Effect of Sublethal Concentrations of Imidacloprid and Precocene on Green Peach Aphid, *Myzus Persicae* (Sulzer) (Hemiptera: Aphididae): A Study of Hormesis at the Gene, Individual and Population Level

by

Murali Mohan Ayyanath

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ABSTRACT

Effect of Sublethal Concentrations of Imidacloprid and Precocene on Green Peach Aphid, *Myzus Persicae* (Sulzer) (Hemiptera: Aphididae): A Study of Hormesis at the Gene, Individual and Population Level

Murali Mohan Ayyanath
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Advisors:
Dr. Cynthia Scott-Dupree
Dr. Gerald Christopher Cutler

Threshold and non-threshold linear models that govern toxicology are challenged by an alternative model, hormesis. It is defined as low-dose stimulation and high-dose inhibition from a stressor. Insecticide-induced hormesis has been studied in a plethora of insect-insecticide models at biochemical, individual and population levels. This research focuses on the effects of sublethal concentrations of insecticides on reproductive responses of green peach aphid, *Myzus persicae* (Sulzer), at individual and population level besides regulation of stress, dispersal and developmental genes during hormesis.

In laboratory studies, irrespective of the duration and route of exposure, sublethal concentrations of imidacloprid induced stimulations in fecundity of *M. persicae* but the nature of response differed intra- and trans-generationally. Fitness tradeoffs could be rendered due to declined fecundity in successive generations. However, continuous exposure to sublethal concentrations does not compromise overall fitness trans-generationally, considering recovered levels of fecundity as controls in successive generations and the total reproduction after four generations. Greenhouse experiments affirmed uncompromised fitness where reproductive stimulations were noted in aphids exposed to imidacloprid treated potato plants.

Up- and down- regulation of stress, dispersal and developmental genes was noted during imidacloprid-induced hormesis in *M. persicae* that mirrored the reproductive responses in few instances. Global DNA methylation results emphasized the heritability of adapted traits trans-generationally via hypermethylation. Dispersal related genes (*OSD*, *TOL* and *ANT*) that are predominantly expressed in alates (about 2- to 5-fold) were affected in apterous aphids continuously exposed to sublethal concentrations of imidacloprid.

No direct relation with the previously noted fecundity was established implying adaptive cellular stress response pathways might be triggered rather than normal regulatory processes due to low-dose imidacloprid exposure. At a biochemical level, a study noted that imidacloprid-induced hormesis concurrently stimulated juvenile hormone III (JH) production and fecundity in *M. persicae*. Precocene, an anti-JH, at sublethal concentrations induced reproductive stimulations in *M. persicae*. Gene regulation during precocene-induced hormesis mirrored imidacloprid results for few genes including *FPPS*, a JH precursor gene, with a higher magnitude of regulation.

Considering these stimulatory effects that insecticide-induced hormesis at various biological hierarchies, causes for pest resurgence, hormesis could have ramifications from declines in natural enemy population.

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1 LITERATURE REVIEW

1.1 Hormesis

Descriptions of dose-response in toxicology have generally been governed by the threshold model (Figure 1.2A), which states that there is a threshold above which chemical exposure causes an effect and below which no effect occurs (Cox 1987); and the linear non-threshold model, which states that there is no threshold in the dose-response relationship (e.g., carcinogenic agents) (National-Research-Council 2006). However, these models fails to explain reported stimulatory responses due to exposure to low doses below the estimated threshold (Calabrese 2010). Hormesis is an alternate model defined by high-dose inhibition and low-dose stimulation from a stressor, e.g., a poison. Hormesis was first reported in mid-1800 by Rudolf Virchow when working on the ciliae of the tracheal mucous membrane of a deceased human being and low doses of sodium hydroxide or potassium hydroxide (Henschler 2006). Instead of a fading activity in cilia of a deceased human being, a dramatic increase in the power and frequency of beating occurred when exposed to low doses of sodium or potassium hydroxide.

Later in the late 1800s, Hugo Schulz reported that yeast exposed to low doses of mercuric chloride, iodine, bromine, chromic acid, arsenious acid, formate and salicylic acid experienced stimulated carbon dioxide emission via fermentation and a decrease at higher doses (Henschler 2006). The term "hormesis", meaning "to excite" was first coined by Southam and Erhlich who observed that the extracts from heartwood of red cedar, *Thuja plicata* Donn ex D. Don, inhibited metabolism of multiple fungal species when administered at high doses, but enhanced metabolism of the fungi at low doses (Southam and Ehrlich 1943). Even during this period the phenomenon of biological stimulation due to low amounts of stress was not unknown. For example, the concept that "poisons stimulate" was proposed by Hatshepset in 1500 BC; in 1540,

Paracelsus stated that the dose is everything, meaning that the dose determines something to be or not to be poisonous. In 1888, Rudolf Arndt, a psychiatrist, author of the book "the basic biological law" and a passionate propagator of homeopathy and Hugo Schultz together formulated the concept of low-dose stimulation and high-dose inhibition termed as "Arndt-Schultz law" (Henschler 2006). Synonymously, the hormesis concept in the field of bacteriology was known as "Hueppe's rule" named after Ferndende Hueppe and in the area of psychological stress biology as "Yerkes-Dodson Law" (Calabrese et al. 2013). In 1946, Luckey found that antibiotics stimulate growth in germ free chicken at low doses but not at high doses possibly due to stress (Luckey 2008). The term "Hormoligosis" was coined by Luckey in 1959 while studying the effects of antibiotics in adaptation to heat stress. Hormesis has gained widespread attention only since the mid-1980s (Calabrese 2010).

Broadly, hormesis is thought to occur by direct stimulation hormesis (DSH) or overcompensation stimulation hormesis (OCSH) (Calabrese and Baldwin 2002, 2003, Calabrese 2010, Calabrese et al. 2013). Initial disruption of homeostasis due to low doses of stressor, followed by a modest overcompensation to reestablish homeostasis and the adaptive nature of the process are key features of OCSH (Calabrese and Baldwin 2002). Otherwise similar to OCSH, the absence of initial disruption of homeostasis is a key feature of DSH. However, the generality of hormesis across a plethora of stressors and organisms suggests that there is no specific overriding mechanism (Forbes 2000, Parsons 2000, Calabrese 2010). In general, hormetic responses have certain characteristics. The most important feature of these responses is that the magnitude of low-dose stimulation is consistently modest stimulation, reaching a maximum of 30-60% over control group which can be confused with data variability (Figure 1.1). It takes at least 3-4 stimulatory doses below the threshold to obtain hormetic model-fit

responses. The width of the hormetic response (stimulatory responses over control) ranges from 10- to 20-fold below the toxicological threshold and peak stimulation occurs at about an average of 5-fold below the no observable adverse effect level (Calabrese et al. 2013).

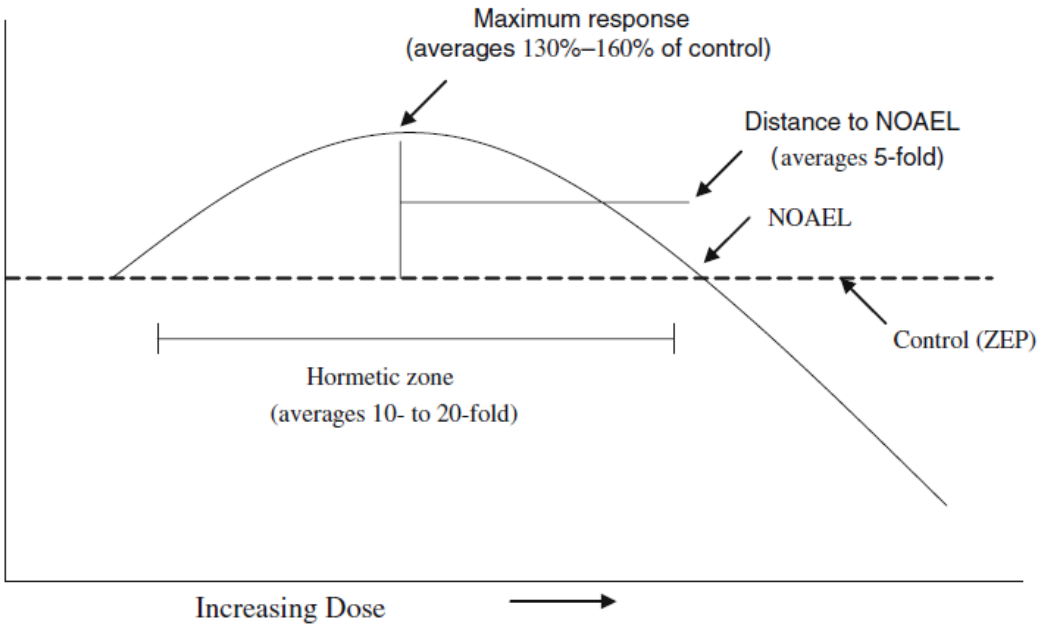

Figure 1.1. Dose-response curve depicting the quantitative feature of hormesis (Calabrese et al. 2013).

There are certain principles generalizing the phenomenon as listed by Calabrese et al. (2013). Depending on the stressor and the endpoint assessed, the hormetic dose-response curve will typically come in two forms. Where stimulation in normal function (e.g., endpoints like survivorship or fecundity) is seen at low doses, but increased dysfunction (e.g., decreased survivorship or fecundity) is seen at high doses, the curve will take on an inverted U-shaped (Figure 1.2B) (Calabrese and Baldwin 2002, 2003). Alternatively, there may be responses where low doses of stressor will reduce dysfunction, but high doses increase dysfunction (Figure 1.2C). The dose response in this second situation will typically take on a J-shaped curve and is frequently observed with agents that induce mutations or cause disease at high doses (Calabrese and Baldwin 2002, 2003). Increased study of hormesis has resulted in development of

mathematical/statistical models to describe the hormetic dose response. Two empirical models that have gained prominence are those developed by Brain and Cousens (1989), and another by Cedergreen et al. (2005). However, it has been shown that these models do not adequately fit all hormetic dose-responses and that more sophisticated models may be needed in some cases (Belz and Piepho 2012).

Many studies have documented hormesis with chemical and non-chemical agents/stressors in many organisms from bacteria to humans using various endpoints (Calabrese 2010). Hormesis has been reported widely in insects, particularly of late (Cutler 2013). For instance, in insecticide toxicology studies: egg production in *Sitophilus granarius* (L.) was stimulated when treated with low doses of DDT (Kuenen 1958); house crickets, *Acheta domesticus* (L.) gained weight when exposed to a number of insecticides at sublethal concentrations (Luckey 1968); increased survival, larval weight and rate of development of *Podisus distinctus* (Stäl) was observed when subjected

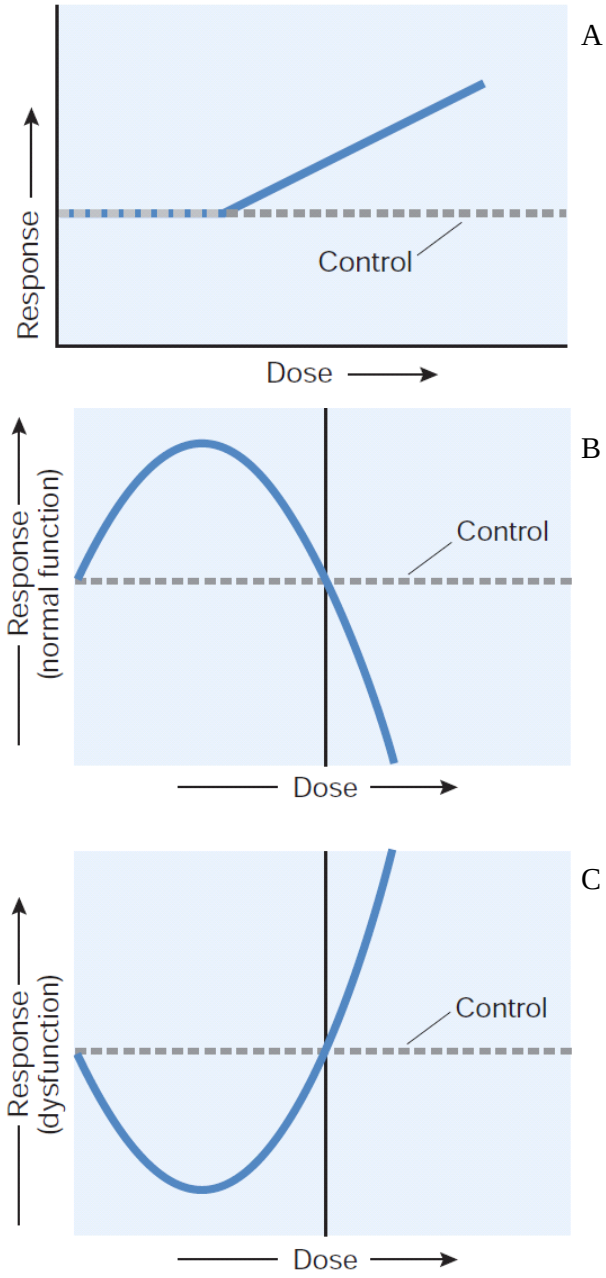

Figure 1.2. Dose-response models: (A) threshold; (B) inverted U-shaped hormetic; (C) J-shaped hormetic (Davis and Svendsgaard 1990).

to sublethal concentrations of permethrin (Magalhaes et al. 2002); and increased F2 production in *Myzus persicae* (Sulzer) following exposure to sublethal doses of imidacloprid (Cutler et al. 2009).

