to a disruption in homeostasis

caused by the initial stress (Calabrese 2001). Hormetic stimulation alone, poses issues for pest management, as increases in life history traits such as reproduction/fecundity, fertility, and lifespan can result in pest outbreaks and allow pests to persist in the environment.

The second response model under the hormesis framework is an adaptive response to conditioning or preconditioning where acute or long-term exposure to sublethal stress enables the organism to withstand subsequent and greater amounts of the same or different stress (Calabrese et al. 2007; Calabrese 2008a). Some of the first research referencing adaptive responses or preconditioning were in the biomedical sciences. Preconditioning in dogs was observed after the animals were subjected to multiple, brief ischemic events, which protected the animals' myocardial tissue, following a prolonged ischemic event (Murry et al. 1986).

Currently, research in preconditioning has expanded to fields related to environmental and agricultural sciences, including pest management. This is because researchers have been more interested in the role of environmental stress and how organisms respond and acclimate to changing environmental conditions (Calabrese 2008a). In agroecosystems, insect pests are frequently exposed to a wide range of chemical pesticides, in some cases over multiple generations, in varying concentrations as they break down in the environment, and are metabolized by plants and microorganisms. It is thus possible these insects will be exposed to low or sublethal doses of insecticides that may precondition them or their progeny to withstand subsequent insecticide applications. Several studies have demonstrated adaptive responses to sublethal exposures to chemical pesticides. After exposing a single generation of

Frankliniella occidentalis (Pergande), the Western flower thrip, to LC_{10} and LC_{25} concentrations of spinosad, negative effects on development time, fecundity, and intrinsic rate of increase were less pronounced in their offspring when also exposed to spinosad (Gong et al. 2015). Also, exposure of several generations of *Myzus persicae* (Sulzer), the green peach aphid, to sublethal concentrations of the insecticide, imidacloprid, primed the aphids to withstand a subsequent nutritional stress (Rix et al. 2016).

The impacts of sublethal stress on biochemical and molecular pathways have shed light on the adaptive responses observed in hormesis and preconditioning. The general mechanism for these adaptive responses is thought to be the activation and upregulation of existing cellular and molecular pathways specific to the organism-stressor model. For instance, *Tribolium castaneum* (Herbst), the brown flour beetle (Yin et al. 2006), and *Cydia pomonella* (L.), the codling moth (Mahroof et al. 2005), subjected to heat treatment had accumulation of heat shock protein 70 (HSP 70), which resulted in increased thermotolerance. Stimulation of fecundity in *M. persicae* exposed to low doses of imidacloprid was associated with increases in juvenile hormone, which is responsible for vitellogenesis (Yu et al. 2010). The response, however, is complex. I have observed transgenerational patterns in up and down regulation of detoxification genes, stress genes and heat shock protein, in association with increases in *M. persicae* reproduction (Rix et al. 2016). Additional pathways in the antioxidant system, glutathione, signal transduction, and epigenetic inheritance are also potentially involved in the complex adaptive response to stress (Zhao and Wang 2012; Ayyanath et al. 2014).

1.2 Stress, Gene Mutations, and Pesticide Resistance

Despite recent research examining adaptation to and the effects of stress on biochemical and molecular pathways in various organisms (Mahroof et al. 2005; Poupardin et al. 2008; Ahsan et al. 2010; Zhao and Wang 2012; Jeon and Kim 2013; Komatsu et al. 2014; Janmohammadi et al. 2015; Dawkar et al. 2016; Zhang et al. 2016; Zhu et al. 2016), little research has specifically focused on how adaptation to low doses of stress could be related to pesticide tolerance/resistance and increased gene mutations. In stressed conditions, mutations could also increase since DNA repair enzymes compete with detoxification enzymes for ATP, and therefore damage to DNA and mutations in DNA could easily generate in the organism while recovering (Gressel 2011). It has been demonstrated that low doses of stress (in particular chemical stress) increase mutations and cause damage to DNA. For example, earthworms exposed to concentrations of the herbicide, paraquat, well below the LC_{50} resulted in direct damage to DNA in immune cells in the coelomic cavity (Muangphra et al. 2014). Exposure of *Crassostrea gigas* (Thunberg), the Pacific oyster, to sublethal pesticide mixtures resulted in the formation of DNA adducts, biomarkers for oxidative stress and induced DNA damage (Geret et al. 2013). Chromosomal abnormalities were observed in pig oocytes exposed to low doses of imidacloprid (Ishikawa et al. 2015). Significant increases in mutation frequencies were observed in human T lymphocytes exposed to low doses of malathion (Pluth et al. 1996).

Traditional modeling of the evolution of pesticide resistance suggested high dose exposures led to major target-site resistance conferred by mutations in the target site of the pesticide, and lower pesticide doses delayed the onset of target site resistance, due to reduced selection pressure on the organism (Gardner et al. 1998; Gressel 2011). Successive exposures of an organism to high doses of a pesticide will generally only lead

to target site resistance, however exposure to low doses of pesticides can lead to multiple types of resistance. In some situations, where exposure doses are low, resistance manifests as a slow shift in the population to increasingly higher mean resistance in each successive generation, often resulting from an accumulation of small allelic mutations, or gene amplifications, that result in successive increases in pesticide metabolism over generations (Gardner et al. 1998; Gressel 2011). This slow shift in increased resistance was observed in *Phalaris minor* Retz. (bunchgrass), which became increasingly more resistant to the herbicide, isoproturon, in fields consistently under dosed (Gardner et al. 1998), and has also been observed in *Lolium rigidum* Gaud. (annual ryegrass) treated with the herbicide, diclofop-methyl, where incremental increases in the dose level of resistance were observed (Heap 1991). Molecular evidence for this metabolic type of resistance has been seen in insecticide resistant populations of *M. persicae* and *Culex quinquefasciatus* Say, exhibiting increased amplification of esterases (Mouches et al. 1986; Field et al. 1988; Philippou et al. 2010) and cytochrome P450 monooxygenases (Puinean et al. 2010b; Gong et al. 2013). Other evidence has also been found in plants, where increases in amplification of 5-enolpyruvylshikimate-3-phosphate synthase were seen in glyphosate tolerant tobacco cells (Wang et al. 1991).

It is usually assumed that in situations described above that low doses simply select for more tolerant individuals, which then become increasingly resistant over time. This is not always the case. There is also some evidence that target-site mutations can occur at lower exposure rates. A study conducted across several locations in Denmark, Lithuania, and Sweden by Wieczorek et al. (2015) examined the effects of DMI (14α-demethylation inhibitor) fungicides on the cyp51 gene (encoding 14α- demethylase) in

Zymoseptoria tritici (Fuckel), which causes Septoria leaf blotch in wheat. Fungicides were applied at half the standard rates over three seasons. Mutations in CYP51 were seen at the end of all three seasons, and varied depending on population and treatment combination. Some instances of these target site mutations occurring at lower exposure rates can result in resistance. *L. rigidum* populations in Australia, where lowest herbicide cut rates were used, were exposed to the reduced rates of the herbicide, sethoxydim, over three seasons, resulting in mutations altering the shape of acetyl coenzyme A carboxylase (ACCase), which sethoxydim inhibits as its mode of action. This additionally resulted in cross-resistance to aryloxyphenoxypropionate, which also shares ACCase as its target site (Tardif et al. 1993; Gressel 2011). Additionally, another population of *L. rigidum* resistant to ACCase inhibitors by means of metabolic methods, but known to still have susceptible ACCase (ACCase inhibitor target site), was subjected to a low dose herbicide selection pressure which resulted in the formation of ACCase inhibitor resistance by means of target site modification (Tardif and Powles 1994).

While there is a precedent for the development of mutations of genes important in pesticide resistance following exposure to low doses of pesticide – usually metabolically over time, but even by means of target site mutations - the question remains as to how low an exposure can result in the development of mutations, particularly those in target sites.

1.3 *Myzus persicae* and Insecticide Resistance

Insects are among the most abundant organisms on earth, comprising around 75% of the world's recorded fauna. While the majority of insects are beneficial, many insect species compete with humans, wreaking havoc on agriculture, stored products and human health

(Loxdale 2016). Insecticides are powerful tools to supress insect pests, with worldwide sales estimated to be above 11.1 billion dollars U.S. (2007 data) (Grube et al. 2011).

Myzus persicae is one of the most economically important pests worldwide. It is highly polyphagous, capable of feeding on over 400 species across 40 different plant families (Bass et al. 2014), such as Chenopodiaceae, Cruciferae, Cucurbitaceae, Solanaceae. It is also known to transmit more than 100 plant viruses (vanEmden and Harrington 2007).

The life cycle of *M. persicae* is heavily dependent upon climate. In temperate regions, where autumn and winter temperatures drop below 20°C, *M. persicae* is holocyclic and heteroecious (vanEmden and Harrington 2007). In autumn and winter, *M. persicae* typically reproduces sexually on its primary host, *Prunus persica* or *Prunis nigra*, overwintering as eggs. Emergence occurs during the warmer summer months, and adults reproduce parthenogenetically (asexually) on available secondary hosts. This is known as cyclical parthenogenesis (Blackman 1974). In regions where temperatures remain around or above 20°C for the duration of the year, anholocyclic lifecycles predominate, whereby aphids reproduce continuously throughout the year by parthenogenesis (Blackman 1974; vanEmden and Harrington 2007). Anholocyclic life cycles, combined with short generation time, allow aphids to reproduce rapidly, and significantly impact population genetics and resistance development (Bass et al. 2014).

Myzus persicae control has relied mainly on extensive use of chemical pesticides. As such, aphids have developed widespread resistance to most classes of insecticides. Carbamates and organophosphate resistance developed by way of overproduction of carboxylesterases, which hydrolyse the insecticide. This was as a result of amplification

of the structural genes for E4 and FE4 esterases (Devonshire and Moores 1982; Field et al. 1988). Dimethyl carbamate resistance resulted from a point mutation in the acetylcholinesterase enzyme (AChE), which changed a serine amino acid to a phenylalanine, preventing the insecticide from interacting with AChE (Nabeshima et al. 2003; Benting and Nauen 2004). This mutation also reduces sensitivity to pyrethroid insecticides, although the main mode of pyrethroid resistance is through mutations in voltage-gated sodium channels, altering the ability of the sodium channel to open, or reducing polar interactions between pyrethroids and the binding site. (Martinez-Torres et al. 1999; O'Reilly et al. 2006; Eleftherianos et al. 2008; Bass et al. 2014; Du et al. 2014; Silver et al. 2014).

In the 1990's, the neonicotinoid class of insecticides and the active ingredient imidacloprid were introduced to the marketplace. Multiple neonicotinoid insecticides have been developed (e.g., acetamiprid, dinotefuran nitenpyram, clothianidin, thiamethoxam, thiacloprid, and imidacloprid) and these insecticides now make up more than 25% of global insecticide sales (Jeschke and Nauen 2008; Bass et al. 2015). Neonicotinoids have become one of the most important classes of insecticides due to their low binding affinity to vertebrate receptors and subsequently, selectivity for arthropods, and systemic movement through plants (Simon-Delso et al. 2015). However, since 2000, the number of cases of resistance to neonicotinoids has greatly increased. Resistance in Hemiptera such as *M. persicae* has been well documented (Bass et al. 2015).

1.4 Nicotinic Acetylcholine Receptors and *Myzus persicae*

Neonicotinoids are agonists of nicotinic acetylcholine receptors (nAChR), binding to receptor subunits and inducing continuous excitation of the neuronal membranes, which leads to exhaustion of cell energy, paralysis and death (Simon-Delso et al. 2015). nAChRs are members of the Cys-loop ligand-gated ion channel superfamily, acting as fast acting receptors for acetylcholine (ACh), and in insects, are located throughout the nervous system (Matsuda et al. 2001). The general structure of all nAChRs consists of a central ion channel, surrounded by five subunits: α subunits and non-α subunits (β, γ, δ, ε). α subunits consist of two adjacent cysteine residues in loop C, non-α subunits do not possess these adjacent cysteine residues (Figure 1.2) (Jones and Sattelle 2010). Loops A-C are supplied by α subunits, whereas loops D-F are supplied by either a non-α subunit or an α subunit. The ACh-binding site is located at the interface between these regions (Figure 2). Subunit composition dictates the functional properties of the receptor. In a given organism, multiple subunit-encoding genes generate diversity in the receptor (Jones and Sattelle 2010). For example, in vertebrate species, seventeen subunits have been identified (α1- α10, β1- β4, γ, δ, ε), whereas in insects nAChRs generally consist of ten distinct subunits (α1- α7, β1- β3) (Millar and Lansdell 2010). Thus far, four α subunits (Mpα1, Mpα2, Mpα3, Mpα4) and one β subunit (Mpβ1) have been fully sequenced (with published primers). A fifth α subunit (Mpα5) has been partially sequenced (Matsuda et al. 2001; Puinean et al. 2010b).

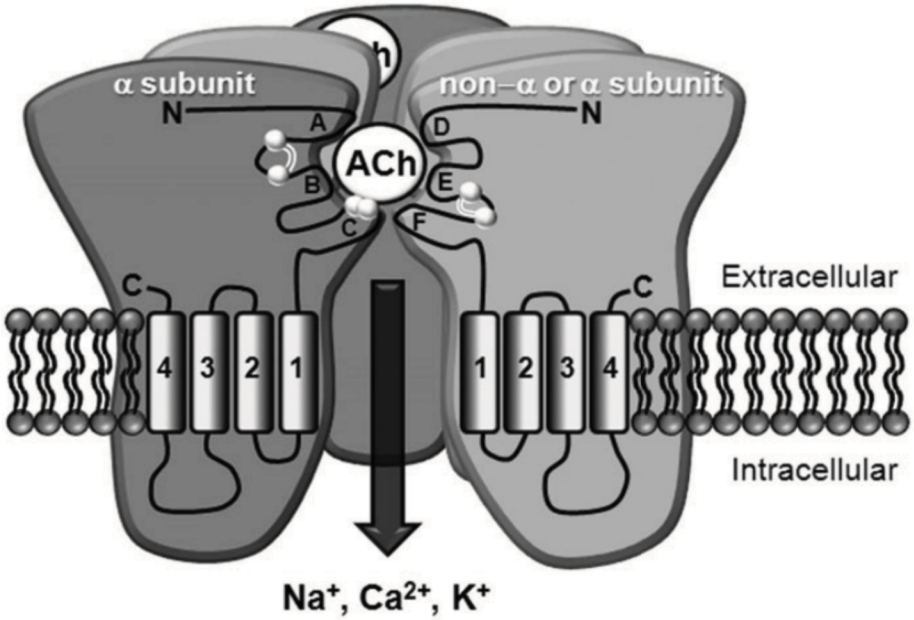

Figure 1.2: General structure of the nAChR. α subunits are depicted in dark grey, non-α subunits are depicted in light grey. Cys-loops are two white spheres connected by white lines. White spheres in loop C represent cysteines, differentiating α subunits from non-α subunits. Binding loops A-F are shown along with bound acetylcholine (ACh) molecules. The subunits making up the receptor are arranged around a central ion channel (Jones and Sattelle 2010).

The α subunits 1 and 3, as well as β subunit 1, appear to be targets for mutations, conferring resistance to neonicotinoids in Hemiptera. Two mutations in the β1 subunit of the pea aphid, *Aphis gossypii* Glover, are associated with resistance to neonicotinoids; one (L80S) changes a leucine amino acid to a serine amino acid, and the other (R81T) changes an arginine amino acid to a threonine amino acid. Both mutations impact the affinity of imidacloprid to the binding site (Bass et al. 2015; Kim et al. 2015). In brown planthopper, *Nilaparvata lugens* (Stal), a mutation in both α1 and α3 subunits (Y151) resulted in replacement of a tyrosine with a serine residue, which resulted in substantial

reduction in imidacloprid binding (Liu et al. 2005). In *M. persicae*, neonicotinoid resistance has thus far been associated with the R81T mutation in the β1 subunit. While amplification of the structural *CYP6CY3* gene has also been implicated in neonicotinoid resistance and sensitivity, the main mode of resistance is through the R81T mutation (Puinean et al. 2010b; Bass et al. 2011).

1.5 Objectives and Hypothesis

The ability of an organism to respond and acclimate to changing environmental conditions is essential to its survival. Hormesis is thought to be an adaptive, evolutionary response to stress. The stimulatory effects induced by hormetic exposures to pesticides can have ramifications in terms of pest outbreaks, but also in terms of adaptation to pesticide exposure. Through preconditioning hormesis, it has been shown that acute or long-term exposure to sublethal stress can enable an organism to withstand subsequent and greater amounts of the same or different stress (Calabrese et al. 2007; Calabrese 2008a). This has been demonstrated in many organisms, including insects (Gong et al. 2015; Rix et al. 2016). Adaptation to stress could also involve gene mutations. Under stressed conditions, mutations can be induced or increased as a result of DNA repair enzymes competing with detoxification enzymes for ATP (Gressel 2011). Several examples of low doses of chemical stress resulting in mutations and changes in DNA have been demonstrated in the literature (Geret et al. 2013; Muangphra et al. 2014), including some evidence to suggest that susceptible populations can develop target site mutations after relatively short exposures (Tardif et al. 1993; Wieczorek et al. 2015).

Preconditioning hormesis has been previously demonstrated in *M. persicae*, as well, sublethal exposure to insecticides have also resulted in changes at the molecular

level (Ayyanath et al. 2014; Rix et al. 2016). It is unclear, however, if preconditioning hormesis can be transgenerational, and whether concentrations in and around the hormetic zone can be stressful enough to cause mutations in target sites.

I reared *M. persicae* for up to four generations on leaf discs treated with sublethal concentrations of the neonicotinoid insecticide imidacloprid, testing whether prior exposure would make subsequent generations less susceptible to imidacloprid. I also tested if prior exposure to imidacloprid would render successive generations of *M. persicae* less susceptible to spirotetramat, an inhibitor of lipid biosynthesis in aphids. I ran probit experiments with imidacloprid in 4^{th} generation conditioned aphids to further determine if prior exposure to imidacloprid at sublethal doses reduces susceptibility to imidacloprid. I hypothesized that hormetic exposure to imidacloprid would enhance aphid ability to withstand subsequent insecticide exposure with each successive generation, as evidenced through significantly reduced mortality across generations. I also hypothesized that hormetic exposure to imidacloprid would reduce susceptibility to imidacloprid in 4^{th} generation aphids as evidenced through significant differences in probit lines constructed from a dose response assay.

I additionally sequenced Mpα1- Mpα4, and Mp β1 genes in first generation and fourth generation aphids reared on hormetic and sublethal concentrations of imidacloprid, and examined these sequences for mutations. I hypothesized that we would observe mutations or changes in gene sequences in aphids exposed to hormetic concentrations not observed in controls.

CHAPTER 2 TRANSGENERATIONAL EFFECT OF LOW DOSE IMIDACLOPRID PRECONDITIONING ON *MYZUS PERSICAE* (HEMIPTERA: APHIDIDAE) EXPOSED TO SUBSEQUENT INSECTICIDAL STRESS

2.1 Introduction

Hormesis is a phenomenon in biology whereby exposure of organisms to sublethal amounts of stress results in the stimulation of various biological processes in the organism. Hormesis is an evolutionarily conserved, adaptive response to stressful conditions (Calabrese et al. 2007). In general, the hormetic response has been shown to stimulate many biological processes in an organism, such as increasing reproduction, fecundity, and lifespan. This has been demonstrated in many insect species, in response to various chemical, physical and biological stressors (Cutler 2013). Included in the hormetic dose-response relationship is the concept of preconditioning, whereby exposure to a low dose of a stressor or toxic agent, reduces susceptibility with subsequent exposure to a harmful dose of the same, similar, or even dissimilar stressor or toxic agent (Calabrese 2016b). Evidence of preconditioning hormesis has been demonstrated in microbes (Samson and Cairns 1977), mammals (cells) (Murry et al. 1986), plants (Shao et al. 2016; Silva et al. 2016), and insects (Gong et al. 2015; Alptekin et al. 2016; Rix et al. 2016).

Insect pests are likely to be exposed to low and sublethal stressors, such as chemical pesticides, as these chemicals break down in the environment (Cutler 2013). Hormesis thus potentially poses challenges to pest management, as organisms continue to reproduce and persist, even when environmental conditions are unfavorable (Calabrese 2008b; Costantini 2014). The effects of preconditioning hormesis could have additional consequences, particularly if the effects of preconditioning can be transgenerational; that

is, if organisms are exposed to low dose/sublethal stress over several generations and toxicity of subsequent stress is reduced with each successive generation.

It was previously shown exposure over several generations to hormetic concentrations of imidacloprid primed *M. persicae* to withstand subsequent food/water stress, but not a subsequent insecticide stress in the form of exposure to an LC20 of spirotetramat, an insect lipid biosynthesis inhibitor (Rix et al. 2016). It was unclear whether failure to see conditioning to spirotetramat exposure was a result of: the imidacloprid preconditioning concentration being too low to provide sufficient priming for an additional chemical stress; the subsequent spirotetramat exposure concentration being too low or high to observe decreased susceptibility; or whether it was a result of spirotetramat being a different mode of action than imidacloprid.

In the present experiments, I examined if multigenerational exposure of *M. persicae* to reproductively hormetic concentrations of imidacloprid resulted in reduced susceptibility of this insect to imidacloprid, or spirotetramat, across generations. I hypothesized that low dose exposure to imidacloprid would result in reduced transgenerational mortality of *M. persicae* when subsequently exposed to imidacloprid or spirotetramat.

2.2 Materials and Methods

2.2.1 Insect Rearing

Myzus persicae were obtained from a wild greenhouse population living on cabbage plants at the Faculty of Agriculture, Dalhousie University. This population has been reared in the laboratory since 2010, without exposure to insecticides. Aphids for experiments were reared on excised cabbage leaves inserted into plastic water floral

picks, and placed into Tupperware® bins (37 x 24 x 14 cm), lined with dry paper towel. Aphids were kept in a growth chamber at 22 ± 2° C, 16:8 L:D, and 65 ± 5 % RH. Twice per week, fresh leaves in floral picks were placed into the containers to allow aphids to move to a new, fresh food source. Old leaves in floral picks were removed after approximately 80% of aphids had transferred to new leaves. Paper towel was also replaced when new leaves in floral picks were added.

2.2.2 Treatment Solutions

Imidacloprid (Admire® 240 SC, 240 g active ingredient (AI) L^{-1}; Bayer Crop Science Canada, AB, Canada) or spirotetramat (Movento® 240, 240 g AI L^{-1}; Bayer CropScience Canada) were suspended in deionized water to make 1000 mg AI L^{-1} stock solutions that were then diluted to make working solutions for specific experiments.

2.2.3 Determination of Hormetic Concentration

Preconditioning bioassays required that I determine the concentration(s) of imidacloprid that resulted in reproductive hormesis in *M. persicae*, as previously reported (Ayyanath et al. 2013). In these bioassays, five first-instar aphids were placed on 1-cm leaf discs, in 5-cm petri dishes lined with filter paper. Leaf discs were dipped for 5 s in 0, 0.025, 0.1, 0.25, or 1.0 µg L^{-1} of imidacloprid, and air-dried. Three leaf discs of the same treatment were placed in each dish. Petri dishes were sealed with Parafilm®, randomly placed within a single Tupperware bin, and kept in an environmental chamber at 22 ± 2° C, 16:8 L:D, and 65 ± 5 % RH. Leaf discs were replaced three times per week and aphids were exposed for approximately 20 days total, which consisted of approximately 10 days to reach adulthood, followed by 10 days of reproduction. The total number of young in each petri dish was recorded each day, and these young were removed so aphids did not

accumulate on leaves. Data analysis was conducted on the overall total number of young. There were three replicate petri plates for the 0, 0.1, 0.25 and 1.0 μg L^{-1} treatments, and two replicates for the 0.025 μg L^{-1} treatment, as aphids in the third replicate were parasitized by *Aphidius ervi* Haliday. Significant differences in reproduction among treatment were determined through analysis of variance (ANOVA) in SAS using the PROC MIXED procedure (SAS 2013). Means were separated with a Tukey test. The hormetic concentration was confirmed to be 0.1 μg L^{-1} (see Results).

2.2.4 Lethal Dose-Response Bioassays

I wanted to test whether aphids could be preconditioned to better survive subsequent exposure to LC_{10} and LC_{50} concentrations of imidacloprid, and the LC_{50} concentration of spirotetramat. To establish LC values of each insecticide, leaf discs were dipped for 5 s in 0, 0.1, 0.3, 0.5, 0.7, 0.9, 1.1 or 1.3 mg L^{-1} of imidacloprid, or 0, 0.3, 0.5, 0.7, 1.0, 3.0, 5.0, 10.0, 15.0, or 20.0 mg L^{-1} of spirotetramat. When dry, three leaf discs of a single treatment were placed in a 5 cm petri dish lined with a filter paper, and seven first-instar aphids were added. Petri dishes were then sealed with Parafilm and placed in Tupperware bins, which were kept in an environmental chamber at 22 ± 2^{o} C, 16:8 L:D, and 65 ± 5 % RH. Mortality was recorded at 48 h. Aphids were considered dead if they did not move after being lightly prodded with forceps. There were three replicate petri plates of aphids per treatment for both insecticides. Probit analysis was run in SAS using PROC PROBIT to determine LC_{10} and LC_{50} of imidacloprid, and the LC_{50} of spirotetramat. For imidacloprid, the LC_{10} was 0.21 mg L^{-1} and the LC_{50} was 0.5 mg L^{-1} (see Results). The LC_{50} of spirotetramat was 2.3 mg L^{-1} (see Results). The LC_{10} concentration of

spirotetramat was also determined (see Results), however only the LC_{50} was used in subsequent insecticide exposure experiments due to aphid numbers.