The energetic resources being limited in an organism, tradeoffs occur for the expended investments towards attributes such as increased reproduction, longevity, weight etc (Costantini et al. 2010). Depending on the number of traits involved, these tradeoffs could be of two kinds (Agrawal et al. 2010). They are one-trait tradeoff which occurs when there is an opposing selection on a single trait and a multiple-trait tradeoff which occurs when multiple traits share a limiting resource. According to the principle of resource allocation, tradeoffs should exist in hormesis but examples of this are unknown in insecticide models. Also unclear is whether fitness increases, decreases or is neutral due to hormesis. Hormesis studies supporting increased fitness (Calabrese and Baldwin 2003, Cutler et al. 2009, Costantini et al. 2010, Costantini et al. 2012) and neutral fitness (Forbes 2000) exist.

Pesticides can provide fast and effective control of pests in integrated pest management but temporal and spatial drift in insecticide applications could lead to unevenness in their distribution. This uneven distribution could lead to the exposure of pests and other non-target insects to sublethal concentrations. Hormesis is significant for pest management since it may be a mechanism for pest resurgence (Chelliah and Heinrichs 1980, Morse and Zareh 1991, Wang et al. 2008, Cutler et al. 2009, Cutler 2013), and thus might result in increased crop damage and additional pesticide treatments, potentially exacerbating non-target impacts, insecticide resistance development and environmental contamination (Cutler et al. 2009).

There have been only a few detailed examinations into the mechanisms of pesticide-induced hormesis in insects. Surviving populations of insecticide treated areas

(organophosphorus and carbamate) possessed greater energy reserves and vigor than untreated, a hormetic response, in the form of increased total Ca^{++} and proteins in spruce budworm, *Choristoneura fumiferana* (Clemens) (Smirnoff 1983). Using an azadirachtin – *Tribolium castaneum* (Herbst) model, it was shown that there was new synthesis and/or suppression of isoesterases due to increased activity of enzymes or the induction of multiple forms adapted to stressor that might contribute to hormesis (Mukherjee et al. 1993). A recent study found that stimulated fecundity in *M. persicae* in response to sublethal doses of imidacloprid was correlated with increased Juvenile hormone (JH) III production (Yu et al. 2010), which is essential for Vitellogenin (Vg) synthesis (Hartfelder 2000). No other work has examined mechanism of hormesis in insects, despite the fact that these insect-insecticide models offer an excellent opportunity to "dig deeper" into questions of hormesis mechanisms, both in terms of the phenomenon in general, but also for more specific applications in pest management (Cutler 2013).

Heat stress-insects have been used to study gene regulation during hormetic phenotypic response. For example, oxidative stress adaptations (hormesis) in the fruit fly *Drosophila melanogaster* Meigen, is achieved by up-regulating protective systems such as the proteasomal proteolytic pathway to eliminate oxidized proteins (Pickering et al. 2013). Increased longevity resulted due to heat- and starvation-stress in *Oomyzus sokolowskii* (Kurdjumov) females (Zhang et al. 2012), due to heat-stress in *Caenorhabditis elegans* Maupas (Rodriguez et al. 2012) and in *D. melanogaster* (Hercus et al. 2003) is now coupled with gene regulatory mechanisms such as nuclear factor – κB (NF-κB) (Le Bourg et al. 2012). It is now known that regulation of gene expression is implicit in hormetic adaptive responses which in turn is the activation of adaptive cellular stress response pathways assisted by transcription factors such as antioxidant response

element (ARE), forkhead box O (FOXO), heat-shock factor (HSF) and NF-κB (Son et al. 2010). The activation of these pathways probably is dictated by specific stimuli induced by stressors. Insects use the HSF pathway for maintenance of homeostasis. For example, heat treated codling moth, *Cydia pomonella* (L.) (Yin et al. 2006) and *T. castaneum* (Mahroof et al. 2005) accumulated heat inducible heat shock protein (Hsp)70 resulting in thermotolerance. Other Hsps in *M. persicae* are essential for survival and other vital processes such as serving as chaperones that assist protein folding and stabilization, removal of degraded proteins, and serving as signaling proteins during immune responses (Ramsey et al. 2007). Generally Hsps have been shown to be up-regulated after septic injury and microbial infections (Pockley 2003).

Besides heat-induced stress, insects are subjected to other forms of abiotic and biotic stress. Biotic stress could be due to overcrowding or natural enemies, and abiotic stress could result from radiation, artificial chemicals, toxicants, or metals. Insects have adapted various mechanisms to evade, tolerate or resist these conditions. Stress evasion is successfully managed by migration and especially in insects such as aphids, possible via wing development for flight. In aphids such as *Myzus persicae* (Sulzer), of the 31 genes overexpressed in alates (winged forms) over apterous (non-winged forms), three are predominant: take-out-like (*TOL*)/JH binding proteins, olfactory segment D (*OSD*), and mitochondrial adenine nucleotide translocase (*ANT*) (Ghanim et al. 2006). Similar genes are deployed in other insects for similar purposes (Zhang et al. 1999, Sarov-Blat et al. 2000, So et al. 2000, Rikhy et al. 2003, Wanner et al. 2004).

Hormesis has been attributed to the adaptive-response genes due to long-lasting epigenetic memory (Scott et al. 2009). Epigenetic inheritance, defined as inherited changes in gene expression caused by mechanisms other than changes in DNA sequence, plays a vital role in mediating gene regulation and phenotypic plasticity (Bonasio et al. 2010) that has been

observed via mechanisms such as transcriptional regulation, posttranslational modification, and DNA methylation (Zhang and Meaney 2010, Weiner and Toth 2012). DNA methylation involves the addition of a methyl group onto cytosines in DNA, and is associated with silencing of gene expression (Bird 2007). In vertebrates, "programming" effects (adaptation) could be derived from gene-environment interactions that lead to structural alteration of the DNA (Zhang and Meaney 2010). In insects, insecticide-resistance is associated with amplification and methylation of esterase genes (Ono et al. 1999, Field 2000, Field et al. 2004). It is suggested that insects serve as an excellent model system for studying evolution of DNA methylation (Glastad et al. 2011, Vaiserman 2012).

1.2 Insecticides

Pests may destroy one-third of the world's food crops (Ware and Whitacre 2004). Insecticides are powerful tools for control of pest populations as they can be deployed quickly, are often broad spectrum, and are economical (Frisbie 2006). They are one of the most important tactics used in insect pest management, with every dollar spent on pesticides providing $4 in returns (Cooper and Dobson 2007). It is predicted that globally there would be a 50% reduction in crop production if pesticides were eliminated from current agricultural conditions (National-Research-Council 2000).

Organophosphorus, carbamate, synthetic pyrethroid, and neonicotinoid insecticides account for approximately three-fourths of global insecticidal sales (Nauen 2006). Neonicotinoids are now the most important insecticide class in terms of overall sales and use, having a global market value of 1.6 billion USD per year accounting for 17% global insecticide market in 2006 (Jeschke and Nauen 2008). Imidacloprid is the leading active ingredient with widespread use against sucking insects in most countries (Elbert et al. 1998, Nauen et al. 2001,

Jeschke and Nauen 2008), mainly aphids, leafhoppers and whiteflies (Elbert et al. 1991, Jeschke and Nauen 2008), but also defoliating insects such as the Colorado potato beetle (Sladan et al. 2012).

Biochemically, neonicotinoids mimic acetylcholine (Liu and Casida 1993) and bind irreversibly to postsynaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects (Bai et al. 1991, Liu and Casida 1993, Nauen et al. 2001, Jeschke and Nauen 2008). This affinity for nicotinic receptors, which occur in much higher levels in arthropods compared to vertebrates, accounts for the selectivity of neonicotinoids in favor of mammals (Mullins 1993, Nauen 1995) and other non-target organisms (Tomizawa and Casida 2003, Casida and Quistad 2004). Resistance to neonicotinoids does occur and can arise either through expression of nAChR subtypes, detoxification mechanisms and/or structural alterations of target-site proteins (Thany 2010). Metabolically, enhanced oxidative detoxification of imidacloprid by over-expressed P450 monooxygenases can occur (Karunker et al. 2008). Recently it was shown that amplification of the cytochrome P450 gene is associated with resistance to imidacloprid in the green peach aphid (Puinean et al. 2010).

Besides neurotoxins, insecticidal compounds that affect developmental processes in insects have been discovered, mainly the juvenile hormone mimics, ecdysone agonists, and chitin synthesis inhibitors (Ishaaya and Horowitz 1998). Juvenile hormone (JH) is a very well-studied insect hormone (Riddiford 1994). It is a sesquiterpenoid manufactured and secreted by the corpora allata, and neuropeptides synthesized by neurosecretory cells control the secretion of JH. JH synthesis is inhibited and stimulated by allatostatins and allatotropins, respectively. JH is present during peak ecdysone titers in preparation for molting but disappears shortly after the last molt. JH also regulates various reproductive processes (Wyatt and Davey 1996). In aphids

it controls sex determination process (Mittler et al. 1979) and high concentration of JH aids in development of apterous (winged) rather than alate (wingless) females (Mittler 1991). JH III is the most common form of JH and is derived from a C15 precursor, farnesyl diphosphate (FPP), an important intermediate of the mevalonate pathway (Schooley et al. 1973) where the biosynthesis of FPP is catalyzed by *FPPS* (Zhang and Li 2008). In general, cholesterol is the end product of this pathway. However, in insects, which do not produce cholesterol, FPP is used in the production of other end products like JH. In most insects JH levels are controlled by JH esterase which degrades JH.

Natural and synthetic JH analogues (juvenoids), including fenoxycarb, pyriproxyfen and methoprene have been designed to cause hyperjuvenism (Williams 1967), although the molecular mechanism is unclear. In addition, blockers or disrupters of JH biosynthesis through enzyme inhibition have been identified from natural sources such as precocenes and brevioxime, and synthetic sources such as fluoromevalonate (Dhadialla et al. 2005). Due to poor efficacy and/or high toxicity they are not commercially available for pest management. Precocenes I and II, extracted from *Ageratum houstonianum* Mill. are powerful antiallatotropins that prevent the corpora allata from producing JH (Bowers 1976, Bowers et al. 1976).

1.3 *Myzus persicae* (Sulzer) – Green peach aphid

Myzus persicae is a polyphagous pest that is frequently found in several agroecosystems especially in areas with a moderate climate (Blackman 1974, Blackman and Eastop 1995, 2000). Its life cycle involves alternation of parthenogenetic generations in secondary hosts with migration to the primary host for sexual reproduction (holocyclic). However, various patterns exist such as obligate parthenogenetic morphs that lack sexual reproduction (anholocyclic), obligate parthenogenetic morphs that produce only males (androcyclic) and an intermediate kind,

where parthenogenetic forms produce females that migrate, invade the primary host and then produce sexual forms (Blackman 1974). Factors affecting the morph production mainly include availability of the primary host and the adversity of prevailing environmental conditions (Blackman and Eastop 2000). In general, it has a heteroecious (requires at least two hosts) holocyclic life cycle (Figure 1.3) in temperate regions where *Prunus persica* (L.), *P. nigra, P. tanella* and peach-almond hybrids act as the primary host. Economically important crops belonging to about 40 additional families act as secondary hosts (Blackman 1974). Apterous adults are small-medium sized, whitish green, pale yellow green, mid-green, pink or red (Blackman and Eastop 1995). Alates of *M. persicae* have a black central dorsal patch on the abdomen. Both forms measure 1.2-2.3 mm in length. This aphid probably originated from Asia like *Prunus spp*. but currently is worldwide in its distribution.