2.2.5 Insecticide Preconditioning

Aphids were exposed to imidacloprid for up to four generations. First instar aphids were randomly selected from stock colonies and placed on 1-cm diameter leaf discs dipped for 5 s in 0 (control), 0.1, 5, or 10 μg L^{-1} of imidacloprid, and air-dried; 0.1 μg L^{-1} was the hormetic concentration determined in section 2.2.3, and the 5 and 10 μg L^{-1} concentrations were mildly toxic. Three leaf discs of a single concentration were placed in a Petri dish, which was lined with filter paper. Five aphids were placed on leaves in each petri dish, and dishes were wrapped with Parafilm. Eight Petri dishes per treatment were placed in Tupperware bins and reared for either 1 generation (G1), two generations (G2), three generations (G3) or 4 generations (G4). A total of three to four bins of petri dishes were preconditioned for each generation for each experiment. Bins were kept in an environmental chamber at 22 ± 2° C, 16:8 L:D, and 65 ± 5 % RH. Three times per week, filter paper and leaf discs were replaced.

2.2.6 Subsequent Insecticide Exposure Experiments

In order to determine if exposure to low doses of insecticides subsequently reduced susceptibility to greater insecticide exposure, over generations, offspring of exposed aphids were subjected to selected concentrations of imidacloprid or spirotetramat. As female aphids reproduced on treatments described in section 2.2.5, seven first-instar nymphs from each treatment were selected from G1, G2, and G3, and then randomly transferred on to three 1 cm leaf discs in petri dishes lined with filter paper. Prior to transfer of offspring, leaf discs were dipped for 5 s in control, LC_{10}, or LC_{50} treatment

concentrations of imidacloprid, or a LC_{50} treatment of spirotetramat (see section 2.2.4). Petri dishes were wrapped in Parafilm and placed into Tupperware bins. Bins were stored in a growth chamber at 22 ± 2° C, 16:8 L:D, and 65 ± 5 % RH. Mortality was recorded at 48 h. Aphids were considered dead if they did not move after being lightly prodded with forceps.

For subsequent exposure to imidacloprid LC_{10} or LC_{50} concentrations, 26-27 replicate plates of aphids were used from each preconditioning treatment per generation. For subsequent exposure to the spirotetramat LC_{50} concentration, 37-38 replicate plates of aphids from the preconditioning treatment were used from G1, 15-20 replicate plates from G2, and 17-18 replicate plates from G3.

Mortality data were analyzed as factorial designs, with imidacloprid concentration (Imid Conc), generation (Gen) and subsequent exposure treatment (0, LC_{10}, LC_{50} imidacloprid (LCI), or 0, LC_{50} spirotetramat (LCS)) as factors, in SAS (SAS 2013) using the PROC MIXED procedure. Highest order significant interactions ($\alpha < 0.05$), were further analyzed through multiple means comparison using LS MEANS with Tukey adjustment (SAS 2013).

2.2.7 Dose-Response Experiments

In addition to subsequently exposing imidacloprid treated aphids to LC_{10} or LC_{50} concentrations of insecticide, I also subjected fourth generation aphids to an imidacloprid dose response assay, to more clearly determine, over a range of concentrations, if several generations of imidacloprid exposure resulted in reduced susceptibility of *M. persicae* to imidacloprid. Fourth generation aphids reared on 0, 0.1, 5 and 10 μg L^{-1} imidacloprid were used in dose response experiments. As their mothers reproduced, seven first-instar

aphids from each of the four treatments were placed on three leaf discs in petri dishes lined with filter paper. Discs were dipped for 5 s in 0, 0.3, 0.6, 0.9, 1.2, or 1.5 mg L^{-1} imidacloprid. Petri dishes were wrapped in Parafilm and placed into Tupperware bins. Bins were stored in a growth chamber at 22 ± 2° C, 16:8 L:D, and 65 ± 5 % RH. Mortality was recorded at 48 h. Aphids were considered dead if they did not move after being lightly prodded with forceps.

There were 5-6 replicate Petri plates of aphids per treatment (0, 0.3, 0.6, 0.9, 1.2, or 1.5 mg L^{-1} imidacloprid) used to generate dose-response curves from 4th generation aphids from each preconditioning treatment (0, 0.1, 5, or 10 µg L^{-1}). Data were analyzed using the PROC PROBIT procedure in SAS (SAS 2013) to create dose mortality data. Differences b/w slopes of the control line were compared with individual treatment lines using ANCOVA (analysis of covariance) in the PROC GLM procedure, in SAS (SAS 2013). A ratio test (Wheeler et al. 2006) was also preformed to determine if treatment LC_{50} values were significantly different from the control.

2.3 Results

2.3.1 Determination of Hormetic Concentration

Reproduction of *M. persicae* on leaf discs was affected by imidacloprid treatment ($F_{4,9}$ = 15.48; $P < 0.0001$). Although reproduction of aphids on discs treated with 0.025, 0.25, and 1.0 µg L^{-1} imidacloprid was not different from that on control leaf discs, reproduction of aphids on leaf discs treated with 0.1 µg L^{-1}imidacloprid was 37% greater than that of controls after 10 days (Table 2.1). This confirmed that the 0.1 µg L^{-1}imidacloprid treatment caused reproductive hormesis in *M. persicae*.

Table 2.1. Reproduction of M*yzus persicae* on imidacloprid-treated leaf discs after exposure of 5 adults per replicate for 20 days. *n* = number of Petri plates containing treated leaf discs. Data were analyzed using ANOVA (α= 0.05), and means separated with Tukey's.

Treatment ($\mu g\ L^{-1}$)	***n***	**Mean Total Number of *M. persicae* (+/- SD)**
0	3	19.16 (± 2.72) bc*
0.025	2	19.26 (± 0.59) bc
0.1	3	30.09 (± 1.83) a
0.25	3	25.43 (± 2.80) ab
1.0	3	17.66 (± 2.17) c

*Means with the same letter are not significantly different.

2.3.2 Lethal Dose-Response Bioassays

The LC_{10} and LC_{50} concentrations of imidacloprid, and the LC_{50} concentrations of spirotetramat that *M. persicae* would be exposed to during subsequent exposure bioassays were confirmed by the probit analysis. Results are displayed in Table 2.2. The LC_{10} value for spirotetramat is recorded, however only LC_{50} was used in subsequent insecticide exposure experiments due to aphid numbers. χ^2 values for both spirotetramat and imidacloprid were not significant, indicating the probit model was an appropriate fit for the data. The LC_{10} values for imidacloprid and spirotetramat were similar. The LC_{50} value for spirotetramat was almost 5-fold larger than the LC_{50} value for imidacloprid, indicating that imidacloprid is more effective as a pesticide.

Table 2.2. Contact toxicity of imidacloprid and spirotetramat to first instar *Myzus persicae* exposed on treated leaf discs for 48 h.

Insecticide	*n*	LC_{10} (mg L^{-1}) (95% FL)	LC_{50} (mg L^{-1}) (95% FL)	Slope (± SE)	χ^2 *(P)*
Imidacloprid	24	0.21 (0.01-0.36)	0.50 (0.13-0.61)	3.44 (± 1.19)	15.32 (0.60)
Spirotetramat	30	0.28 (0.08-0.57)	2.30 (1.35-3.56)	1.39 (± 0.21)	16.23 (0.91)

2.3.3 Subsequent Insecticide Exposure Experiments: Imidacloprid

Main effects of imidacloprid exposure (Imid Conc), generation (Gen), and subsequent imidacloprid exposure (LCI) significantly affected aphid mortality (Table 2.3). The interaction between imidacloprid exposure and generation was significant (Table 2.3), indicating that significant effects on aphid mortality were dependent upon the combinations of prior imidacloprid exposure and generation. I was primarily interested in potential differences across generations within each of the four exposure concentrations (0 (Control), 0.1, 5, 10 μg L^{-1}), not differences between the concentrations (which are to be expected), thus only statistical comparisons across generations within a given exposure concentration were reported (Table 2.4). There were no significant differences in mortality across generations for aphids reared on control leaves, and subsequently exposed to 0, LC_{10}, and LC_{50} concentrations of imidacloprid (Table 2.4, Fig. 2.1). For aphids reared on 0.1 μg L^{-1} of imidacloprid, no significant differences in mortality after subsequent imidacloprid exposure were seen between G1 and G2, or G2 and G3. However, mortality in G3 was significantly lower than mortality in G1 (Table 2.4, Fig. 2.1). Mortality after subsequent imidacloprid exposure in aphids reared on leaves treated with 5 μg L^{-1} of imidacloprid was marginally higher in G1 than G2 and G3 (Table 2.4, Fig. 2.1). There were no significant differences in mortality between G2 and G3 (Table 2.4, Fig. 2.1). For aphids exposed to 10 μg L^{-1} of imidacloprid, mortality was significantly higher in the first generation compared with G2, but not significantly different from G3. G2 mortality was also not significantly different from G3 (Table 2.4, Fig. 2.1). Thus, only transgenerational exposure of aphids to a hormetic concentration of

imidacloprid reduced susceptibility to higher, more toxic concentrations of imidacloprid (LC_{10}, LC_{50}), and only after several generations of exposure to imidacloprid.

Table 2.3. Statistics for main effects of concentration (Conc), generation (Gen) and subsequent imidacloprid exposure (LCI) on mortality in *Myzus persicae*.

Effect	Statistics
Imid Conc	$F_{3,286} = 3.82$; $P = 0.01$
Gen	$F_{2,286} = 15.01$; $P < 0.01$
LCI	$F_{2,286} = 484.82$; $P < 0.01$
Imid Conc*LCI	$F_{6,286} = 0.61$; $P = 0.72$
Imid Conc*Gen	$F_{6,286} = 2.27$; $P = 0.04$
Gen*LCI	$F_{4,286} = 0.13$; $P = 0.97$
Imid Conc*Gen*LCI	$F_{12,286} = 1.37$; $P = 0.18$

M. persicae were exposed for 1, 2, or 3 generations to leaf discs treated with 0, 0.1, 5, or 10 μg L^{-1} of imidacloprid, and subsequently exposed to leaf discs treated with 0, LC_{10} or LC_{50} concentrations of imidacloprid for 48 h.

Table 2.4. Comparisons (LSMEANS) of significant interaction of analyzing of subsequent imidacloprid exposure and generation on *M. persicae* mortality. Stars (*) highlight significant differences.

Generational Comparison [a]	**Concentration (µg L^{-1})**			
	0 (Control)	**0.1**	**5**	**10**
Gen 1: Gen 2	$t_{286} = 0.34$ $P = 1.00$	$t_{286} = 1.28$ $P = 0.98$	$t_{286} = 2.24$ $P = 0.52$	$t_{286} = 3.84$ $P < 0.01$*
Gen 1: Gen 3	$t_{286} = 1.37$ $P = 0.97$	$t_{286} = 4.32$ $P < 0.01$*	$t_{286} = 2.24$ $P = 0.52$	$t_{286} = 2.72$ $P = 0.22$
Gen 2: Gen3	$t_{286} = 1.05$ $P = 0.99$	$t_{286} = 3.04$ $P = 0.10$	$t_{286} = -0.00$ $P = 1.00$	$t_{286} = -1.12$ $P = 0.99$

[a] Comparisons show significant or non-significant differences in *M. persicae* mortality across generations within each of the four exposure concentrations, not differences between concentrations.

M. persicae were exposed for 1, 2, or 3 generations to 0, 0.1, 5, or 10 µg L^{-1} of imidacloprid on treated leaf discs and subsequently exposed to 0, LC_{10} or LC_{50} of imidacloprid on leaf discs for 48 h.