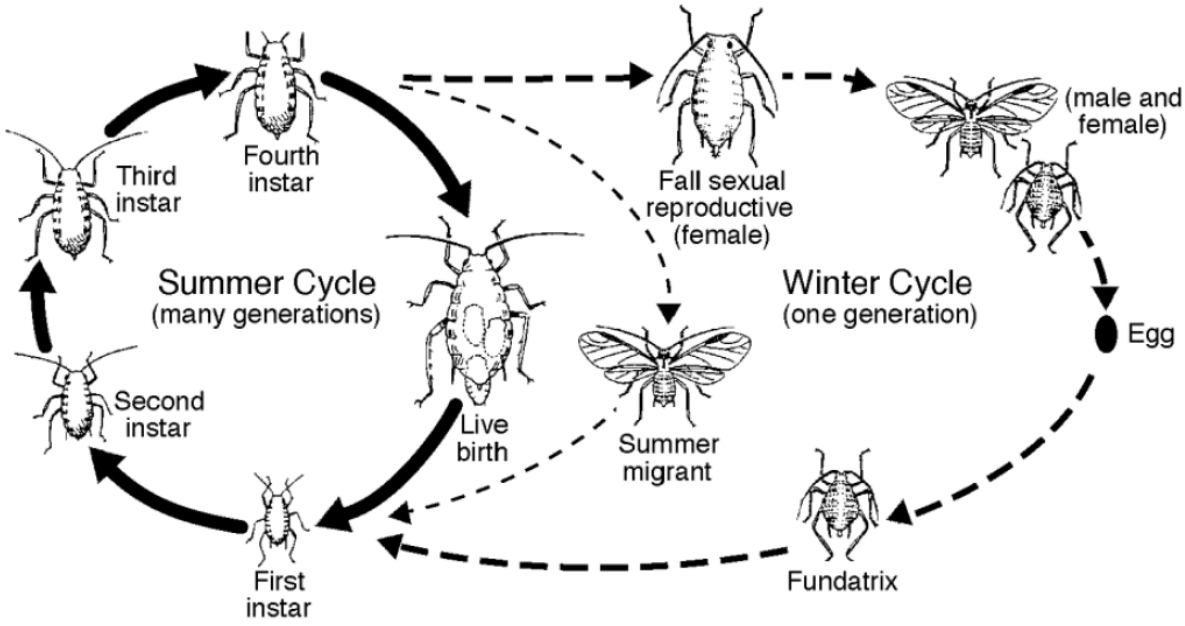

Figure 1.3. The reproduction cycles of *Myzus persicae*, the green peach aphid (www.ucdavis.edu).

Myzus persicae is a pest of global significance, causing direct feeding damage and indirect damage through transmission of more than 100 plant viruses (Kennedy et al. 1962, Blackman and Eastop 2000). This insect is thus subject to numerous insecticides and has in turn developed resistance to multiple insecticides classes (Blackman and Eastop 2000).

1.4 Objectives and Hypothesis

A large number of basic questions concerning hormesis remain to be answered at the molecular, individual and population levels. Insects are an excellent model to address such questions: they are easily reared at high numbers; there is extensive background work in insect ecology, toxicology, biochemistry and molecular biology; and there few ethical issues when using them in experiments. Moreover, hormesis has practical relevance within in the context of crop protection and insect pest management (Cutler 2013).

Hormesis is a general phenomenon that occurs in an organism exposed to any stressor (Calabrese 2010). However, not all chemicals that are toxic induce stimulation at low doses (Belz et al. 2008). Further, it is not clear whether hormesis manifests in insects the same way (i.e., endpoint expression) when exposed to chemical stressors with very different modes of action. The principle of resource allocation predicts tradeoffs during changes in physiological responses to stress. When in stressful conditions an organism will allocate most resources to critical functions, and the remaining energy to relatively less important processes (Sibly and Calow 1986).

Chemical hormesis was studied using the *M. persicae* – potato model and two chemical stressors, imidacloprid and precocene I, compounds that are lethally toxic to *M. persicae* but with very different modes of action. As discussed, *M. persicae* reproduces rapidly and has asexual, parthenogenetic reproduction, which limits heterogeneity. It is easy to rear, and

economically important to a large number of crops, including potato. Green peach aphid is well studied with many investigations of insecticide toxicology and molecular biology, and related genes have been identified for this insect.

Useful targets to examine, as they relate to the various doses of chemical stressors in and around the stimulation peak of the hormetic curve, are the mevalonate pathway and its related enzymes like *FPPS* (Farnesyl diphosphate synthase). These are important regulators in JH production, which has strong implications for insect development and reproduction. JH and JH-related genes (JH precursor gene *FPPS1*; *TOL* /JH binding proteins (Brisson 2010)), *M. persicae* genes involved in wing dimorphisms (*OS-D*) (imidacloprid induced stimulation has been associated with wing dimorphism in *M. persicae* (Wang et al. 2008)), and gene involved in respiration (*ANT* (Ghanim et al. 2006)) could be considered as a indicators for the occurrence of this phenomenon. Analyzing genes in *M. persicae* such as those related to stress response (*Hsp60*), dispersal (*OSD, TOL*, and *ANT*) and or development (*FPPS* 1) following exposure to sublethal concentrations of different insecticide stressors could provide a clearer understanding of molecular foundations of chemical hormesis.

1.4.1 Hypotheses

The overriding hypothesis tested in this thesis research is that insecticide-induced hormesis can manifest in insects at multiple levels. I predicted that, based on previous experiments, hormetic responses would be seen in individual insects, but might vary with life stage and generation. I also predicted that hormetic effects could be seen at the population level, under exposure scenarios one might expect to encounter in an agricultural situation. Finally, I expected that phenotypic hormetic responses seen in insects could be explained by changes in

expression of genes linked to stress responses and corresponding life history traits such as fecundity, longevity etc.

2 TRANSGENERATIONAL SHIFTS IN REPRODUCTION HORMESIS IN GREEN PEACH APHID EXPOSED TO LOW CONCENTRATIONS OF IMIDACLOPRID

2.1 ABSTRACT

Hormesis is a biphasic phenomenon that in toxicology is characterized by low-dose stimulation and high-dose inhibition. It has been observed in a wide range of organisms in response to many chemical stressors, including insects exposed to pesticides, with potential repercussions for agriculture and pest management. To address questions related to the nature of the dose-response and potential consequences on biological fitness, I examined trans-generational hormesis in the green peach aphid, *Myzus persicae*, when exposed to sublethal concentrations of the insecticide imidacloprid. A hormetic response in the form of increased reproduction was consistently observed and a model previously developed to test for hormesis adequately fit my data. However, the nature of the dose-response differed within and across generations depending upon the duration and mode of exposure. Decreased reproduction in intermediate generations confirmed that fitness tradeoffs can be a consequence of the hormetic response. However, recovery to levels of reproduction equal to that of controls in subsequent generations and significantly greater total reproduction after four generations indicated that biological fitness was increased by exposure to low concentrations of the insecticide, even when insects were continuously exposed to the stressor. This was especially evident in a greenhouse experiment where the instantaneous rate of population increase almost doubled and total aphid production more than quadrupled when aphids were exposed to potato plants systemically treated with low amounts of imidacloprid. My results show that although fitness tradeoffs do occur with the hormetic responses, this does not necessarily compromise overall biological fitness.

2.2 INTRODUCTION

Hormesis is a biological phenomenon whereby a stressor can have inhibitory effects at high exposure levels, but at low levels can stimulate biological processes (Calabrese 2008, Mattson 2008). It has been observed in a plethora of organisms responding to a wide range of chemical, physical, and biological stressors. Oddly, although the consideration of a time component is thought to be critical in understanding hormesis (Calabrese and Baldwin 2002, Carelli and Iavicoli 2002), the vast majority of hormesis studies incorporate only a single time point into their experimental designs (Calabrese 2005, 2008).

From an evolutionary perspective, only through inclusion of multiple time points in experimental designs can hypotheses related to biological fitness be fully examined. The *principle of allocation* states there are fitness tradeoffs in the allocation of resources among different physiological processes. Increased energy allocation to certain processes (possibly observed as hormesis) is predicted to result in decreased allocation of energy to other processes or traits, and shifts over time in tradeoff expression are expected. Differences in the expression of tradeoffs due to hormesis might vary within and across generations depending on the exposure scenario or duration. However, there is debate as to whether the hormetic response can translate into increased overall fitness, or if there are inherent tradeoffs that render the response to be effectively neutral over the long term (Forbes 2000, Parsons 2000, 2001, Costantini et al. 2010, Jager et al. 2012). It has been suggested that in certain situations and for certain species hormesis might be less energetically demanding than expected, and may come at no fitness cost, even if a stressor is not encountered again or encountered at low levels (Costantini et al. 2010).

The consequences of hormetic responses on biological fitness have been observed in invertebrates over multiple generations, with variable results. When exposed to food and

temperature stress, *Hydra magnipapillata* Ito asexual reproduction was positively affected without clear tradeoffs, suggesting stresses can have a beneficial impact on the fitness-related phenotypical traits in this species (Schaible et al. 2011). Humic substances that act as mild chemical stressors modified life-history traits of the cladoceran *Moina macrocopa* (Straus), favoring its persistence in fluctuating environments through increased lifespan and promotion of transgenerational resistance to salt stress (Suhett et al. 2011). When *Daphnia magna* Straus was exposed to low but field-relevant concentrations of fluoxetine, fluvoxamine, and 4-nonylphenol, there was increased offspring production and/or juvenile developmental rates, with different responses depending on life stages and food availability (Campos et al. 2012). On the other hand, 21-day exposures of *Daphnia carinata* King to sublethal concentrations of chlorpyrifos resulted in reproductive hormetic effects in the second generation, but reduced reproduction in the first generation and increased pesticide sensitivity in the third generation (Zalizniak and Nugegoda 2006). Similarly, when the rotifer *Brachionus calyciflorus* Pallas was exposed to low concentrations of dimethoate, increased population growth of the F0 generation was followed by reduced population growth of the F1 and F2 generations, suggesting long-term fitness was compromised by the initial hormetic response (Guo et al. 2012).

Degradation of pesticides over time and uneven pesticide distribution within the plant canopy make it a virtual certainty that insects will be exposed to low pesticide concentrations in most agricultural fields. Hormesis has been shown to accelerate pest population growth, and pesticide-induced arthropod pest resurgences have been well documented (Dittrich et al. 1974, Chelliah et al. 1980, Morse and Zareh 1991, Morse 1998, Cutler 2013). Hormesis may also have ramifications for insecticide resistance development (Guedes et al. 2010), and applications for management of beneficial insects (Zanuncio et al. 2003, Guedes 2009, Ramanaidu and Cutler

2013). In the present study I examined the hormetic and transgenerational effects of exposure to sublethal concentrations of imidacloprid on green peach aphid, *Myzus persicae* (Sulzer). *Myzus persicae* is a major worldwide insect pest that is useful in the study of pesticide-induced hormesis in insects (Cutler et al. 2009, Yu et al. 2010). Ecologically it is considered an *r*-selected species that occupies unstable environments (e.g., agro-ecosystems), has high fecundity, and reproduces quickly. I hypothesized that duration and route of exposures to sublethal concentrations of the pesticide would differentially affect the hormetic response in this insect. I expected that continuous exposure to the stressor would result in prolonged hormesis (multiple generations) at lower concentrations, whereas temporary exposure to the stressor would result in short-term hormesis (single generation) at higher concentrations. Through laboratory and greenhouse experiments, effects were studied for up to four generations. I also predicted that although a transgenerational shift in the hormetic response and biological tradeoffs would occur, and might vary with the exposure scenario, there would be no effects on the overall fitness of this *r*-selected species.

2.3 METHODS AND MATERIALS

2.3.1 Plant and insect maintenance

Potato, *Solanum tuberosum* L. (cv. Kennebec), was grown in 12.5 cm diameter pots containing Pro-Mix® (Halifax Seed, Halifax, Nova Scotia, Canada) potting soil. Plants were watered as needed. Foliage from these plants was used for insect rearing and experiments. *Myzus persicae* was obtained from a wild population infesting broccoli plants (*Brassica oleracea* L.) in a greenhouse at the Faculty of Agriculture, Dalhousie University. Aphid cohorts were maintained on excised leaves in clear plastic boxes (37 L x 24 W x 14 H cm) lined with deionized water-moistened paper towels. Boxes were held in a growth chamber (22 ± 2^{o} C, 16:8 L:D, 65 ± 5 %

RH) and every second day a layer of freshly excised leaves was placed on one end of the box. The infested foliage on the opposite end was discarded when ca. 80% aphids moved to fresh foliage. Paper towels were replaced every 10 days.

2.3.2 Chemicals

Imidacloprid (Admire® 240 SC, 240 g a.i. L^{-1}; Bayer CropScience Canada, Calgary, Alberta, Canada) was suspended in deionized water to obtain a 1000 µg a.i. L^{-1} stock solution. Only the working solutions contained 0.15% Triton X 100 (BDH Chemicals, Toronto, Ontario, Canada) as an emulsifier. Sublethal insecticide concentrations (as determined in preliminary bioassays) of 0.025, 0.1, 0.25, 1.0, 2.5, 10, and 25 µg a.i. L^{-1} were used in leaf-dip exposure experiments, and concentrations of 0.2, 0.6, 2.0, 6.0, 20, 60 and 200 µg a.i. L^{-1} were used in topical exposure experiments. Controls in these experiments consisted of water and 0.15% Triton only. For greenhouse experiments, insecticide solutions of 0 (control), 0.025, 0.1, 0.25, 1.0, 2.5, 10, and 25 µg a.i. L^{-1} were prepared in distilled water. Fresh solutions were prepared for every bioassay replicate.