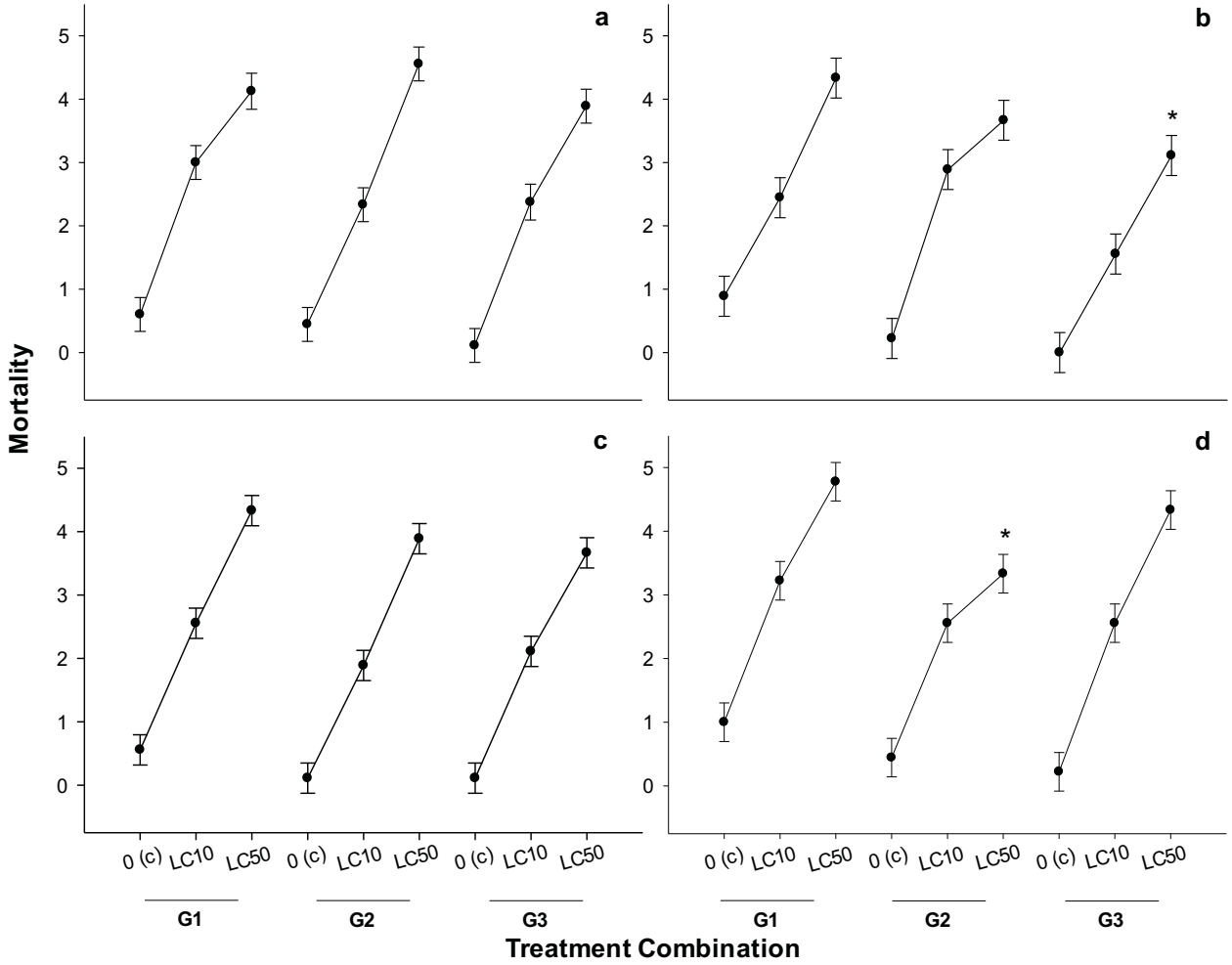

Figure 2.1. Mortality (out of 7) of *Myzus persicae* exposed to 0 (control) (a), 0.1 (b), 5 (c) and 10 (d) µg L^{-1} of imidacloprid over three generations (G1, G2, G3), after subsequent exposure to 0 (control), LC_{10} (10) and LC_{50} (50) concentrations of imidacloprid. Stars represent significant effects on mortality shown in Table 2.4.

2.3.2 Subsequent Insecticide Exposure Experiments: Spirotetramat

Main effects of imidacloprid exposure (Imid Conc), generation (Gen), and subsequent spirotetramat exposure (LCS) significantly affected aphid mortality (Table 2.5). The interaction between imidacloprid exposure, generation, and subsequent spirotetramat exposure (Table 2.5) was significant, indicating that effects on aphid mortality were dependent upon the combinations of prior imidacloprid exposure, generation, and subsequent spirotetramat. Given that I was mainly interested in potential differences across generations within each of the four imidacloprid exposure concentrations (0 (Control), 0.1, 5, 10 μg L^{-1}), not differences between the four concentrations (which are to be expected), I reported only statistical comparisons across generations, within a given exposure concentration (Table 2.6). There were no significant differences in aphid mortality across generations when reared on control, 0.1, and 5 μg L^{-1} imidacloprid-treated leaves, and subsequently exposed to 0 and LC_{50} concentrations of spirotetramat (Table 2.6, Figure 2.2). That is, transgenerational exposure of aphids to low or hormetic doses of imidacloprid did not reduce the susceptibility of progeny to a toxic dose (LC50) of spirotetramat. For aphids reared on leaves treated with 10 μg imidacloprid L^{-1} and subsequently exposed to LC_{50} concentrations of spirotetramat, mortality was significantly lower in G1 aphids compared with G2 aphids, but no other significant differences were observed (Table 2.6, Figure 2.2).

Table 2.5. Main effects of imidacloprid concentration (Imid Conc), generation (Gen), and subsequent spirotetramat exposure (LCS) on mortality in *Myzus persicae*.

Effect	Statistics
Imid Conc	$F_{3,258} = 4.71$; $P < 0.01$
Gen	$F_{3,286} = 3.62$; $P = 0.028$
LCS	$F_{1,258} = 733.18$; $P < 0.01$
Imid Conc*LCS	$F_{3,258} = 10.03$; $P < 0.01$
Imid Conc*Gen	$F_{6,258} = 1.18$; $P = 0.32$
Gen*LCS	$F_{2,258} = 7.75$; $P < 0.01$
Imid Conc*Gen*LCS	$F_{6,258} = 2.22$; $P = 0.04$

M. persicae were exposed for 1, 2, or 3 generations to leaf discs treated with 0, 0.1, 5, or 10 μg L^{-1} of imidacloprid, and subsequently exposed to leaf discs treated with 0 or LC_{50} concentrations of spirotetramat for 48 h.

Table 2.6. Comparisons (LSMEANS) of the significant interaction of imidacloprid concentration, subsequent spirotetramat exposure, and generation on *Myzus persicae* mortality. Stars (*) highlight significant differences.

Generational Comparison [a]	**Concentration (µg L^{-1})**			
	0 (Control)	**0.1**	**5**	**10**
Gen 1 (0): Gen 2 (0)	t_{226} = 0.54 P = 1.00	t_{226} = 1.44 P = 0.99	t_{226} = 2.06 P = 0.92	t_{226} = 2.01 P = 0.94
Gen 1 (LC_{50}): Gen 2 (LC_{50})	t_{226} = 1.38 P = 0.99	t_{226} = -1.26 P = 0.99	t_{226} = 0.65 P = 1.00	t_{226} = -3.81 P = 0.03*
Gen 1 (0): Gen 3 (0)	t_{226} = 0.49 P = 1.00	t_{226} = 1.91 P = 0.96	t_{226} = 2.55 P = 0.64	t_{226} = 2.59 P = 0.60
Gen 1 (LC_{50}): Gen 3 (LC_{50})	t_{226} = 0.48 P = 1.00	t_{226} = -0.05 P = 1.00	t_{226} = 0.97 P = 1.00	t_{226} = -1.63 P = 0.99
Gen 2 (0): Gen 3 (0)	t_{226} = -0.03 P = 1.00	t_{226} = 0.46 P = 1.00	t_{226} = 0.51 P = 1.00	t_{226} = 0.41 P = 1.00
Gen 2 (LC_{50}): Gen 3 (LC_{50})	t_{226} = -0.75 P = 1.00	t_{226} = 1.01 P = 1.00	t_{226} = 0.28 P = 1.00	t_{226} = 1.98 P = 0.95

[a] Comparisons show significant or non-significant differences in aphid mortality across generations between 0 or LC_{50} concentrations (given the 3-way interaction), within each of the four exposure concentrations, not differences between concentrations.

M. persicae were exposed for 1, 2, or 3 generations to 0, 0.1, 5, or 10 µg L^{-1} of imidacloprid on treated leaf discs and subsequently exposed to 0 or LC_{50} of spirotetramat on leaf discs for 48 h.

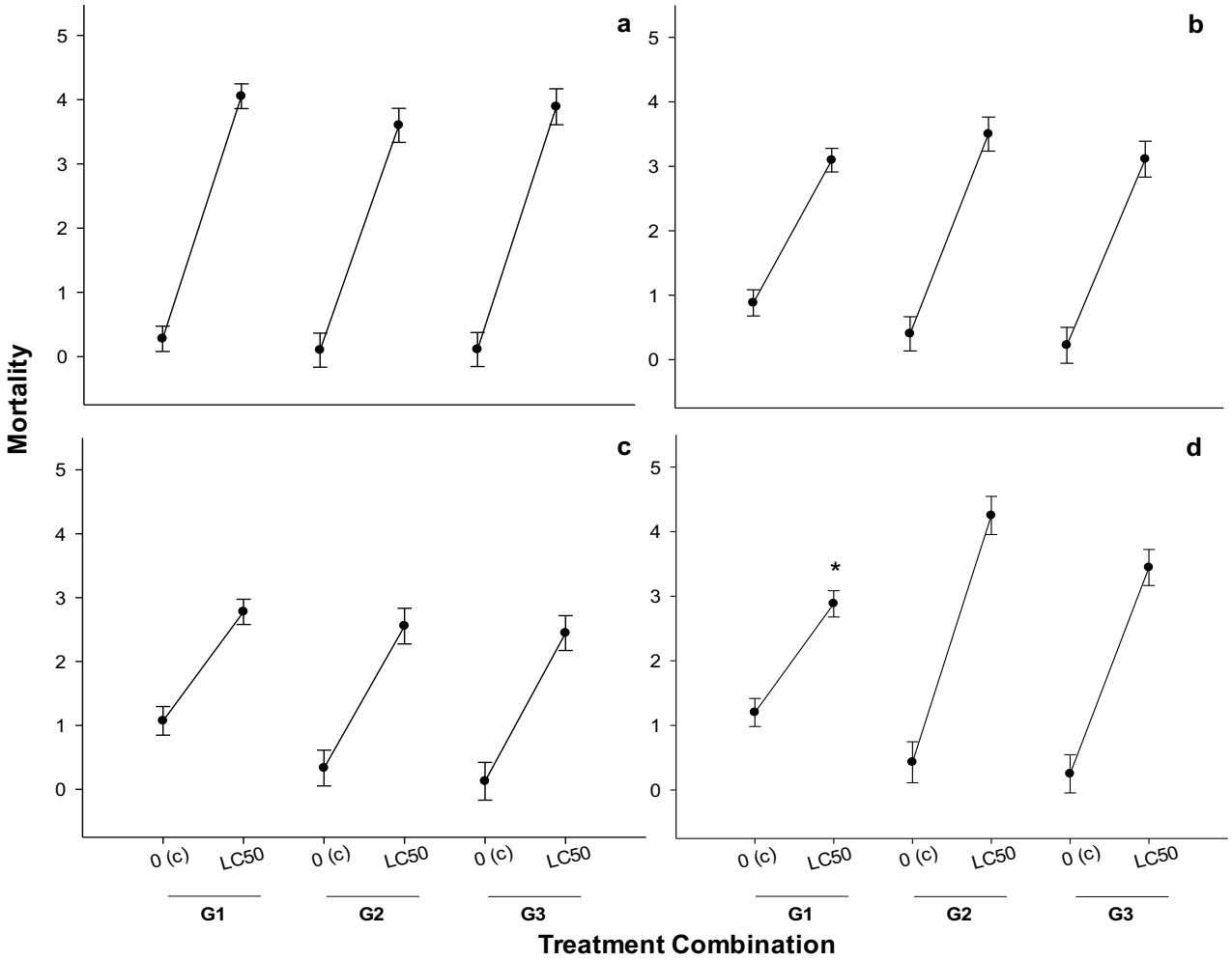

Figure 2.2. Mortality (out of 7) in *M. persicae* exposed to 0 (control) (a), 0.1 (b), 5 (c) and 10 (d) µg L^{-1} of imidacloprid over three generations (G1, G2, G3), after subsequent exposure to 0 (control) and LC_{50} concentrations of spirotetramat. Stars represent significant effects on mortality shown in Table 2.6.