2.3.3 Leaf-dip exposure

Potato leaf discs (1.8 cm diameter) were excised using a stainless steel cork borer. Using forceps, the leaf discs were dipped in control or insecticide solutions for 5 seconds, air-dried for 1 h, and then placed individually in 5.5 cm Petri plates lined with a dry Whatman No. 1 filter paper. In order to avoid cross-contamination, controls were treated first followed by sequential treatment of lowest to highest concentrations of insecticide. Five first instar *M. persicae* (ca. 24 h old) were transferred to each treated leaf disc. Dishes were covered with a Petri plate lid, and placed in sealable plastic containers and held in growth chamber at 22 ± 2^{o} C, 16:8 L:D, and 65 ± 5 % RH.

Depending on the experiment, leaf discs were replaced every second day according to one of two scenarios: a continuous exposure to treated leaf discs where founding first instars were exposed to and reared on treated leaf discs for four generations (G0-G3); or a one-time treatment where founding first instars were exposed to treated leaf discs for two days and thereafter reared on untreated leaves for four generations. In all experiments nymph production was recorded every second day. In the continuous exposure experiment, adult aphid length and longevity were also recorded in the first experimental block. The length of 72 h old adults from each generation was measured from the anterior end of the head to the tip of the distal abdominal segment using a microscope and ocular micrometer.

For each exposure scenario, the experiment was a randomized complete block design, with imidacloprid concentration being the main factor of interest. Each bioassay had five imidacloprid concentrations, and for each there were five Petri dishes with five aphids per dish. Each bioassay was considered an experimental block, and was conducted three times. Repeated measures analyses were conducted using Proc Mixed in SAS (SAS 2008), with the error terms assumed to be normal with constant variance but not to be independent. Autoregression (AR (1)) represented the appropriate type of dependence for covariance structure. Residuals were used to verify the assumptions of normal error distribution and constant variance. Data were log-transformed as needed to meet the assumptions. If means were significantly different, they were separated using a LSD test ($\alpha = 0.05$). Backtransformed means are reported as required.

2.3.4 Topical exposure

Five first instars *M. persicae* were placed in a clean glass Petri plates (9 cm diameter) and sprayed in a Potter tower (Burkard Scientific, Uxbridge, United Kingdom) at 78 kPa with 2 ml of control or insecticide solution. After each treatment, aphids were transferred to plastic Petri

plates (5.5 cm diameter) lined with Whatman No. 1 filter paper containing two untreated potato leaf discs (1.8 cm diameter). Leaf discs were replaced every second day with freshly excised leaf discs. Holding conditions were as described above, except aphids were maintained for two generations only. The experimental design and statistical analysis was as in the leaf-dip exposure experiment.

2.3.5 Exposure to systemically treated plants

Potatoes were sown in 7.5 cm diameter pots containing Pro-Mix. Approximately two weeks after germination (plant height about 5-7 cm), 50 ml of distilled water or insecticide solution was poured on to the soil surface. Three days later, five first instar *M. persicae* were randomly collected from the stock colony and transferred on to a single randomly selected leaf in the middle of the potato plant. Immediately after transfer of aphids, plants were individually covered with perforated plastic bread bags containing ca. 4-5 holes per cm^{-2} (Prism Pak Inc., Pennsylvania, USA) and secured with an elastic band around the top of the pot. There were three replicates per treatment. Pots were arranged in a completely randomized design in a greenhouse and watered as needed for 21 days. After 21 days in the greenhouse, the total number of aphids per plant was counted.

The greenhouse experiment was repeated three times, and each repetition was considered an experimental block in time. For each potted plant, the instantaneous rate of population increase (r_i) was determined as:

$$r_i = \frac{\ln\left(\frac{N_t}{N_0}\right)}{t}$$

where N_t was the final number of aphids per plant, N_0 was the initial number of aphids introduced and t was the number of elapsed days during the experiment (Walthall and Stark

1997, Stark and Banks 2003). Calculated r_i values for each test plant were subjected to mixed model analysis of variance using Proc Mixed followed by a LSD mean separation test ($\alpha = 0.05$) (SAS 2008). Treatment was a fixed factor, and plant and experimental block were considered random factors in the model.

2.3.6 Dose-Response Modeling

In addition to analysis of variance methods, I used a four-parameter logistic model developed by Cedergreen et al. (2005) to test for hormesis and to assess the dose at which maximal hormetic response occurs. This was done for G0 and G1 fecundity data of the continuous leaf-dip exposure experiment, and the whole plant greenhouse experiment. The following equation was used:

$$y = c + \frac{d - c + f\exp\left(-1/x^{\alpha}\right)}{1 + \exp\left\{b\left[\ln(x) - \ln(e)\right]\right\}}$$

where *d* represents the untreated control; α governs the rate at which the hormetic effect manifests; *c* is the lower limit (0) of the dose-response curve; *b* represents the steepness of the curve after the maximal hormetic effect; *e* provides a lower bound on the ED50 level; and *f* measures the rate of stimulation. Parameter *f* cannot be considered a direct representation of the extent of hormesis, but $f > 0$ suggests presence of hormesis. The statistical test for the presence of hormesis is represented by analyzing if $f > 0$ ($P < 0.1$) (Cedergreen et al. 2005).

Normal distribution and constant variance assumptions on the error terms were verified by examining the residuals of reproductive responses. Data that did not meet these assumptions were square-root transformed ($\sqrt{x + 0.5}$) before fitting to the nonlinear model. The dose-response curve with hormetic term *f* was used and all analyses were done using R statistical

software with an add-on package drc (http://www.bioassay.dk) (Cedergreen et al. 2005). All data were described with models using a lower limit of zero.

2.4 RESULTS

2.4.1 Leaf-dip exposure

Exposure to sublethal concentrations of imidacloprid on leaf discs had a significant effect on *M. persicae* fecundity (Table 2.1). When exposed to treated leaves, the treatment effect was significant in the first three generations but not the fourth, irrespective of whether the treatment exposure was continuous or only for the first two days of the experiment. Except in the foundress generation (G0) of the continuous exposure scenario, there was no significant effect of bioassay replicate in any experiment.

Continuous Exposure. When first instars were continuously exposed to treated leaf discs, significant stimulations in fecundity were noted in G0 and G1 at different concentrations (Table 2.2). However, these stimulations were absent in G2 and G3. In this exposure scenario, peak G0 reproductive stimulation occurred at 0.025 µg L^{-1} and resulted in a doubling of the number of G0 nymphs compared to the controls. In G1 the peak hormetic response shifted to a higher concentration of 0.1 µg L^{-1}. Continuous exposure to 10 µg L^{-1} of imidacloprid resulted in G0 fecundity similar to that seen in the control, reduced fecundity in G1 and G2, and fecundity similar again to that of the control in G3. By the end of the experiment, the total number of progeny produced was significantly greatest in the 0.025 and 0.1 µg L^{-1} treatments, with progeny output in other treatments being equal to or less than that of the control (Table 2.2).

With continuous exposure, treatment had no effect on aphid longevity except in G1 (G0: $F_{7,32} = 1.32$; $P = 0.27$; G1: $F_{7,32} = 2.5$; $P = 0.036$; G2: $F_{7,32} = 1.77$; $P = 0.13$; G3: $F_{7,32} = 0.51$; $P = 0.82$). However, there were strong differences in adult aphid longevity across generations

Table 2.1. *P*-values for a multigenerational (G) experiment examining effects of imidacloprid concentration and experimental replicate (blocking factor) on *Myzus persicae* fecundity under laboratory conditions. Effects requiring further multiple means comparisons are in bold.

Source of Variation	**Generation**				**Total**
	G0	**G1**	**G2**	**G3**	
Continuous leaf-dip exposure					
Concentration	**0.0001**	**0.0001**	**0.0001**	0.5343	**0.0001**
Bioassay replicate	0.0001	0.0547	0.0710	0.3712	0.7853
One-time leaf-dip exposure					
Concentration	**0.0001**	**0.0001**	**0.0001**	0.4129	**0.0001**
Bioassay replicate	0.2950	0.4101	0.4993	0.1082	0.8371
One-time topical exposure					
Concentration	**0.0001**	**0.0003**	-	-	**0.0001**
Bioassay replicate	0.4758	0.5065	-	-	0.6562

($F_{3,128}$ = 17.58; P < 0.0001) (Figure 2.1A). There was no significant treatment-generation interaction on adult longevity ($F_{21,128}$ = 1.51; P = 0.083), but a trend was evident; whereas exposure to concentrations of 0.025 and 0.1 µg L^{-1} tended to increase the longevity of adults above control levels in G0 and G1, longevity seemed to be reduced at these concentrations in G2 and G3 (Figure 2.1A).

With continuous exposure, there were significant treatment effects on the body length of G0 – G2 adults (G0: $F_{7,42}$ = 4.47; P = 0.0009; G1: $F_{7,57}$ = 2.97; P = 0.010; G2: $F_{7,42}$ = 3.75; P = 0.0031) but not G3 adults ($F_{7,42}$ = 0.91; P = 0.51). Adults exposed to 25 µg L^{-1} imidacloprid were shorter than controls, but no significant hormetic/stimulatory effect on adult *M. persicae* length was seen at any concentration or generation. There were also significant differences in body length across generations ($F_{3,162}$ = 2.91; P = 0.036) (Figure 2.1B), but no significant treatment-generation interaction effect on adult body length ($F_{21,162}$ = 0.98; P = 0.49).

Two-Day Exposure. In the experiment where only G0 foundress aphids were exposed to treated leaf discs for two days, aphid fecundity was stimulated at imidacloprid concentrations of 0.25, 1.0 and 10 µg L^{-1} in G0, with 2-3 times more progeny being produced compared to controls (Table 2.3). In G1, only the 0.1 and 1.0 µg L^{-1} treatments had stimulated aphid reproduction, approximately 1.5-fold above that seen in the control. By G2, no imidacloprid treatments were stimulatory, and the 0.25 and 10 µg L^{-1} treatments, which were stimulatory in G0, gave fewer progeny than the control. Unlike the continuous exposure scenario, the lowest concentration used (0.025 µg L^{-1}) resulted in 3-fold lower nymph production than controls in the first generation, and gave the lowest overall fecundity (Table 2.3). By the end of the experiment, only the 1.0 µg L^{-1} treatment produced more aphids than the control (Table 2.3). G0 nymphs exposed to leaf discs treated with 25 µg L^{-1} imidacloprid did not survive to adulthood.

2.4.2 Topical Exposure

Topical exposure to imidacloprid had a significant effect on *M. persicae* reproduction, but there was no difference among bioassay replicates (Table 2.1). A significant increase in reproduction over the control was found at 0.6 µg L^{-1} in G0, but fecundity for all other treatments and time points was equal to or below that of the control (Table 2.4).

2.4.3 Exposure to Systemically Treated Plants

The concentrations of imidacloprid applied to soil had a significant effect on r_i ($F_{7,62} = 6.59$; $P < 0.0001$) and total number of *M. persicae* per plant ($F_{7,62} = 17.28$; $P < 0.0001$). The difference among experimental blocks was significant for r_i ($F_{2,62} = 3.27$; $P = 0.045$), but there was no block effect for total number of aphids per plant ($F_{2,62} = 1.95$; $P = 0.15$). Only the 0.25 µg L^{-1} treatment resulted in a r_i significantly greater than the control, although both the 0.25 and 2.5 µg L^{-1} treatments resulted in significantly more total aphids per plant after 21 days. The 1.0 µg L^{-1} treatment had a significantly lower r_i and fewer total aphids per plant than the control (Table 2.5).

In all experiments, depending on the exposure scenario, we found that fecundity outputs at concentrations just below or above the hormetic peak concentration, were significantly below those in the control, but increased again at higher concentrations to levels equal to or exceeding the control (Tables 2.2-2.5). I found these effects to be highly reproducible in my experiments.

2.4.4 Dose-Response Modeling

When *c* was set to zero and α set at 0.25, *f* was significantly different from zero for fecundity of G0 ($P < 0.001$) and G1 ($P = 0.0637$) *M. persicae* adults exposed continuously to sublethal concentrations of imidacloprid, and for r_i ($P = 0.0003$) with exposure to potato plants treated with sublethal concentrations of imidacloprid (Table 2.6). The model found that maximum

Table 2.2. Least-squares means of multigenerational (G0, G1, G2 and G3)[a] fecundity following continuous exposure of *Myzus persicae* to sublethal concentrations of imidacloprid.

Concentration (μg L^{-1})	**Generation[b]**				**Total**
	G0	**G1**	**G2**	**G3**	
0	4.32 bc	4.03 cd	4.01 a	4.43 a	94.52 b
0.025	8.55 a	6.54 ab	1.22 bc	2.27 ab	117.45 a
0.1	5.64 b	8.33 a	1.54 b	2.83 ab	122.14 a
0.25	3.32 cd	2.63 de	1.27 bc	2.53 ab	77.72 bc
1.0	4.10 bc	2.55 e	3.86 a	2.23 ab	90.39 b
2.5	2.82 d	5.30 bc	0.76 cd	3.19 ab	90.90 b
10	3.86 cd	1.82 ef	0.50 d	2.31 ab	63.03 c
25	0.63 e	1.10 f	0.63 d	1.99 b	32.45 d
SEM	0.09	0.12	0.11	0.21	6.68

[a] 24 h old nymphs were placed on treated potato leaf discs and fecundity of each resulting adult was recorded every 2 days until it died. In succeeding generation, 5 randomly selected 24 h old nymphs were tracked and fecundity of the resulting adult was recorded every 2 days until it died. G0 is initial generation, G1 is progeny of G0, G2 is progeny of G1, and G3 is progeny of G2. Leaf discs were replaced every two days over all generations.