2.3.3 Dose-Response Experiments

To more clearly elucidate potential effects of several generations of exposure to hormetic or sublethal concentrations of imidacloprid on the ability of *M. persicae* to tolerate subsequent imidacloprid exposures, dose-response bioassays were produced for fourth generation aphids after rearing of previous generations on 0, 0.1, 5 and 10 μg L^{-1} of imidacloprid. Results from the probit analysis are outlined in Table 2.7. The slope of the probit line for the 0.1 μg L^{-1} treatment was significantly lower than the slope of the control probit line, indicating that the aphid response to the treatment was more heterogeneous than that of the control (Table 2.7, Figure 2.3). Slopes for the 5 μg L^{-1} and the 10 μg L^{-1} lines were not significantly different from controls (Table 2.7, Figure 2.3). There was no significant difference between the control line intercept and the 0.1 μg L^{-1} line intercept, however the intercepts for the 5 μg L^{-1} and 10 μg L^{-1} lines were significantly higher than that of the control line. Ratio tests indicated that LC_{50} values for all treatment probit lines were not significantly different from controls (Table 2.7, Figure 2.3). While the slope of the 0.1 μg L^{-1} line was significantly lower than the control, observations of individual data points on probit lines showed that mortality began to dip below control values around/after the LC_{50} point (Figure 2.3).

Table 2.7. Dose response assays conducted on *Myzus persicae* adults exposed to 0, 0.1, 5 and 10 µg L^{-1} of imidacloprid for four generations, and statistics from ANCOVA comparing slopes and intercepts, and ratio tests comparing LC_{50} values, from the control probit line with treatment probit lines.

Probit Line (µg L^{-1})	***n***	**Slope (± SE)**	**Intercept (± SE)**	**LC_{50} mg L^{-1} (95% FL)**	**Slope Comparison**	**Intercept Comparison**	**Ratio Test *Z, P***
Control	36	3.64 (± 0.52) χ^2 = 49.64	-9.47 (±1.44) χ^2= 43.23	0.40 (0.32-0.47)	----	----	----
0.1	34	2.95 (± 0.53) χ^2= 31.43	-7.63 (±1.54) χ^2= 24.60	0.38 (0.24-0.51)	F_1 = 29.06, *P* <0.01	F_1 = 0.78, *P* = 0.38	*Z*= 0.02, *P*= 0.98
5	35	3.42 (± 0.48) χ^2= 50.78	-9.33 (± 1.39) χ^2= 43.37	0.53 (0.43-0.62)	F_1 = 2.52, *P* = 0.12	F_1 = 67.21, *P* <0.01	*Z*= 0.00, *P*= 0.99
10	36	3.43 (± 0.58) χ^2= 34.44	-9.07 (± 1.68) χ^2= 29.01	0.44 (0.31-0.54)	F_1 = 2.41, *P* = 0.13	F_1 = 8.60, *P* <0.01	*Z*= 0.00, *P*= 0.99

Slopes, intercepts, and LC_{50} values from treatment lines were compared individually with the control line only.

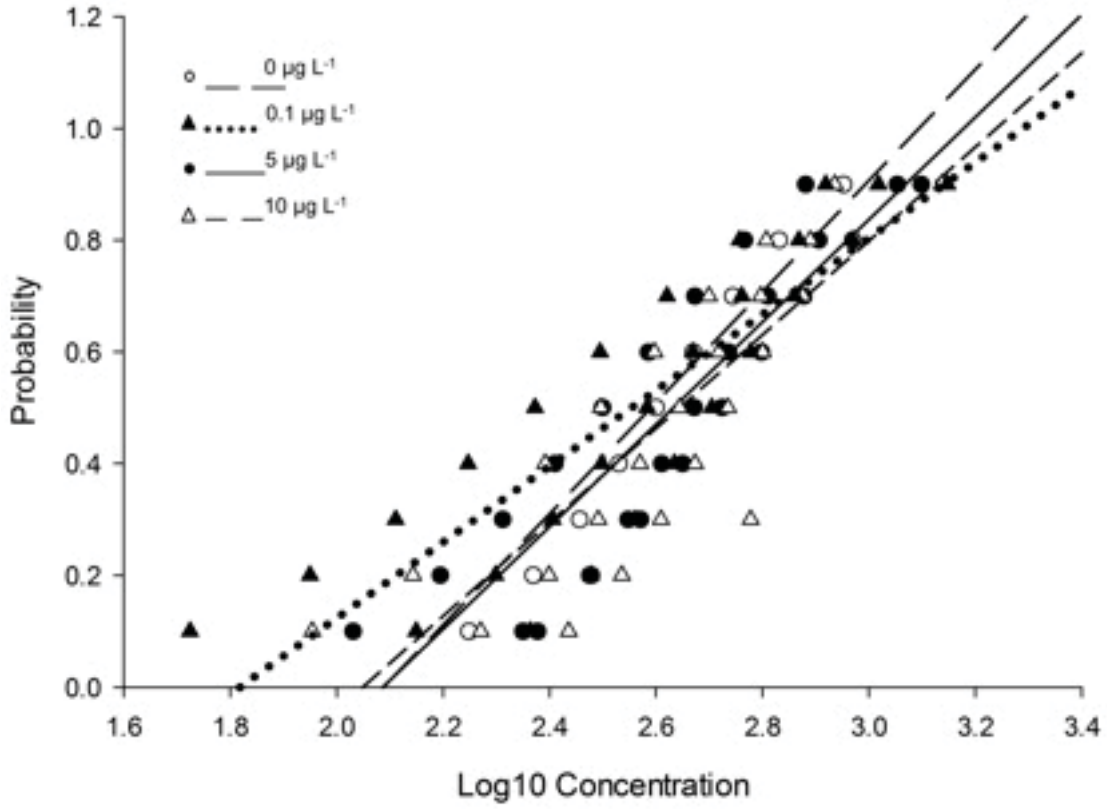

Figure 2.3. Imidacloprid probit lines from dose response assays conducted on fourth generation aphids reared on 0 (control), 0.1, 5 and 10 µg L^{-1} of imidacloprid. In dose response assays, fourth generation, first instar aphids were exposed to 0, 0.3, 0.6, 0.9, 1.2, or 1.5 mg L^{-1} of imidacloprid.

2.4 Discussion

The environmental conditions and stressors to which organisms are exposed help to fine-tune the adult phenotype. Parental environments, as well, can be good indicators of the environments in which offspring will live, thus parental acclimations to stress may be passed on to progeny (Mousseau and Dingle 1991; Costantini et al. 2010; Costantini et al. 2012; Ismaeil et al. 2013). Exposure of parental generations to stressors, such as chemical stress, might therefore influence responses to stress in offspring.

It was previously demonstrated that hormetic exposure to imidacloprid over several generations primed *M. persicae* to better withstand a subsequent food and water stress, but not a subsequent chemical stress, in the form of spirotetramat (Rix et al. 2016). Given these past findings, I wanted to determine if imidacloprid exposure over several generations at a hormetic concentration, or at levels above the hormetic zone, could prime aphids to better survive increased exposure to the same insecticide, or relatively greater exposure to a different insecticide, spirotetramat. I also wanted to determine if response of aphids to chemical stress changed with each successive generation.

The concentration of imidacloprid that induced increased reproduction in aphids on treated leaf discs was similar to that previously described by Ayyanath et al. (2013), demonstrating good reproducibility of the hormetic response in this test system. Multi-generational exposure of aphids to concentrations of imidacloprid exceeding the hormetic concentration did not result in changes in mortality in aphids following exposure to imidacloprid or spirotetramat, indicating no preconditioning occurred. However, mortality was significantly reduced after three generations of exposure to the hormetic concentration of 0.1 $\mu g\ L^{-1}$, when subsequently exposed to higher concentrations of

imidacloprid. Further analysis, in the form of probit, in fourth generation aphids demonstrated that although the intercept and LC_{50} value for hormetically exposed aphids was not significantly different from the control, the slope was significantly lower. Mortality in 0.1 μg L^{-1} exposed aphids appeared to become reduced over control mortality above the LC_{50} level. This suggests that some aphids exposed to the hormetic concentrations of the insecticide were less susceptible to the insecticide, thereby reducing the slope. Additionally, as in a previous study (Rix et al. 2016), prior exposure to hormetic concentrations of imidacloprid did not reduce *M. persicae* susceptibility to spirotetramat.

The effects of stress on an organism and its progeny appear to vary depending on the organism, endpoint studied, and the level of stress (Costantini 2014). This is evident given that I only observed reductions in mortality under hormetic conditions, and only when aphids were exposed to subsequent imidacloprid stress. The effect of stress on an organism can be impacted by the nature of the stress, and/or a subsequent stress to which the organism is exposed. Another study examined the trans-generational effects of multiple stressors on *Trichoplusia ni*. (Hubner), the cabbage looper. Larvae were subjected to a nutritional stress for five instars, and then further exposed to sublethal concentration of *Bacillus thuringiensis* Berliner (additional stressor). Offspring were subsequently exposed to one of six increasing concentrations of *B. thuringiensis*, or subjected to one of five increasing doses of a naturally occurring DNA virus (Shikano et al. 2015). Offspring resistance to *B. thuringiensis* was increased over controls when parents had been previously exposed to the pathogen stress. Nutritional stress resulted in increased resistance to the DNA virus, but prior pathogen stress (*B. thuringiensis*

exposure) had no impact of resistance to the DNA virus (Shikano et al. 2015). In the nematode, *Caenorhabditis elegans* (Maupas), short exposures to a heat challenge, an oxygen challenge, and a chemical challenge (juglone), resulted in subsequent resistance to the same challenge. Cross-tolerance between oxygen and juglone was also observed. Exposures to ultraviolet light and ionizing radiation, however did not result in subsequent resistance or cross-tolerance to stress (Cypser and Johnson 2002). Thus not all prior stressors will buffer the effects of subsequent stress in an organism or its offspring. I suspect this is likely the reason why I saw no adaptation to spirotetramat stress, following imidacloprid preconditioning. Even conditioning with the same stress experienced in subsequent exposure scenarios does not always guarantee adaptation to that stressor. Exposure of *Trialeurodes vaporariorum* Westwood to nitenpyram for seven generations, as discussed above, resulted in decreased resistance to the same stressor (Liang et al. 2012).

Adaptation to stress can also be associated with the amount of stress an organism encounters. An analysis of preconditioning by Calabrese (2016b) revealed that the optimal preconditioning doses, which resulted in the highest levels of protection against subsequent stress, were the doses that also induced hormesis. When stresses are small, it is possible the organism has the metabolic ability to adapt more quickly, particularly given that hormesis is known to induce increases in metabolic activities (Calabrese 2016b). Adaptation to levels of stress above the hormetic zone may require more generations of conditioning, or perhaps significant trade-offs (Costantini et al. 2010; Costantini et al. 2012). It is possible that this is why I saw evidence of reduced susceptibility to imidacloprid, only after three and four generations at the hormetic level

of exposure (0.1 μg L^{-1}), and no reduced susceptibility with the highest levels of exposure (5 and 10 μg L^{-1}). Susceptibility in aphids exposed to 5 μg L^{-1} did not significantly change over three generation and susceptibility in aphids exposed to 10 μg L^{-1} was reduced in second generation, but then increased again in the third generation. This, along with the fact the intercepts of 5 and 10 μg L^{-1} fourth generation probit lines were significantly higher than control intercepts and slopes were not significantly different from controls, could indicate that these concentrations were too stressful to result in adaptation, at least after several generations of exposure. Several studies have shown that low stress resulted in timely adaptation to subsequent stress. For example, *Taeniopygia guttata* Reichenbach *(*zebra finches) were exposed to control, low, or high heat stresses early in life, and as adults were all exposed to high heat stress (Costantini et al. 2012). Birds exposed to high heat stress in early life experienced the highest levels of oxidative damage, whereas birds that experienced mild heat stress in early life had no increased oxidative damage after high heat stress later in life, suggesting that too much stress in early life resulted in further oxidative damage into adulthood (Costantini et al. 2012). Adaptation to higher levels of stress can occur when the organism is exposed for many generations, such as with pesticide selection, but no or negative effects can occur when higher levels of exposure are experienced for a shorter period of time.

Different organisms may react differently to similar stresses. For instance, when two species of wheat aphid, *Rhopalosiphum padi* (L.) and *Sitobion avenae* Fabricius were exposed to LC_{25} concentrations of primicarb, decreases in fecundity were observed in parental generations of *R. padi*, and offspring experienced increased development, reproductive period and longevity, but no effects of primicarb were observed in parental

generations or offspring of *S. avenae* (Xiao et al. 2015). It is thus possible that exposure to imidacloprid could prime another insect species to withstand subsequent spirotetramat exposure. The most reasonable explanation, however, for why I again, under different circumstances, did not observe preconditioning after subsequent exposure to spirotetramat lies with my previous findings. I observed hormetic concentrations of imidacloprid increased expression of heat shock proteins (*Hsp60*) in exposed aphids (Rix et al. 2016). Heat shock proteins are known to help insects subjected to dehydration stress (Zhao and Jones 2012), which was likely why I previously saw that green peach aphid exposed to hormetic concentration of imidacloprid were able to withstand subsequent food/water stress, but not subsequent spirotetramat stress, as heat shock proteins simply did not protect the aphids from a subsequent chemical exposure (Rix et al. 2016). In my current work, some aphids hormetically exposed to imidacloprid may be becoming resistant to the insecticide, thus I observed evidence of preconditioning, however, as in my previous findings, the metabolic and molecular changes induced by hormetic concentrations of imidacloprid, may not help the aphids to withstand exposure to a different chemical stressor in the form of spirotetramat. Thus the lack of evidence of evidence of preconditioning after exposure to a subsequent and different stress may simply be a result of the type of additional stress to which the green peach aphids were exposed.