[b] Progeny per adult were log transformed before analysis. Backtransformed means are presented. Values followed by different letters are significantly different (LSD, $\alpha = 0.05$). SEM values are not backtransformed.

Table 2.3. Least-squares means of multigenerational (G0, G1, G2 and G3)[a] fecundity following two-day exposure of *Myzus persicae* to sublethal concentrations of imidacloprid.

Concentration (µg L^{-1})	**Generation[b]**				**Total**
	G0	**G1**	**G2**	**G3**	
0	3.68 de	3.05 b	2.99 a	2.60 a	61.15 b
0.025	1.13 f	1.10 c	1.57 b	1.90 a	38.13 c
0.1	4.54 cd	5.07 a	3.09 a	1.83 a	68.17 b
0.25	6.22 bc	3.52 ab	1.17 b	1.78 a	59.98 b
1.0	8.61 ab	5.30 a	2.78 a	2.44 a	86.88 a
2.5	2.55 e	1.20 c	1.16 b	2.31 a	41.56 c
10[c]	10.52 a	2.56 b	1.43 b	1.61 a	72.89 ab
SEM	0.11	0.13	0.13	0.12	5.33

[a] 24 h old nymphs were placed on treated potato leaf discs and fecundity of each resulting adult was recorded every 2 days until it died. In succeeding generation, 5 randomly selected 24 h old nymphs were tracked and fecundity of the resulting adult was recorded every 2 days until it died. G0 is initial generation, G1 is progeny of G0, G2 is progeny of G1, and G3 is progeny of G2. G0 nymphs were exposed to treated discs for two days and all aphids were thereafter exposed to untreated leaf discs.

[b] Progeny per adult were log transformed before analysis. Backtransformed means are presented. Values followed by different letters are significantly different (LSD, $\alpha = 0.05$). SEM values are not backtransformed.

[c] G0 nymphs did not survive to adulthood when treated with 25 µg L^{-1}. This concentration was not included in the analysis.

Table 2.4. Least-squares means of two-generational (G0 and G1)[a] fecundity following topical exposure of *Myzus persicae* to sublethal concentrations of imidacloprid.

Concentration (µg L^{-1})	**Generation[b]**		**Total**
	G0	**G1**	
0	3.65 b	3.37 a	35.02 ab
0.2	1.14 e	1.01 c	12.72 d
0.6	7.69 a	1.09 cd	41.86 a
2	2.01 cde	2.39 ab	23.64 c
6	1.85 de	1.51 bcd	19.71 cd
20	1.41 de	2.36 ab	23.72 c
60	2.40 bcd	2.77 a	27.96 bc
200	3.21 bc	2.04 abc	29.08 bc
SEM	0.13	0.14	3.49

[a] 24 h old nymphs were topically treated and thereafter reared on untreated potato leaf discs. Fecundity of each resulting adult was recorded every 2 days until it died. In the succeeding generation, 5 randomly selected 24 h old nymphs were tracked and fecundity of the resulting adult was recorded every 2 days until it died. G0 is initial generation, G1 is progeny of G0.

[b] Progeny per adult were log transformed before analysis. Backtransformed means are presented. Values followed by different letters are significantly different (LSD, $\alpha = 0.05$). SEM values are not backtransformed.

Table 2.5. Least-squares means of instantaneous rate of increase (r_i) and total number of aphids after 21 days following infestation of *Myzus persicae* on to potato plants treated with sublethal concentrations of imidacloprid in a greenhouse.

Concentration (µg L^{-1})	r_i	**Total**[a]
0	0.094 bcd	38.53 cd
0.025	0.101 bcd	42.27 bcd
0.1	0.062 de	21.96 de
0.25	0.167 a	173.34 a
1.0	0.034 e	8.93 e
2.5	0.121 ab	72.28 b
10	0.112 bc	54.81 bc
25	0.077 cde	43.30 bcd
SEM	0.016	0.72

[a] Data were square root transformed before analysis. Backtransformed means are presented. SEM values are not backtransformed.

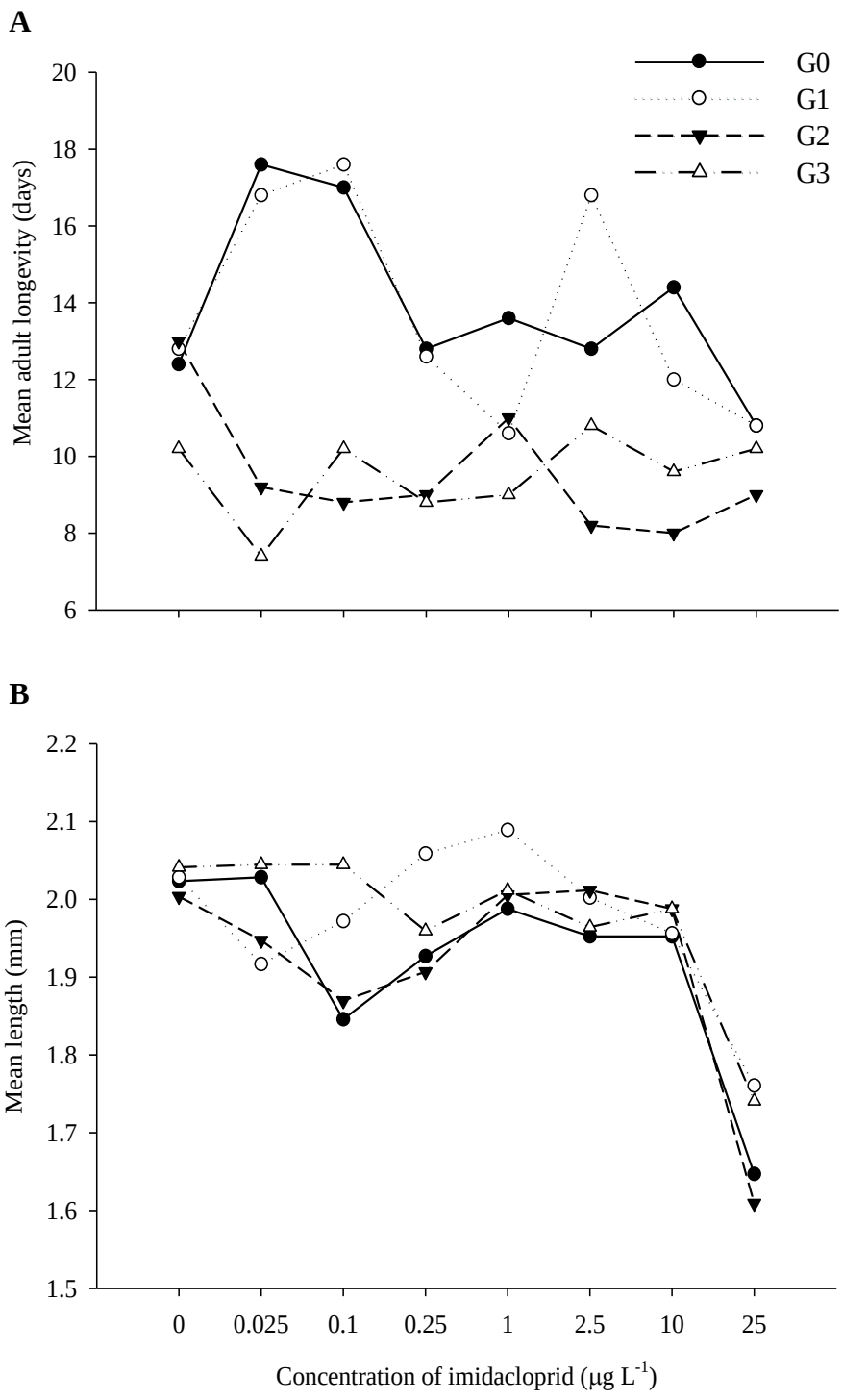

Figure 2.1. Multigenerational effects of low doses of imidacloprid on aphids

Multigenerational effects of continuous exposure to sublethal concentration of imidacloprid on the (A) longevity and (B) length of adult *M. persicae.*

stimulation (9.42 nymphs per adult) was obtained at 0.08 µg imidacloprid L^{-1} in G0, and at 0.18 µg imidacloprid L^{-1} (8.05 nymphs per adult) in G1 of aphids. The maximum r_i of 0.123 was obtained at 1.44 µg imidacloprid L^{-1} for *M. persicae* exposed on whole potato plants (Figure 2.2).

2.5 DISCUSSION

One of the key questions for scientists that study hormesis within the context of environmental toxicology is, what is the consequence of the hormetic response on biological fitness (Forbes 2000, Parsons 2000, 2001, Costantini et al. 2010, Jager et al. 2012)? Using the aphid *M. persicae* and insecticide imidacloprid as a model, I implemented various exposure scenarios over multiple generations as a unique approach to examine the temporal nature and biological consequences of the hormetic dose-response. When first instar *M. persicae* were continuously exposed to sublethal concentrations of imidacloprid on leaf discs for four generations, fecundity doubled in the first two generations at certain concentrations, with a shift to a higher peak hormetic concentration from the first to second generation. This was countered by significant reductions in fecundity at the same concentrations in third generation adults, and recovery to fecundity outputs equal to that of controls in the fourth generation. This demonstrates that tradeoffs in resource allocation occurred (Forbes 2000, Zalizniak and Nugegoda 2006, Guo et al. 2012, Jager et al. 2012).

In an attempt to identify potential intra-generational tradeoffs, aphid length and longevity were recorded for one experimental block in the continuous exposure scenario. No inhibition of longevity or length was found at the hormetic concentrations in the first two generations, and there was a trend (not significant) towards stimulation of longevity. Tradeoffs might have

Table 2.6. Regression parameters of model-fitting hormetic responses (G0, G1 fecundity and r_i) in *Myzus persicae* exposed to sublethal concentrations of imidacloprid.

Generation	Parameter[a]	Estimate	SE	*t*-value	*P*
G0[b]	*B*	0.483	0.031	15.754	0.0000
	D	7.721	0.784	9.850	0.0000
	E	0.003	0.001	3.852	0.0001
	F	327.710	63.221	5.184	0.0000
	RSE	6.064			
	Df	476			
G1[bc]	*B*	0.424	0.037	11.451	0.0000
	D	2.692	0.194	13.902	0.0000
	E	0.421	0.514	0.818	0.4140
	F	10.553	5.677	1.859	0.0637
	RSE	1.643			
	Df	476			
r_i[cd]	*B*	0.238	0.033	7.286	0.0000
	D	0.782	0.017	45.954	0.0000
	E	672.740	572.848	1.174	0.2466
	F	0.475	0.121	3.934	0.0003
	RSE	0.043			
	Df	44			

[a] *b*, steepness of the curve after the maximal hormetic effect; *d*, untreated control; *e*, lower bound on the ED50 level; *f*, rate of stimulation; RSE, residual standard error; *df*, degrees of freedom (Cedergreen et al. 2005).

[b] denotes fecundity of *M. persicae* adults continuously exposed to sublethal concentrations of imidacloprid.

[c] data were square-root transformed before analysis.

[d] r_i is the instantaneous rate of increase of a *M. persicae* population exposed to low-dose imidacloprid treated potato plants.

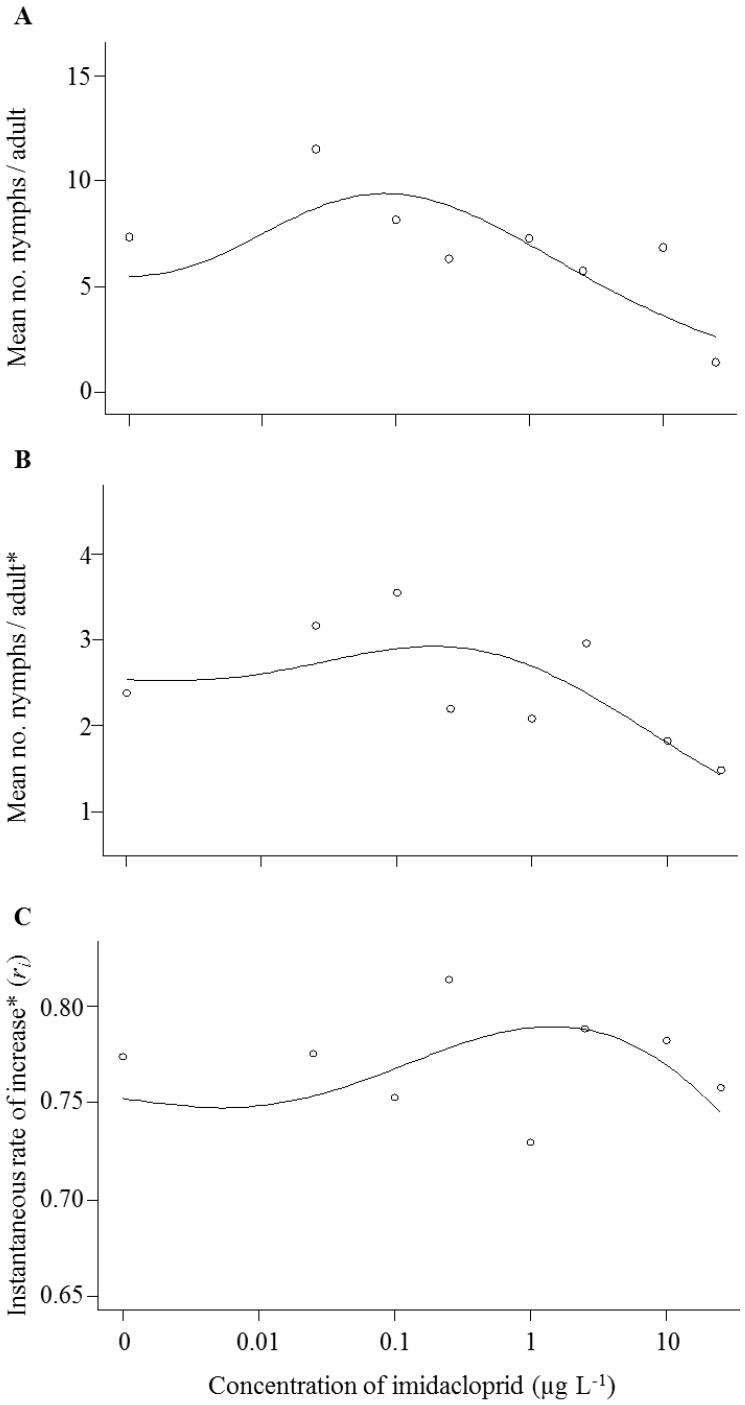

Figure 2.2. Hormesis model-fitting of low doses of imidacloprid on fecundity and r_i of aphids

Four-parameter biphasic model (Cedergreen et al. 2005) for reproductive hormetic responses of *M. persicae* in an initial (A) and second (B) generation when continuously exposed to sublethal concentrations of imidacloprid on potato leaf discs, and (C) the instantaneous rate of increase (r_i) of *M. persicae* populations developing on whole potato plants treated with sublethal concentrations of imidacloprid. * indicates data were square-root transformed before analysis.

occurred in these initial generations through other phenotypic or physiological traits not measured. Concurrent hormetic responses of multiple traits without obvious biological tradeoffs has similarly been reported in eucalyptus plants exposed to low dose glyphosate (Velini et al. 2008), and *M. persicae* exposed to low concentrations of imidacloprid (Cutler et al. 2009). However, I did observe differences among generations with these endpoints, particularly with a tendency towards reduced longevity in generations three and four. This likely reflects a fitness tradeoff experienced due to increased reproductive outputs (hormesis) and energy expenditures in early generations (Forbes 2000, Jager et al. 2012).

Despite significantly reduced reproductive outputs and tradeoffs at hormetic concentrations, the total number of aphids at the end of the continuous exposure experiment was significantly greater than that of controls. Total reproductive outputs equal to or exceeding control levels were found at hormetic concentrations in my other experiments as well. This was especially the case in the greenhouse experiment where treatment of plants with 0.25 µg imidacloprid L^{-1} resulted in a significant increase in r_i and 4.5-fold more total aphids than in controls. Previous greenhouse experiments involving sublethal concentrations of imidacloprid and *M. persicae* that did not detect hormesis (Janmaat et al. 2011) probably used inappropriate concentrations to detect the effect.

These results suggest there was no long-term fitness cost for the stimulatory response in early generations, supporting one of my hypotheses and results of other multigenerational studies with invertebrates exposed to sublethal amounts of stress (Schaible et al. 2011, Suhett et al. 2011, Campos et al. 2012). Although there may be negative energetic consequences (tradeoffs) associated with a hormetic response, energy intake could be slightly increased when an organism is exposed to low levels of a stressor (i.e. in the 'hormetic zone') to optimize tradeoffs between

self-maintenance and other activities, such as reproduction (Costantini et al. 2010). The hormetic response might confer a new/adapted normal state that in essence primes or conditions the organism to better cope with higher levels of the stressor when encountered on subsequent occasions (Costantini et al. 2010, Schaible et al. 2011, Suhett et al. 2011). I speculate that with continued exposure to low levels of the stressor, late generation aphids could better survive imidacloprid exposure than unexposed individuals. This could manifest through induced up-regulation at hormetic concentrations of detoxification enzymes such as esterases (Mukherjee et al. 1993) or developmental enzymes and proteins (Smirnoff 1983). Ultimately, hormetic responses to stress in insects could be a precursor for insecticide resistance development as stress is a general enhancer of mutation rates, which might include mutations leading to pesticide resistance (Gressel 2011).

Dose and temporal patterns of hormesis were somewhat different when founding nymphs were exposed to imidacloprid on leaf discs for only 2 days. At the lowest concentration of 0.025 µg L^{-1}, intra-generational and overall reproduction was well below that of the control. Low reproduction at the lowest concentration was also seen in the topical exposure and greenhouse experiments, and I had previously observed this in continuous exposure leaf-dip experiments (M-M.A. unpublished data). High first generation fecundity at 0.025 µg L^{-1} under continuous exposure resulted in a doubling in reproductive output when insects reached adulthood. In contrast, with the shorter 2-day or topical exposure, by the time 0.025 µg L^{-1} treated nymphs reached adulthood there was no longer any exposure to the insecticide and the hormetic response to the stressor was complete. Thereafter, the insects entered the tradeoff phase of the response, represented by lower reproductive output. This supports my prediction that short-term exposure

to the stressor would result in short-term hormesis expressed at higher concentrations relative to that seen with continuous exposure to the stressor.

Hormetic responses occurred at several concentrations in aphids of the first two generations following the 2-day exposure, at concentrations higher than in the continuous exposure, again reflecting what I believe is the insect's ability to better tolerate higher concentrations when exposed for shorter periods. The occurrence of pre-hormetic toxicity (reduced fecundity) that I observed with short-term exposure has previously been observed in insects (Luckey 1968) and in plants (Belz and Piepho 2012). In my experiments, at the lowest imidacloprid concentration, nymphs might have been able to allocate an adequate amount of resource towards coping with the stress before reaching adulthood. If this were the case, by the time these nymphs reached adulthood, they would have been in the tradeoff phase of the response, observed as lowered reproduction in adults. This was not seen in the continuous exposure scenario because aphids were coping with the stressor right up to adulthood, and the tradeoff was not observed until subsequent generations following hormesis. With short-term exposure at higher concentrations, nymphs required more resources and time to cope with the stress, resulting in hormesis and higher reproduction in first generation adults. Low-dose stimulatory effects seen as hormesis are likely not the only toxicological phenomenon occurring in the low dose range (Sinkkonen et al. 2011, Belz and Piepho 2012, Cutler 2013).

The four-parameter logistic model developed by Cedergreen et al. (2005) detected reproductive hormesis in first and second generation aphids exposed continuously to imidacloprid on leaf discs, and in r_i from my greenhouse experiment, corroborating my analysis of variance and population growth analyses. The predicted hormetic peak was slightly lower than that found in my experiments. This is probably partially because the model requires 4-5

hormetic doses to adequately describe the hormetic response (Cedergreen et al. 2005), whereas my data allowed for only 1-2 hormetic doses. Although the model provides a significant fit for hormesis, I feel it masks certain results in some of the data. Most importantly, the current model does not adequately address the pre-hormetic toxicity that I characterized as "dips" in the dose-response, a limitation that has previously been noted in statistical models that describe hormesis (Belz and Piepho 2012). I consistently found pre-hormetic toxicity in several of my experiments. The occurrence and mechanisms of this phenomenon should be explored further, along with refinement of models that better take into account such pre-hormetic toxicity.

In conclusion, the hormetic response in *M. persicae* exposed to low doses of imidacloprid was robust and highly reproducible. However, intra- and transgenerational reproductive responses differed depending on the exposure scenario. Despite tradeoffs in transgenerational reproduction, this did not adversely affect total reproductive output after four generations, suggesting that overall fitness was not adversely affected. In some situations fitness tradeoffs due to hormesis may render the phenomenon evolutionary neutral (Forbes 2000, Jager et al. 2012), and my results show that hormetic response need not come at the expense of biological fitness. Indeed, hormesis is likely a critical adaptation for organisms that allows them to adjust to fluctuations in their environment, possibly acting as a 'conditioning' mechanism that enables the organism to better cope with subsequent exposure to higher levels of the stressor (Calabrese et al. 2007). Costantini et al. (2010) suggested that in certain situations such conditioning could increase biological fitness, even if the stressor was not encountered again, or encountered at low levels. As I predicted, this would seem particularly important for *r*-selected species that are specialized for high and rapid reproduction in unstable and unpredictable environments. Insect

pests that are targets of frequent pesticide applications in agroecosystems clearly represent such a scenario.

3 GENE REGULATION DURING IMDACLOPRID-INDUCED HORMESIS

3.1 ABSTRACT

Imidacloprid-induced hormetic stimulation in *Myzus persicae* fecundity was noted in the first two generations of a continuous exposure scenario. However, the molecular foundation of this phenotypic response, gene expression, was seldom addressed in insects. Here, stress response (*Hsp60*), dispersal (*OSD*, *TOL* and *ANT*) and developmental (*FPPS 1*) genes were examined over four time points (G0 nymph, G0 adult, G1 nymph and G1 adult stages) in order to correlate with the previously noted phenotypic response, fecundity. Also, using global DNA methylation the hypothesis that gene regulation during imidacloprid-induced hormesis is inherited to succeeding generations was tested. My results showed that the gene regulation mirrored the fecundity data in several instances that was found to be inherited to succeeding generations probably via hypermethylation, silencing of genes. Interestingly, dispersal related genes that are predominantly expressed in alates (about 2- to 5-fold) were up- or down-regulated when apterous aphids (about 2- to 10-fold) were exposed to sublethal concentrations of imidacloprid. Alternation of up- and down-gene regulation in successive life stages suggest trade-offs occurring during chemical hormesis which was not evident at individual level. Although no direct relation with the previously noted fecundity was established, gene regulation for several genes had tight hormetic dose-response. This suggests that adaptive cellular stress response pathways might be triggered rather than normal regulatory processes due to low-dose imidacloprid exposure.

3.2 INTRODUCTION

Hormesis is low-dose stimulation coupled with high dose inhibition of a stressor-induced response, and is an alternative to the threshold model in toxicology. Insecticide-induced hormesis is widespread across many biological groups and has been studied in medical, evolutionary and ecological contexts (Calabrese and Baldwin 2003). The biochemical and molecular underpinnings of hormetic responses have been studied using several biological models (Calabrese and Baldwin 2003) but only seldom using insect-insecticide models. A hormetic response in the form of stimulated total calcium and proteins was seen in *Choristoneura fumiferana* (Clemens) exposed to sublethal doses of organophosphorous and carbamate insecticides (Smirnoff 1983). It was suggested that basic metabolites such as sugars, lipids and total proteins can be measured in addition to organismal and population endpoints to give clues into how metabolism in the animal changes during hormesis (Smirnoff 1983). Larval weight gain and changes in isoesterases profiles were seen in *Tribolium castaneum* L., exposed to low concentrations of azadirachtin that induced hormesis (Mukherjee et al. 1993). Juvenile hormone III (JH III) titers were correlated with stimulated fecundity in green peach aphid, *Myzus persicae* (Sulzer), when exposed to low doses of imidacloprid (Yu et al. 2010). Transcriptional responses of gossypol-induced hormesis were suggested to be a specific transcriptional adaptation rather than general stress response (Celorio-Mancera et al. 2011).

Regulation of gene expression is implicit in the hormetic adaptive responses (Son et al. 2010), activating the adaptive cellular stress response pathways assisted by transcriptional factors such as antioxidant response element (ARE), forkhead box O (FoxO), heat-shock factor (HSF) and nuclear factor – κB (NF-κB). The HSF pathway can be triggered by elevated temperatures, heavy metals, infection, and chemical toxicants (Son et al. 2010). In agriculture, insecticides are

used to kill insect pests, but these insecticides can initiate the HSF pathway in survivors for maintenance in response to the toxicant (Yoshimi et al. 2002). Other abiotic stressors that disrupt homeostasis can also initiate the HSF pathway. For example, *Chironomus tentans* Fabricius (Karouna-Renier and Zehr 1999), *Tribolium castaneum* L. (Mahroof et al. 2005), *Cydia pomonella* (L.) (Yin et al. 2006) and *Liriomyza huidobrensis* Blanchard (Huang et al. 2007) accumulated heat shock proteins (Hsp) following heat stress, resulting in thermotolerance. Nutrition and heat stress induced higher transcription of Hsp70 than heat stress alone (Salvucci et al. 2000). Hsp in *M. persicae* are essential for survival and other biological processes, such as detoxification of xenobiotics (Figueroa et al. 2007, Ramsey et al. 2007).