It is important to acknowledge that within a population, individuals will vary in their genetic makeup, and thus not all individuals will respond equally to stressors (Costantini 2014). Belz and Sinkkonen (2016) observed that hormetic effects varied amongst fast and slow growing individuals in experiments with lettuce (*Lactuca sativa*

L.) exposed to PCIB. They observed that the slowest growing individuals were stimulated at the very lowest levels of stress, whereas the fastest growing individuals were stimulated at the threshold of toxic effects. This would indicate that individuals at the tails of normal distributions can have a large impact on measurable hormetic effects. The slopes of fourth generation imidacloprid probit lines for aphids exposed to hormetic (0.1 μg L^{-1}) concentrations of imidacloprid, were significantly lower than the slopes of control lines. This appeared to be the result of some aphids which were less susceptible to the imidacloprid after exposure to hormetic concentrations of imidacloprid. The 0.1 μg L^{-1} probit line intercept was not significantly different from the control intercept and fiducial limits (FL) of points at the lower portions of this line closely aligned with those of the control. For example: control LC_{20} = 0.23 mg L^{-1} (0.16 mg L^{-1}- 0.30 mg L^{-1}); 0.1 μg L^{-1} LC_{20}= 0.2 mg L^{-1} (0.089 mg L^{-1}- 0.3 mg L^{-1}). Above the LC_{50}, mortality in the 0.1 mg L^{-1} probit line began to dip below that of the control, in other words higher concentrations of imidacloprid were required to kill the hormetically exposed aphids compared with the control aphids. In looking at the upper portion of the hormetic and control probit lines, the line points and corresponding FL in the hormetic line deviated more from the control line than they did in the lower portions of the line. For example: the control LC_{90} = 0.90 mg L^{-1} (0.76 mg L^{-1}- 1.13 mg L^{-1}); 0.1 μg L^{-1} LC_{90}= 1.04 mg L^{-1} (0.87 mg L^{-1}- 1.40 mg L^{-1}). Thus, it would appear the effect of preconditioning observed in the third and fourth generations of hormetically exposed aphids, was as a result of some aphids at the upper tail portion of the distribution, not the entire population of exposed aphids becoming less susceptible to imidacloprid.

2.5 Conclusion

Prior exposure to stress can reduce the effects of subsequent exposure to stress. It has also been suggested that low doses of stress may hasten the evolution of resistance; stressors strengthening populations with each successive generation (Gressel 2011). I had hypothesized that exposure to sublethal concentrations of imidacloprid over several generations would result in increased ability to withstand subsequent stress with each generation. My results indicate that exposure to sublethal imidacloprid stress that also induces hormetic effects on reproduction in *M. persicae*, can result in reduced susceptibility to subsequent imidacloprid stress after several generations of exposure, but this may only be true for some individuals in the population. Higher levels of exposure do not result in preconditioning, nor was their evidence of preconditioning after subsequent exposure to spirotetramat stress; under my conditions. These findings demonstrated that preconditioning was transgenerational, however not with each subsequent generation. It took two generations of priming for preconditioning to be observed. I also saw that chemical preconditioning does not always result in adaption to the same or different chemical stress, as is also evident in the literature. My findings additionally substantiated previous work which showed that hormetic imidacloprid exposure several generations did not prime aphids to withstand a subsequent spirotetramat stress (Rix et al. 2016). This finding was not a result of preconditioning concentrations being too low, but rather likely as a result that imidacloprid stress simply does not prime aphids to withstand spirotetramat stress.

While it has been observed in the literature that hormesis plays a role in preconditioning (Calabrese 2016b), it is clear the extent to which low dose stress

exposure primes an organism to withstand subsequent stress is dependent upon the prior stress, the subsequent stress, and the organisms being affected. Variation within populations may additionally impact whether adaption is observed at the population level (Belz and Sinkkonen 2016). Because the effects of preconditioning appear to be highly variable, and because preconditioning does not appear to be entirely transgenerational, this could indicate that exposure to low dose stress may not have a profound effect on adaptation to stress and resistance development

CHAPTER 3 CAN MULTIGENERATIONAL EXPOSURE TO LOW DOSES OF IMIDACLOPRID CAUSE MUTATIONS IN NICOTINIC ACETYLCHOLINE RECEPTORS IN *MYZUS PERSICAE* (HEMIPTERA: APHIDIDAE)?

3.1 Introduction

Hormesis, which manifests as stimulatory effects after low dose exposure to biological, physical and chemical stress, is considered to be evolutionarily conserved, adaptive response to stress (Calabrese 2008a; Calabrese 2008b; Costantini 2014). Stress, however, plays an important role in the development of mutations. Under stressed conditions, an organism can accumulate mutations, when detoxification enzymes compete with DNA repair enzymes for ATP and other nucleotide triphosphates. Although mutations can be deleterious to individuals, there can an evolutionary advantage to mutation development. Greater variability via mutations can be beneficial for populations that have to adapt to changing environmental conditions (Gressel and Levy 2010; Gressel 2011).

Insecticide-induced hormesis may pose challenges to pest management. Resulting stimulatory effects such as increased fecundity, fertility, and lifespan from low-dose insecticide exposure can allow pests to continue to thrive, even under pesticide pressures (Guedes and Cutler 2014). If low or hormetic doses of insecticides were stressful to insects such that they also induced mutations in pesticide target sites, which could lead to pesticide resistance, this could be a bigger concern for pest management. However, the extent to which low doses of stress lead to mutations is not clear. Traditional modeling of the evolution of pesticide resistance shows that high dose exposures to pesticides can lead to selection of individuals that express phenotypes for resistance. Low dose exposures can select for more tolerant individuals, which, over time, become increasingly resistant to pesticides (Georghiou and Taylor 1977; Gressel and Segel 1978; Gardner et al. 1998;

Gressel 2011). However, traditional models may not consider that in stressed conditions rates of mutations increase (Gressel and Levy 2010; Gressel 2011). It thus could be possible that under low or hormetic stresses, mutations could accumulate, perhaps even in target sites. There is some evidence to suggest that mutations in target sites can occur at lower doses (Wieczorek et al. 2015), some even being involved in resistance development (Tardif et al. 1993; Tardif and Powles 1994).

Neonicotinoids are agonists of nicotinic acetylcholine receptors (nAChR) in insects (Matsuda et al. 2001). Mutations in nAChRs have been linked to neonicotinoid resistance in several Hemiptera. A mutation (Y151) in both the α1 and α3 subunits of the nAChR conferred resistance to the neonicotinoid insecticide, imidacloprid, in Nilaparvata *lugens*, the brown plant hopper (Liu et al. 2005). Two mutations (L80S, R81T) in the β1 subunit of the nAChR in *Aphis gossypii*, the pea aphid, have been associated with neonicotinoid resistance (Bass et al. 2015; Kim et al. 2015). The R81T mutation in the β1 subunit was also found in neonicotinoid resistant *M. persicae* (Bass et al. 2011).

In this chapter, I examined whether exposure to hormetic and low doses of imidacloprid, over several generations, resulted in mutations in the nAChR subunits α1, α2, α3, α4, or β1, in *M. persicae*, referred to as Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1. These subunits form a significant portion of the binding sites of imidacloprid (Matsuda et al. 2001; Matsuda et al. 2009; Puinean et al. 2010b; Bass et al. 2011). I hypothesized that exposure of aphids to hormetic and sublethal doses of imidacloprid would result in mutations over several generations, not observed in controls.

3.2 Materials and Methods

3.2.1 Insect Rearing

Insects were reared as described in Chapter 2.2.1.

3.2.2 Insecticide Treatments

Imidacloprid (Admire® 240 SC, 240 g active ingredient (AI) L^{-1}; Bayer Crop Science Canada, AB, Canada) was suspended in deionized water to make 1000 mg AI L^{-1} stock solutions that were then diluted to make working solutions of 0 μg L^{-1} (control), 0.1 μg L^{-1}, 5 μg L^{-1}, and 10 μg L^{-1}. The 0.1 μg imidacloprid L^{-1} treatment was confirmed in previous experiments (Chapter 2) to cause reproductive hormesis, and I predicted that prolonged, multi-generational exposure to this treatment would result in mutations in nAChR units responsible for imidacloprid resistance. The two higher concentrations were selected as I wanted to test the hormetic concentration against higher levels of stress, outside the hormetic zone. Through preliminary experimentation, I determined my aphids could be exposed to these concentrations chronically for up to four generations with adequate reproduction for experimentation. They were also outside of the hormetic zone (outside the no observable adverse effects concentration), as reproduction was observed to have been compromised, indicating mildly toxic effects.

3.2.3 Sublethal Insecticide Exposure

Leaf discs were dipped in 0, 0.1, 5, or 10 μg L^{-1} of imidacloprid for 5 seconds and air-dried. Three discs of a given treatment were placed in petri dishes lined with filter paper. Five first-instar aphids were placed on the leaf discs. Lids were then placed over plates and plates were sealed with Parafilm, and placed in Tupperware bins (37 x 24 x 14 cm). Aphids were reared until adulthood for a single generation, and for four generations, G4.

Aphids were reared in separate bins, according to generation (G0, and G4). There were eight replicate petri plates of each concentration in each bin. All first instar aphids placed on leaf discs came from the same cohort of adult aphids. Leaf discs were replaced three times per week. Bins were kept in a growth chamber at 22 ± 2^{o} C, 16:8 L:D, and 65 ± 5 % RH. Thirty adult aphids from each concentration, for each generation (G0 and G4), were randomly collected, and immediately frozen at -80°C, until further molecular analysis. Aphids in each generation were pooled within concentrations to ensure high quality samples.

3.2.4 Sequence Analysis of nAChR Subunits

Total RNA was extracted from samples using an RNeasy® mini kit (Qiagen, ON, Canada), followed by cDNA synthesis from 1 µg of RNA using a QuantiTect® Reverse Transcription kit (Qiagen). PCR amplification was performed with a Taq PCR Master Mix kit (Qiagen), using a nested design, with gene specific primers for Mpα1- Mpα4, and Mpβ1. Primer details are given in Table 3.1. PCR reactions contained 10 µL of Taq PCR Master Mix (consisting of Taq DNA Polymerase, Qiagen PCR Buffer, $MgCl_2$, and dNTP's), 6.5 µL of water, 0.25 µL each of forward and reverse primer, and 3 µL of cDNA template (sample). Cycling was performed for 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, with a final extension step of 5 min at 72°C. Amplicons were verified by gel electrophoresis (1% agarose) (Appendix 1). Samples were then purified using the QIAquick® PCR Purification kit (Qiagen, ON, Canada), and sequenced in both directions using the BigDye mix (Applied Biosystems, Thermo Fisher Scientific Inc., ON, Canada). Sequences were analyzed in Sequencher

(Gene Codes Co., MI, USA), and aligned to published nAChR subunit sequences using MUSCLE (Edgar 2004b; Edgar 2004a).

Table 3.1. Primer sequences (5' to 3') for used to sequence *Myzus persicae* nAChR subunits. A nested primer design was used to amplify all genes thus two sets are listed for each gene. Genes were amplified using the first primer (1), followed by a second amplification with the second primer (2).

Gene (primer)	Forward Sequence	Reverse Sequence	Reference/ Accession Number
Mpα1 (1)	GGTCAGCAACAACACCGACACC	TCGGCAGGCAGATAGAAGACCA	(Puinean et al. 2010b) X81887
Mpα1 (2)	CGACACCGTCCTGGTCAAGC	GACCAGCACCGACAGGTAAG	(Puinean et al. 2010b) X81887
Mpα2 (1)	GCCAAACGGCTTTACGACGATC	GAACACCAGCACGGACAGGAAC	(Puinean et al. 2010b) X81888
Mpα2 (2)	CGACGATCTGCTGAGCAACTAC	GACAGGAACGATATACCCACGC	(Puinean et al. 2010b) X81888
Mpα3 (1)	CGGATGCAAAAAGGCTGTACGA	GGTAAAACACAAGTATCGTGAG	(Puinean et al. 2010b) AJ236786
Mpα3 (2)	GGCTGTACGACGACCTTTTATC	GAAACGATATCCCCATACAAGG	(Puinean et al. 2010b) AJ236786
Mpα4 (1)	GATGACCTTCTGAGCAACTAC	GTCAGTCCGACACAAGGGATG	(Puinean et al. 2010b) AJ236787
Mpα4 (1)	CAACTACAATCGGCTGATCAG	GTCCGACACAAGGGATGATC	(Puinean et al. 2010b) AJ236787
Mpβ1 (2)	GTCCAGAACATGACCGAAAAAG	CGCACAGGAAAGATATAAGGAC	(Puinean et al. 2010b) AJ251838
Mpβ1 (2)	GACCGAAAAAGTCAATGTCCAG	AAGATATAAGGACCGTGGGCAG	(Puinean et al. 2010b) AJ251838

3.3 Results

Sequence alignments (Appendix 3) showed no mutations in the regions of nAChR subunits Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1 in my population of *M. persicae* exposed to 0, 0.1, 5, or 10 μg L^{-1} of imidacloprid for up to four generations (Table 3.2). I observed what appeared to be several genotypes in Mpα4 in all concentrations of exposed aphids in both G1 and G4. Overlapping peaks were observed on chromatograms at base pair 654, which corresponded to amino acid 190. At this location two genotypes of glutamine (CAG and CAA) were observed (Appendix 2). So this would not be confused with a mutation, all sequences were aligned with the CAG genotype (Appendix 3).

Table 3.2. Number of mutations observed in sequenced regions of the nAChR (Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1) in *Myzus persicae* exposed to hormetic and sublethal concentrations of imidacloprid. G0 represents aphids exposed to imidacloprid as first instar nymphs, continuously until adulthood. G4 represents aphids exposed continuously until fourth generation offspring reached adulthood (see Appendix 3).

Gene	Concentration ($\mu g\ L^{-1}$)	G0 Number of Base Pairs	G0 Number of Mutations	G4 Number of Base Pairs	G4 Number of Mutations
Mpα1	0.0	534	0	598	0
	0.1	439	0	546	0
	5.0	466	0	496	0
	10.0	496	0	519	0
Mpα2	0.0	564	0	555	0
	0.1	577	0	510	0
	5.0	582	0	529	0
	10.0	549	0	538	0
Mpα3	0.0	484	0	554	0
	0.1	557	0	540	0
	5.0	499	0	548	0
	10.0	521	0	550	0
Mpα4	0.0	541	0	555	0
	0.1	565	0	548	0
	5.0	557	0	549	0
	10.0	553	0	548	0
Mpβ1	0.0	515	0	586	0
	0.1	560	0	511	0
	5.0	502	0	460	0
	10.0	481	0	567	0

3.4 Discussion

Hormesis is an evolutionary adaptation to stress (Calabrese 2008b), and stress plays an important role in the development of mutations (Galhardo et al. 2007; Gressel 2011). Thus, low levels of stress theoretically could induce mutations or other forms of mutant DNA. However, in my experiments, aphids that were exposed continuously to hormetic and mildly toxic concentrations of imidacloprid for up to four generations had no mutations in nAChR subunits.

There are examples of low levels stress inducing mutations. Studies using insects have shown that low doses of radiation stress resulted in reductions in the frequencies of mutations. This was observed in germ cells of *Drosophila melanogaster* Meighen, the fruit fly, and may have been a result of stress-induced upregulation of DNA repair (Koana et al. 2007; Ogura et al. 2009). DNA damage was observed in *Eisenia fetida* (Savigny), earthworm, after exposure to very low concentrations of imidacloprid (Wang et al. 2016).

Gressel (2011) suggested that mutations could be induced after exposure to low levels of stress as a result of detoxification enzymes and DNA repair enzymes competing for limited energy resources. However, it is possible that low levels of stress upregulate protective metabolic processes, but not such that these processes compete for energy resources, thus protecting organisms from accumulating mutations. Thus, mutations may not be induced by low levels of stress. Greater amounts of stress may be needed to trigger such mutation development, which may be why I did not observe mutations even after continuously exposing aphids for four generations to low dose imidacloprid stress. Several studies conducted with chemical pesticides have demonstrated that chemical

stressors may not induce mutations, even when those concentrations are at toxic levels. Exposure to mild to moderately toxic concentrations (those below LC_{50}) of the insecticides spinosad and deltamethrin did not induce somatic cell mutations in *D. melanogaster* (Akmoutsou et al. 2011). A similar study examined somatic cell mutation frequency after acute and chronic exposure of *D. melanogaster* to imidacloprid concentrations below the $LC_{50,}$ and to very high, but non-toxic concentrations of the herbicide, acetochlor. Neither of these exposures induced mutations (Frantzios et al. 2008). Additionally, certain mutations might only occur after long term exposure and/or selection with the pesticide. Li et al. (2012) characterized permethrin concentration thresholds at which mutations in sodium channels of the mosquito, *Culex quinquefasciatus* Say, occurred. They determined that the prevalence of various combinations of mutations (both synonymous and non-synonymous) in the sodium channels corresponded to the level of permethrin selection that the mosquitos underwent (Li and Liu 2010; Li et al. 2012). Although my research did not examine selection for mutations, the above referenced studies demonstrate that some mutations may only occur after certain levels of pesticide selection.

It is possible mutations in nAChRs only develop after long term exposure to or selection with neonicotinoids. *Nilaparvata lugens* (Stal) continuously selected in laboratory on rice plant roots treated with 0.05-3.00 mg L^{-1} of imidacloprid (concentrations were increased over time), only began to show evidence of resistance development after 8 generations of selection, and LD_{50} levels then continued to increase after 22 generations of selection. Subsequent molecular tests after additional selection identified that laboratory-selected resistance was as a result of a point mutation (Y151S)

in both the α1 and α3 subunits of the nAChR (Liu et al. 2003; Liu et al. 2005). Thus a large level of selection pressure over many generations was needed to observe the nAChR mutation in *N. lugens*, which may also be the case for *M. persicae*. A limited number of *M. persicae* populations have been found to possess mutations. A *M. persicae* population (clone 5191A) from Greece possessed a small number of synonymous mutations in α subunits. This population is moderately resistant to neonicotinoids through amplification of cytochrome P450 *CYP6CY3*. High resistance in *M. persicae* (clone FRC from Southern France) is associated with a single point mutation in the β1 nAChR subunit (R81T). Clones with this mutation also possessed a number of synonymous mutations in α subunits (Puinean et al. 2010b; Bass et al. 2011; Bass et al. 2014). A number of resistant *M. persicae* populations in Italy have also been shown to possess the R81T mutation in the nAChR β1 subunit (Panini et al. 2014). Thus it is possible that high levels of neonicotinoid selection pressure are required to result in mutations in nAChRs.

While there is evidence to suggest that use of lower doses of pesticide can lead to populations with target site mutations (Tardif et al. 1993; Tardif and Powles 1994; Wieczorek et al. 2015) (discussed in Chapter 1), this may be a phenomenon that is dependent upon the organism or the gene. Wieczorek et al. (2015) observed mutations in *CYP51* in *Zymoseptoria tritici* (Fuckel) exposed to half field-rates of DMI fungicides. Mutations in this gene have been widely reported in multiple species, and multiple mutations in *CYP51* were observed after a single season of exposure in all populations of *Z. tritici* sampled across several countries (Cools and Fraaije 2013; Wieczorek et al. 2015). Thus, this gene may develop mutations under smaller amounts of selection pressure. Similarly, the reported instances of target site mutations occurring after lower

exposures, conferring, or increasing pesticide resistance are both in populations of *Lolium rigidum* Gaud., in the acetyl coenzyme A carboxylase (ACCase) (after exposure to sethoxydim) (Tardif et al. 1993; Tardif and Powles 1994). *Lolium rigidum* is capable of exhibiting resistance across 12 different herbicide classes by means of 8 different target site mutations (Tardif and Powles 1994). This could suggest that weed species like *L. rigidum* have more of a propensity to develop target site mutations or mutations in general. *Lolium rigidum* is well known for its adaptability (Gressel and Levy 2010). Plants, unlike animals, cannot move to avoid environmental stress, and thus must possess other methods to cope with stress, which may include genetic mechanisms involved in the development of mutations (Gressel and Levy 2010).

While *M. persicae* is well known for its ability to develop resistance to pesticides by means of target site mutations, amongst other methods (Bass et al. 2014), mutations in nAChRs may be an exception. Neonicotinoids, despite their widespread use, have not been as vulnerable to resistance as other insecticide classes such as pyrethroids, organophosphorus and carbamate insecticides (Denholm et al. 2002; Nauen and Denholm 2005; Puinean et al. 2010a), where numerous mutations in their target sites (voltage gated sodium channels, and acetylcholinesterase, respectively) are well documented in many arthropod species (Park et al. 1997; Head et al. 1998; Martinez-Torres et al. 1999; Liu et al. 2000; Baek et al. 2005; Toda and Morishita 2009; Chang et al. 2014; Lee et al. 2015).

Mutation development can occur differentially across populations, and not all populations will respond the same way to selection pressures. Populations of the mosquito, *Aedes aegypti* (L.), responded differently to laboratory permethrin selection. Six populations from southern Mexico all possessing the Ile1,016 allele, having

resistance with a mutation in the voltage gated sodium channel (in frequencies >0.5), were selected in laboratory with LC_{50} concentrations of permethrin. The frequency of the Ile1,016 mutation increased in two of six populations, but remained fixed in the others (Saavedra-Rodriguez et al. 2012). Three populations of the melon aphid, *Aphis gossypii* Glover, were selected for 30 generations at LC_{80} level concentrations of dimethoate. Sequencing of acetylcholinesterase gene *Ace2* revealed that while all three populations possessed the A302S and S431F mutations, only two populations possessed a third mutation (G221A) (Lokeshwari et al. 2016). These populations were under the same high selection pressure in laboratory for many generations, but prior exposures in the field (Lokeshwari et al. 2016) may have additionally impacted the differential effects of laboratory selection. It is possible that if I conducted my research on different populations of *M. persicae*, they could respond differently to the insecticide exposure. Additionally, prior stress exposures in a different population of aphids could yield different effects.

3.4 Conclusion

One of the greatest challenges in insect pest management is successfully managing the pest species while mitigating the development of resistance, particularly where chemical pesticides are heavily relied upon (Hoy 1998). If lower doses of pesticides are also capable of increasing mutations, particularly in target sites and potentially increasing the development of resistance, this could further complicate the management of pest species. I thus examined if low dose imidacloprid exposure, that also resulted in hormetic effects, and low dose exposure just outside the hormetic zone, could result in mutation development in subunits of the nAChR, the target site of imidacloprid. I did not observe any mutations in the regions of the Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1 of the nAChR

that I sequenced in my population of *M. persicae* exposed to imidacloprid for up to four generations. Very low doses of some stressors may not be stressful enough to induce mutations in some regions on the genome, and thus higher levels of exposure or selection are required for some mutations to develop. I suspect mutation development is highly dependent upon the level of stress, the organism, and the gene. While very low doses of stress did not result in mutations in *M. persicae* nAChRs, other genes and other organisms could be more susceptible to mutation development in other regions on the genome. While hormesis and low dose effects of stress pose a challenge to pest management, there may be limitations to the extent with which hormetic and low dose exposure lead to resistance and/or mutation development. This information is important for pest management specialists who are concerned about the sublethal effects of pesticides on pest species in their fields.

CHAPTER 4 GENERAL DISCUSSION

Hormesis is a biphasic dose response phenomenon whereby exposure to low or sublethal doses of chemical, physical, or biological stress can stimulate biological processes in an organism (Calabrese and Baldwin 2002; Calabrese 2008b). Hormesis has been demonstrated in many organisms, in response to numerous stressors (Calabrese and Blain 2005; Calabrese 2013a). Hormesis has also come to the attention of entomologists, particularly those involved in areas of pest management. Stimulatory effects on reproduction, oviposition, and longevity have been well demonstrated in insects exposed to low dose stressors such as chemical pesticides (Cutler 2013). These stimulatory effects are of concern in pest management as they may allow pest insects to persist and proliferate when exposed to the very chemicals used to eliminate them, as these chemicals break down to lower doses in the environment. Additionally, under the umbrella of the hormetic dose response lies another phenomenon known as hormetic preconditioning, whereby acute or long-term exposure to sublethal stress enables an organism to withstand subsequent and higher amounts of the same, similar, or different stressor (Calabrese et al. 2007; Calabrese 2008a; Calabrese 2016b). This could pose additionally problematic in insect pest management as insects may not only be stimulated by hormetic levels of stress, but may also adapt to stress.