M. persicae exhibit dimorphism as alate (winged) and apterous (wingless) forms (Dixon 1998, Braendle et al. 2006). The switch to the alate form in *M. persicae* seems to be triggered by several mechanisms in response to sub-optimal environmental conditions such as high density and poor nutrition (Braendle et al. 2006). Alate production is accompanied by up-regulation of olfactory segment D (*OSD*), take-out like / juvenile hormone (JH) binding protein, and mitochondrial adenine nucleotide translocase (*ANT*) (Ghanim et al. 2006). The role of JH in aphid wing dimorphism is still uncertain (Braendle et al. 2006, Schwartzberg et al. 2008), but is probably governed by environmental and genetic stimuli (Brisson 2010). Imidacloprid-induced reproductive stimulation has been associated with wing dimorphisms in *M. persicae* (Wang et al. 2008). JH biosynthesis (mevalonate pathway) includes a precursor farnesyl diphosphate synthase I (*FPPS I*) gene (Zhang and Li 2008) and its expression may regulate reproduction in insects (Cusson et al. 2006).

When first instar *M. persicae* were continuously exposed to sublethal concentrations of imidacloprid, stimulated fecundity of first and second generation adults was observed (Chapter

2). To understand the molecular underpinnings of this phenotypic response, gene expression in *M. persicae* exposed to hormetic concentrations of imidacloprid for two generations was examined in the present study. Stress response (*Hsp60*) (Stanley and Fenton 2000), dispersal (*OSD*, *TOL*, and *ANT*) and developmental (*FPPS 1*) genes were examined. Based on the *principle to resource allocation*, invested energy into homeostatic maintenance combating low-dose stress could be expended as increased fecundity, I hypothesized that *OSD*, *TOL*, and *ANT* gene expression would correspond to fecundity responses noted in Chapter 2, up-regulated at an initial time point (G0 second instars), but was uncertain of the changes in gene expression during the insect development and over remaining time points (G0 adults and G1 nymphs and adults). I hypothesized that *Hsp60* gene expression would be up-regulated in G0 second instars and alternate between life traits, if the insect adapts to the stressor. I further hypothesized that *FPPS I* gene regulation would be down-regulated during adult exposure corresponding to fecundity responses (Chapter 2) but was uncertain of changes in gene expression during nymphal stages.

In addition, insecticide-induced hormesis in insects, probably an epigenetic process, resulting in heritable changes in gene expression has received meager attention. As DNA methylation is an important epigenetic mechanism in insects that may provide critical contributions to insect developmental and phenotypic variation (Glastad et al. 2011), besides testing its low-dose insecticide adaptive heritability, global DNA methylation was measured in test aphids. I hypothesized hypermethylation at time points where the *Hsp60*, *FPPS I*, *OSD*, *TOL* and *ANT* gene regulations were up-regulating.

3.3 METHODS AND MATERIALS

3.3.1 Plants and insects

Potato, *Solanum tuberosum* L. (cultivar Kennebec), was grown in 12.5 cm diameter pots containing Pro-Mix® (Halifax Seed, Halifax, Nova Scotia) potting soil. Plants were watered as needed. Foliage from these plants was used for rearing and experimental purposes. *M. persicae* was obtained from a wild population infesting broccoli plants (*Brassica oleracea* L) in a greenhouse at the Faculty of Agriculture, Dalhousie University. Aphid cohorts were maintained on excised leaves in clear plastic boxes (37 x 24 x 14 cm high) lined with moistened paper towels. Boxes were held in a growth chamber (22 ± 2^{o} C, 16:8 L:D, 65 ± 5 % RH) and every second day a layer of freshly excised leaves was placed on one end of the box. The infested foliage on the opposite end was discarded when about 80% aphids moved to fresh foliage. Paper towels were replaced every 10 days.

3.3.2 Chemicals

Imidacloprid (Admire® 240 SC, 240 g a.i. L^{-1}; Bayer Crop Science Canada, AB, Canada) was suspended in deionized water to obtain a 1000 µg a.i. L^{-1} stock solution. The working solutions contained 0.15% Triton™ X 100 (BDH Chemicals, ON, Canada) as emulsifier. Insecticide concentrations of 0.025, 0.1, 0.25, 2.5 and 10 µg a.i. L^{-1} were used in the experiments. Controls in these experiments consisted of 0.15% Triton in water.

3.3.3 Leaf-dip exposure

Potato leaf discs (1.8 cm diameter) were excised using a stainless steel cork borer. Using forceps, leaf discs were dipped in control or insecticide solutions for 5 sec, air-dried for 1 h, and then placed individually in 5.5 cm Petri plates lined with a Whatman No. 1 filter paper. In order

to avoid cross contamination, controls were treated first followed by sequential treatment of lowest to highest concentrations of insecticide. Ten first instar *M. persicae* (ca. 24 h old) were transferred to each treated leaf disc. Dishes were covered with a Petri plate cover, placed in sealable plastic containers and held in growth chamber as described above.

The aphids were exposed to and reared continuously on treated leaf discs for two generations (G0, G1). The experiment was a completely randomized design, with imidacloprid concentration being the main factor of interest. Each container represented a biological replicate of a concentration and had five Petri dishes with ten aphids each. For gene expression analyses, five aphids from each Petri plate were randomly collected at day 4, 9, 13 and 17 (representing G0 second instar, G0 adult, G1 second instar and G1 adult stages, respectively). A total of three biological replicates were set up in the experiments. For DNA methylation experiments, the same experimental procedures and design were used.

3.3.4 Sample preparation

3.3.4.1 Gene expression analyses

Collected aphids were flash frozen in liquid nitrogen. Total RNA was isolated using RNeasy® mini kit (Qiagen, ON, Canada). Quality ($A_{260/280} > 2.0$) and quantity of total RNA was assessed with a Nanodrop ND-1000 (NanoDrop Technologies, DE, USA) and gel electrophoresis (rRNA band intensity: 28s = 2X 18s). Later, cDNA was synthesized from 1 µg of total RNA using a QuantiTect® Reverse Transcription kit (Qiagen, ON, Canada) and stored at -20^{o} C until further analyses. The primers (Sigma-Aldrich, ON, Canada) used for quantitative Real-Time (qRT) PCR are listed in Table 3.1. Internal controls with cycle of threshold (ct) values closer to ct of selected genes were chosen to calculate expression fold-change using ΔΔct method (Pfaffl 2001). For quantification of *OSD* and *TOL* genes, the *β-actin* gene was used as an internal

control. *Ace* was used as an internal control for *ANT*, *Hsp60* and *FPPS I* genes. qRT PCR was performed on StepOne™ RT PCR System (Applied Biosystems, ON, Canada) in a 10 µL reaction following the manufacturer's instructions using SYBR green reagent (Applied Biosystems, ON, Canada). The reaction mixture contained 2X SYBR green reagent master mix, 2 µL cDNA, 2.5 µL ultrapure water (Agriculture Campus, Dalhousie University, Truro, Canada) and 0.25 µL each of forward and reverse primers (final concentration of 2.5 mM). Data were analyzed from three independent runs using MIXED model analysis of variance (SAS 2008) and if means were significantly different, they were separated using a LSD test ($\alpha = 0.05$). Relative quantification (RQ) of gene regulation above or below that of controls is reported.

3.3.4.2 Global DNA methylation analysis

Samples were collected as described above, and genomic DNA was isolated using a DNeasy® Blood and Tissue Kit (Qiagen, Toronto, Canada). From aphids of each concentration and time point, 500 ng of digested, using Nuclease P (Sigma-Aldrich, ON, Canada), and phosphorylated, using Alkaline Phosphatase (Sigma-Aldrich, ON, Canada), gDNA was used to examine global DNA methylation changes using DNA Methylation EIA™ kit (Cayman Chemical, MI, USA). A standard curve with $r^2 > 0.9$ was obtained using kit standard and the data analysis was performed as per the manufacturer instructions after calculating the amount of methylated DNA based on the obtained equation from the standard curve. Later, relative methylation of DNA to controls was analyzed using MIXED model analysis of variance (SAS 2008) and if means were significantly different, they were separated using a LSD test ($\alpha = 0.05$).

3.4 RESULTS

3.4.1 Gene expression analyses

Continuous exposure to sublethal concentrations of imidacloprid resulted in up- and down-regulation of the genes analyzed, with intra- and inter-generational differences observed (Table 3.2). Gene expression in treatments is reported as relative quantity (RQ) to that in control aphids and a 2-fold up- or down- regulation was considered as biologically significant.

Hsp60 gene. In the first generation (G0), there was a 2.5-fold down-regulation of *Hsp60* in second instars exposed to 0.25 µg a.i. L^{-1}. In G0 adults, 2.0-fold down-regulation was found in the 0.025 µg a.i. L^{-1} treatment, and minor down-regulation at higher concentrations (Figure 3.1). In G1, there was 3.2-, 2.5- and 5.3-fold up-regulation of the *Hsp60* gene in second instars at 0.25, 2.5 and 10 µg a.i. L^{-1}, respectively. Two- to 3.3-fold down-regulation of the gene was observed at all concentrations in G1 adults.

FPPS I gene. About 2.0-fold down regulation of the *FPPS* I gene was observed in G0 second instars exposed to 0.25 and 2.5 µg a.i. L^{-1} imidacloprid (Figure 3.2), and in G0 adults from the 0.025 µg a.i. L^{-1} treatment. At other imidacloprid concentrations changes in *FPPS* I gene expression was relatively minor in G0. G1 second instars from the 0.025, 2.5 and 10 µg a.i. L^{-1} treatments had 2.2-, 2.7 and 5.0-fold up-regulation, respectively, of the *FPPS* I gene. In G1 adults, ca. 2.0- to 5.0-fold down-regulation was observed across the range of imidacloprid treatments.

OSD gene. When G0 first instar *M. persicae* were exposed to 0.1 µg imidacloprid L^{-1}, 2.3 fold up-regulation of the *OSD* gene was observed in G0 second instars, and at 0.25 µg a.i. L^{-1} ca. 2.0-fold down-regulation was observed (Figure 3.3). Exposure of G0 nymphs to other

Table 3.1 Primer sequences (5`→3`) for selected and internal control genes used to measure gene expression in *Myzus persicae* exposed to sublethal concentrations of imidacloprid.

Symbol	**Forward sequence**	**Reverse sequence**	**Accession# / reference**	**size (bp)**
ANT	GCCGGTAATTTAGCATCAGG	CCTTGGACAAACAGTCTCCA	DQ407505	151
OSD	TCCCGAAGGAGCTGAACTTA	GCTTAGGGTCCCATTTGTCA	AJ634652	164
TOL	AGCGCTTTCTGACGGAAATA	AGCATTCGAAGAAGCGATTG	EB714328	177
FPPS I	CGAACAGGCCATTTACCAGT	GACCCATCGCAGTTTTCATT	EU334430	107
Hsp60	AGCATTGACCATGCCATGTA	AAACATCGGTCATTGCATCA	AJ250348	122
β-actin	GGTGTCTCACACACAGTGCC	CGGCGGTGGTGGTGAAGCTG	(Puinean et al. 2010)	90-120
Ace	TAACGTAGTAGTGCCAAAGC	CACTGTAGAGCCATTAGCTG	(Puinean et al. 2010)	90-120

Table 3.2. *P*-values for a two generation (G) experiment examining gene regulation and global DNA methylation during imidacloprid-induced hormesis in second instar (N) and adult (A) *Myzus persicae*.