I previously demonstrated that exposure of *Myzus persicae* to sublethal doses of imidacloprid that stimulated increases in reproduction, also enabled the insect to withstand a subsequent food/water stress, but not a subsequent insecticidal stress, spirotetramat (Rix et al. 2016). The first objective of my thesis work (Chapter 2) was to further explore the intricacies of chemical preconditioning hormesis. I examined whether

chemical preconditioning could be transgenerational, that is, whether exposure to low doses of imidacloprid over several generations enabled *M. persicae* to better withstand subsequent and higher exposure to the same chemical stress (imidacloprid), or a different chemical stress (spirotetramat, at a level higher than previously studied) with each successive generation. This also enabled me to determine if my previous inability to detect chemical preconditioning after exposure to spirotetramat was a result of the level of prior or subsequent stress or simply a result of spirotetramat having a different mode of action than imidacloprid.

Exposure of *M. persicae* to two mildly toxic doses of imidacloprid outside the hormetic zone (5 and 10 μg L^{-1}), over several generations, did not result in transgenerational preconditioning after exposure to higher doses (LC_{10} and LC_{50}) of the same insecticide, or a higher dose (LC_{50}) of an insecticide with a differing mode of action (spirotetramat) (Chapter 2). Exposure to a hormetic dose (0.1 μg L^{-1}) of imidacloprid (a dose that was shown to induce reproductive hormesis), however, did result in reduced mortality after three generations of exposure. Hormetic preconditioning was not observed after subsequent exposure to higher levels of spirotetramat.

The lack of observed preconditioning after exposure to a much higher concentration of spirotetramat (Chapter 2) confirmed previous findings (Rix et al. 2016), suggesting that imidacloprid exposure does not prime aphids to withstand subsequent exposure to spirotetramat. While there is evidence that one type of stressor can prime an organism to withstand different types of stress (Eggert et al. 2015; Calabrese 2016b; Calabrese 2016a), not all stressors will prime insects to cope with subsequent stress, even in cases where subsequent exposures may be similar, or the same (Cypser and Johnson

2002; Liang et al. 2012; Shikano et al. 2015). I previously observed that hormetic exposure to imidacloprid increased the expression of heat shock proteins (*Hsp60*) (Rix et al. 2016), which are known to help insects cope with dehydration stress (Zhao and Jones 2012). I observed increased ability to cope with dehydration stress when my imidacloprid exposed aphids were subsequently exposed to food/water stress, and mortality was reduced compared with controls (Rix et al. 2016). Given this and the fact that I again did not observe preconditioning after subsequent exposure to spirotetramat, I concluded that low dose imidacloprid exposure simply does not prime the insect to cope with an additional spirotetramat stress, but this may not preclude priming to other forms of stress.

Hormetic levels of imidacloprid exposure resulted in reduced mortality to subsequent and higher exposure to imidacloprid after several generations, but not exposure to two mildly toxic doses of imidacloprid (Chapter 2). It has been consistently seen throughout the literature that hormetic levels of stress are stimulatory, and levels outside the hormetic zone result in no or inhibitory effects (Calabrese and Baldwin 2002; Calabrese and Baldwin 2003; Calabrese 2013a), and that the optimal preconditioning dose is also the dose that induces hormesis (Calabrese 2016b). My results also conform to this pattern seen in the literature. Hormesis is considered to be an evolutionary adaptation to stress, likely as a result of overcompensation in response to a small stress (Calabrese 2001; Calabrese 2013b). Small stresses, thus appear to allow an organism to adapt quickly to incoming stressors (Costantini et al. 2012; Costantini 2014), particularly given that hormesis often induces increases in metabolic activities (Calabrese 2016b). Adaptation to larger amounts of stress thus may require more generations of conditioning or significant trade-offs (Costantini 2014), which is likely why reduced susceptibility to

imidacloprid was not observed in aphids exposed to two levels of imidacloprid outside the hormetic zone. Fourth generation probit lines of imidacloprid exposed aphids further showed that the higher levels of imidacloprid exposure may have been too stressful to result in adaptation, as the intercepts of these lines (5 and 10 μg L^{-1}) were significantly higher than the control line, and LC_{50} values and slopes were not significantly different from control lines (Chapter 2).

While I did observe evidence of preconditioning in my aphids after three generations of hormetic exposure to imidacloprid, fourth generation probit lines showed that not all aphids were exhibiting reduced susceptibility to imidacloprid and thus not all aphids were preconditioned. While the slope of the hormetic probit line was significantly smaller than the control probit line, the intercept and the LC_{50} were not significantly different from the control line (Chapter 2). Mortality in the hormetic line dropped below the control line at concentrations above the LC_{50}, suggesting that a number of aphids were exhibiting some tolerance to imidacloprid, resulting in the reduced slope. Thus some aphids, and not the entire population of hormetically exposed aphids, were preconditioned (Chapter 2). Hormetic effects can vary within a population. Belz and Sinkkonen (2016) observed that slow growing individuals were stimulated at low levels of stress, and fast growing individuals were stimulated at high levels of stress, at the threshold of toxic effects. This indicated that individuals at the tails of normal distributions largely influence the significance of hormetic effects, which was precisely what I saw when further analyzing observed hormetic preconditioning with fourth generation probit lines (Chapter 2).

The second objective of my thesis aimed to connect stress, hormesis, and mutation induction. The stimulatory effects observed in the hormetic dose response, are adaptations to stress (Calabrese 2008a; Calabrese 2008b; Costantini 2014). Stress is a key driver of mutations (Gressel and Levy 2010) and mutations are important driving forces of evolution. Stress can potentially accelerate adaptive evolution by inducing mutations (Galhardo et al. 2007). Thus it stands to reason that mutations would increase in an organism under stress, as greater variability in the genome, as a result of mutations, could help an organism under stressful environmental conditions to adapt (Gressel 2011). If hormetic levels of pesticide stress, in addition to stimulating life history traits and priming insects to better withstand subsequent stressors, can also induce or increase mutations, particularly in insecticide target regions (which could increase the risk of target site resistance development), this could be additionally problematic for pest management. What is unclear, however, is how low of a stressor can result in mutations. Low and sublethal doses of pesticides have induced DNA damage in cells of eukaryotic organisms (Pluth et al. 1996; Geret et al. 2013; Muangphra et al. 2014). Low doses of pesticides have also resulted in mutations in target site regions in fungi (Wieczorek et al. 2015) and weed species (Tardif et al. 1993; Tardif and Powles 1994). It is not known, however, whether chemical stressors as low as hormetic levels can result in mutations in target sites. I thus examined whether exposure of *M. persicae* to the insecticide, imidacloprid, at a hormetic concentration and two mildly toxic concentrations outside the hormetic zone, resulted in mutations in sites on the nAChR which form a significant portion of the imidacloprid binding region (Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1) in *M. persicae*. Mutations in nAChR subunits are known to occur in neonicotinoid resistant

populations of *M. persicae* and other hemipterans (Liu et al. 2005; Bass et al. 2011; Bass et al. 2015; Kim et al. 2015).

Examination of sequenced portions of Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1 in *M. persicae* exposed for one and four generations to control, hormetic and mildly toxic concentrations of imidacloprid showed no evidence of mutations compared with published sequences (Chapter 3). It has been hypothesized that exposure to low levels of stress may result in mutation induction as DNA repair enzymes compete with detoxification enzymes for limited energy sources (Gressel and Levy 2010; Gressel 2011). However, like hormesis and hormetic preconditioning, effects may be dependent upon the type of stress, the level of stress and the organism or gene being studied. Although there is evidence of low doses of stress inducing mutations, there is other evidence in insects showing that low doses of stress can reduce mutation frequencies (Koana et al. 2007; Ogura et al. 2009). Even exposure to moderately toxic and very high levels of stress may not induce mutations, as observed in *D. melanogaster* exposed to moderately toxic concentrations of several chemical pesticides (Frantzios et al. 2008; Akmoutsou et al. 2011) (Chapter 3).

Some mutations may require long term exposure or selection with a pesticide in order to occur. In other words, some mutations may only result after a certain threshold of exposure or selection, which has been demonstrated in sodium channels of *C. quinquefasciatus* (Li and Liu 2010; Li et al. 2012). It is possible that mutations in nAChRs only occur after long term selection. This is the most reasonable explanation for why I did not observe mutations in nAChRs in my aphids. Mutations in two nAChR subunits were observed after 22 generations of selection with imidacloprid in the

hemipteran species, *N. lugens* (Liu et al. 2003; Liu et al. 2005), suggesting a large amount of selection pressure was required for mutations to occur. This may also be true for *M. persicae* as a limited number of populations with mutations in nAChRs have been found (Puinean et al. 2010b; Bass et al. 2011; Bass et al. 2014; Panini et al. 2014). Those incidences of low dose exposure to pesticides leading to mutations (Tardif et al. 1993; Tardif and Powles 1994; Wieczorek et al. 2015) are likely a reflection of the gene and the organism. Some genes are well known to mutate, and some organisms are well known for their adaptability under stress (Chapter 3). Although *M. persicae* is well known to develop resistance to insecticides, they are still largely susceptible to neonicotinoids, as are other insects, despite the widespread use of this insecticide (Bass et al. 2015) (Chapter 3). This may also indicate that large amounts of selection are required for mutations to occur in nAChRs.

Conclusions and Prospects for Future Work

In this thesis, I have further examined the intricacies of the hormetic response, examining hormetic preconditioning over several generations and working to connect hormesis, stress adaptation and responses, and gene mutations. I demonstrated that exposure to imidacloprid at levels that also induce hormetic stimulation on reproduction, can reduce susceptibility to subsequent imidacloprid exposure, but only after several generations of exposure, and only in some individuals. Exposure to mildly toxic concentrations of imidacloprid did not result in preconditioning, nor did prior imidacloprid exposure prime *M. persicae* to withstand a subsequent spirotetramat stress, under my conditions. These findings also indicated that preconditioning was transgenerational, however only after two generations of hormetic imidacloprid exposure. I also demonstrated that exposure to

hormetic and mildly toxic concentrations of imidacloprid over several generations did not induce mutations in nAChR subunits, comprising the imidacloprid binding site, thus failing to connect hormesis and stress to induction of gene mutations. My findings and subsequent literature analysis suggest that the effects of hormetic preconditioning and the occurrence of gene mutations are highly variable, dependent upon the type and level of stress and the organisms being studied. While hormesis and low dose effects of stress pose a challenge to pest management, my findings suggest that the extent to which low dose exposures to stress may lead to pesticide adaptation, over several generations, may be limited.

My study was conducted over four generations, and thus it is certainly possible that more substantial effects could be observed after several more generations of study. My results demonstrated that after three to four generations of hormetic imidacloprid exposure, some individuals in the population were exhibiting some resistance to imidacloprid. Further work could expand on these results, examining additional generations exposed to hormetic levels of imidacloprid and whether this resistance persists, and whether small amounts of resistance in the population, although statistically significant, are also biologically significant. Additionally, other insect-pesticide systems have had more substantial findings than what I observed with a *M. persicae*-imidacloprid model (Liang et al. 2012; Eggert et al. 2015; Shikano et al. 2015). Other pest-stressor models may be advantageous to examine from a pest management perspective, however, examination of stimulatory effects induced by exposure to hormetic levels of stress or hormetic priming in beneficial insects such as natural enemies or pollinators could provide strategies for commercial insect rearing facilities to enhance insect production.

Finally, although low dose imidacloprid exposure did not result in mutations in nAChR subunits, other research at the molecular level has demonstrated that low levels of stress can alter patterns in gene expression, aiding in stress adaptation (Mahroof et al. 2005; Yin et al. 2006; Rix et al. 2016). Hormesis studies at the molecular level could further expand on gene expression work into studies looking at energy trade-offs, examining whether trade-offs counter the stimulatory effects induced by hormetic and low dose stressors. Future research could examine a broader range of the insect transcriptome to more fully comprehend trade-offs. Although my results were largely non-significant, this should not preclude additional study with a *M. persicae*-imidacloprid model, other insect-stressor models, or further examinations of hormesis at the molecular level.

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APPENDIX 1

Confirmation of amplicons from nAChR subunits Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1 by gel electrophoresis. Genes from the five subunits were amplified from pooled samples of 30 aphids exposed for 1 generation (G1), and four generations (G4) exposed to 0, 0.1, 5, and 10 μg L^{-1} of imidacloprid.

Available on Dal Space.

APPENDIX 2

Chromatogram showing overlapping peak at base pair 654 in Mpα4, corresponding to amino acid 190, glutamine (CAG/CAA). Chromatograms from all generations (G1 and G4) and concentrations (0, 0.1, 5, and 10 μg L^{-1} of imidacloprid) of exposed aphids (including controls), possessed this overlapping peak.

Available on Dal Space.

APPENDIX 3

Sequence alignments of nAChR subunits Mpα1, Mpα2, Mpα3, Mpα4 and Mpβ1 for generation (G1), and four generations (G4) exposed to 0, 0.1, 5, and 10 μg L^{-1} of imidacloprid. Individual sequences are aligned to published nAChR subunits. Available on Dal Space.