Source of Variation	**Generation-time point**			
	G0-N	**G0-A**	**G1-N**	**G1-A**
Hsp60	0.0001	0.0001	0.0001	0.0001
FPPS I	0.0004	0.0001	0.0023	0.0001
OSD	0.0035	0.0001	0.0001	0.0001
TOL	0.0001	0.0001	0.0001	0.0001
ANT	0.0469	0.0002	0.0001	0.0001
Global DNA methylation	0.0002	0.0001	0.0040	0.0131

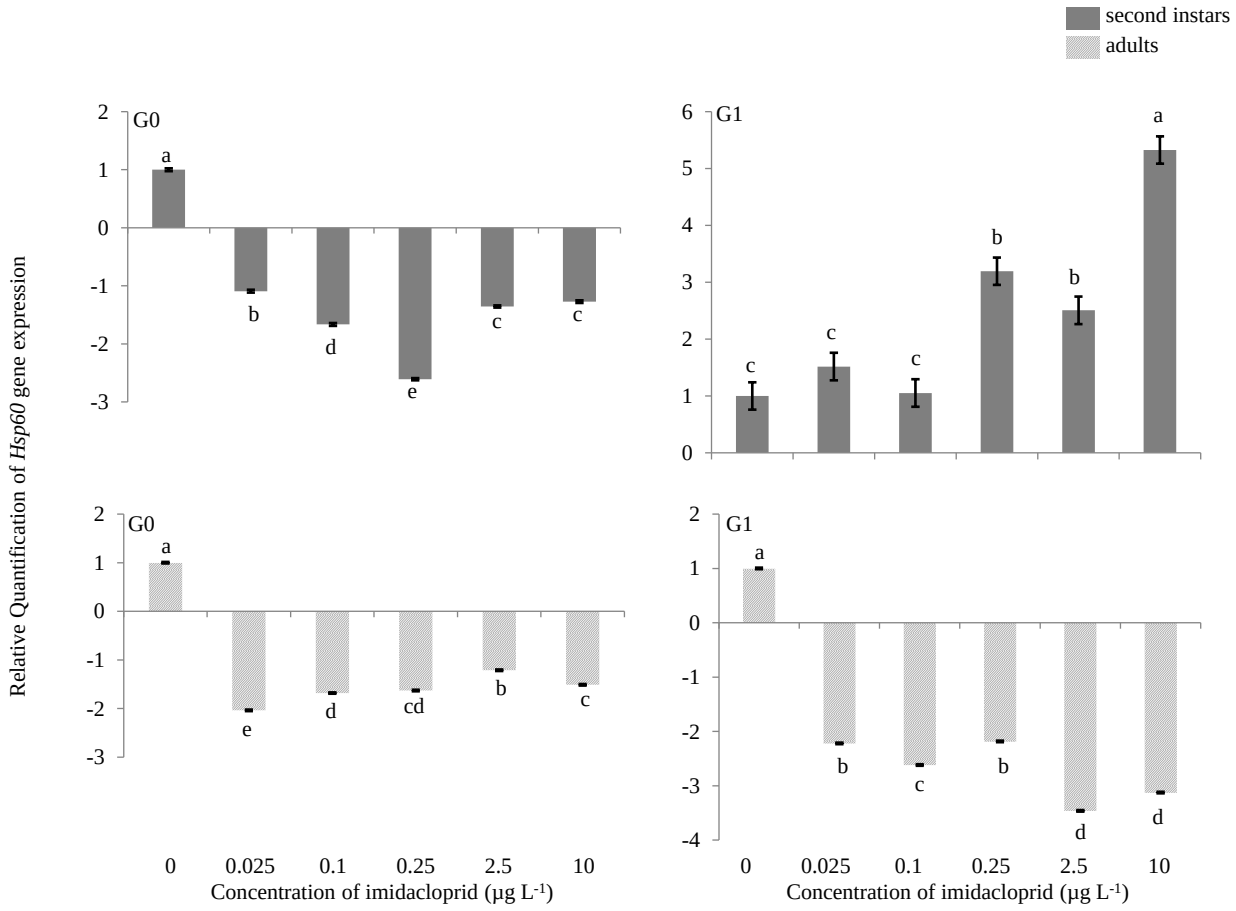

Figure 3.1. Heat shock protein (Hsp) 60 gene regulation during imidacloprid-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing second instars and adults from initial (G0) and succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

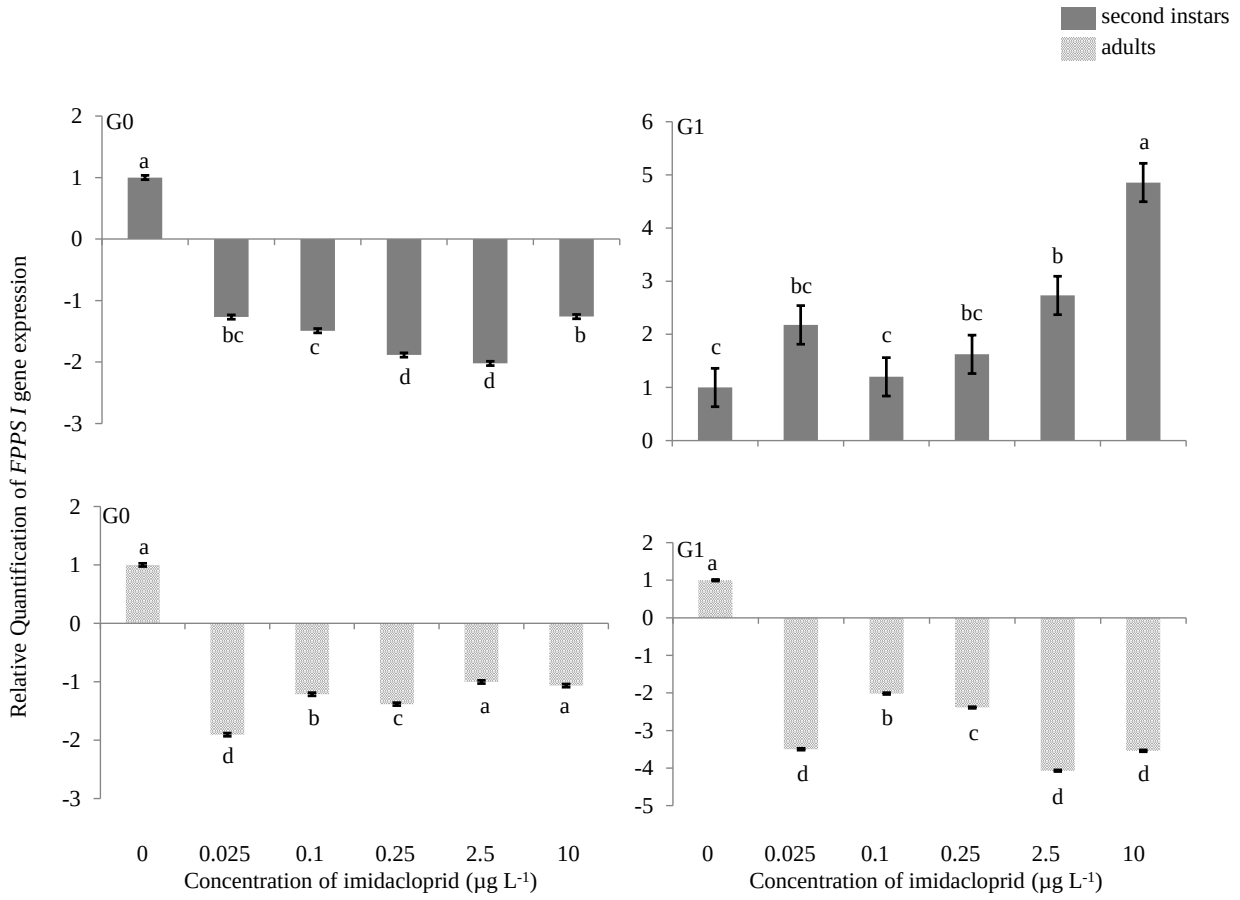

Figure 3.2. Farnesyl diphosphate synthase (*FPPS*) I gene regulation during imidacloprid-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing second instars and adults from initial (G0) and succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

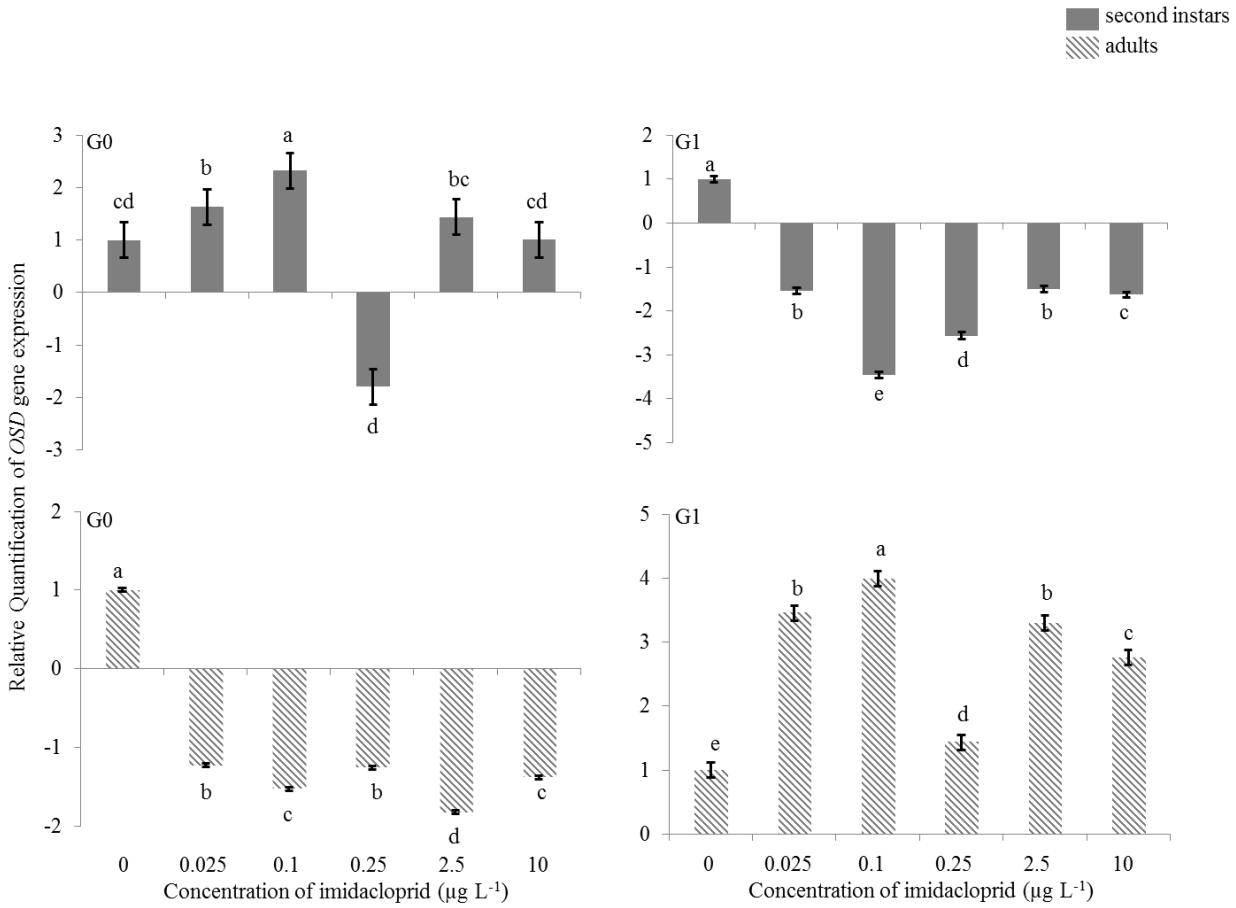

Figure 3.3. Olfactory Segment-D (*OSD*) gene regulation during imidacloprid-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing second instars and adults from initial (G0) and succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

concentrations did not change the expression of the *OSD* gene. In G0 adults, only at 2.5 µg a.i. L^{-1} was a change in gene expression observed, with ca. 2.0-fold down-regulation. G1 second instars exposed to 0.1 and 0.25 µg a.i. L^{-1}, had 3.3- and 2.5-fold down-regulation, respectively. Other concentrations did not alter *OSD* gene expression at that time point. All concentrations except 0.25 µg a.i. L^{-1} up-regulated *OSD* gene expression in G1 adults; 3.5-, 4.0-, 3.3- and 2.8-fold up-regulation of the gene was observed at 0.025, 0.1, 2.5 and 10 µg a.i. L^{-1}, respectively.

TOL gene. There was a 2.0-fold up-regulation of the *TOL* gene in G0 second instars exposed to 0.1 µg a.i. L^{-1} imidacloprid (Figure 3.4), and 3.3- and 10.0-fold down-regulation at 0.25 and 10 µg a.i. L^{-1}, respectively. In G0 adults, a change in *TOL* gene expression (2.0-fold down regulation) was only observed at 10 µg a.i. L^{-1}. In G1 second instars, there was 4.0-, 4.0- and 6.0 fold up-regulation at 0.025, 0.25 and 2.5 µg a.i. L^{-1}, respectively, and 2.0-fold down-regulation at 10 µg a.i. L^{-1}. In G1 adults, 2.6- and 3.0-fold up-regulation of the *TOL* gene was observed at 0.025 and 10 µg a.i. L^{-1} of imidacloprid.

ANT gene. There was no change in expression of the *ANT* gene in G0 nymphs or adults (Figure 3.5). In G1 second instars, there was 4.5-, 2.0-, 6.4-, 4.2- and 4.2-fold up-regulation at 0.025, 0.1, 0.25, 2.5 and 10 µg a.i. L^{-1}. In G1 adults, 2.5- to 5.0-fold down-regulation was observed at all imidacloprid exposure concentrations.

3.4.2 Global DNA methylation analysis

When first instar *M. persicae* were exposed to sublethal concentrations of imidacloprid and G0 second instars were analyzed for global DNA methylation, ca. 25, 10 and 20% hypermethylation above the control was observed at 0.025, 0.1 and 0.25 µg a.i. L^{-1}, respectively, and about 10% hypomethylation was observed at 2.5 µg a.i. L^{-1} (Figure 3.6).

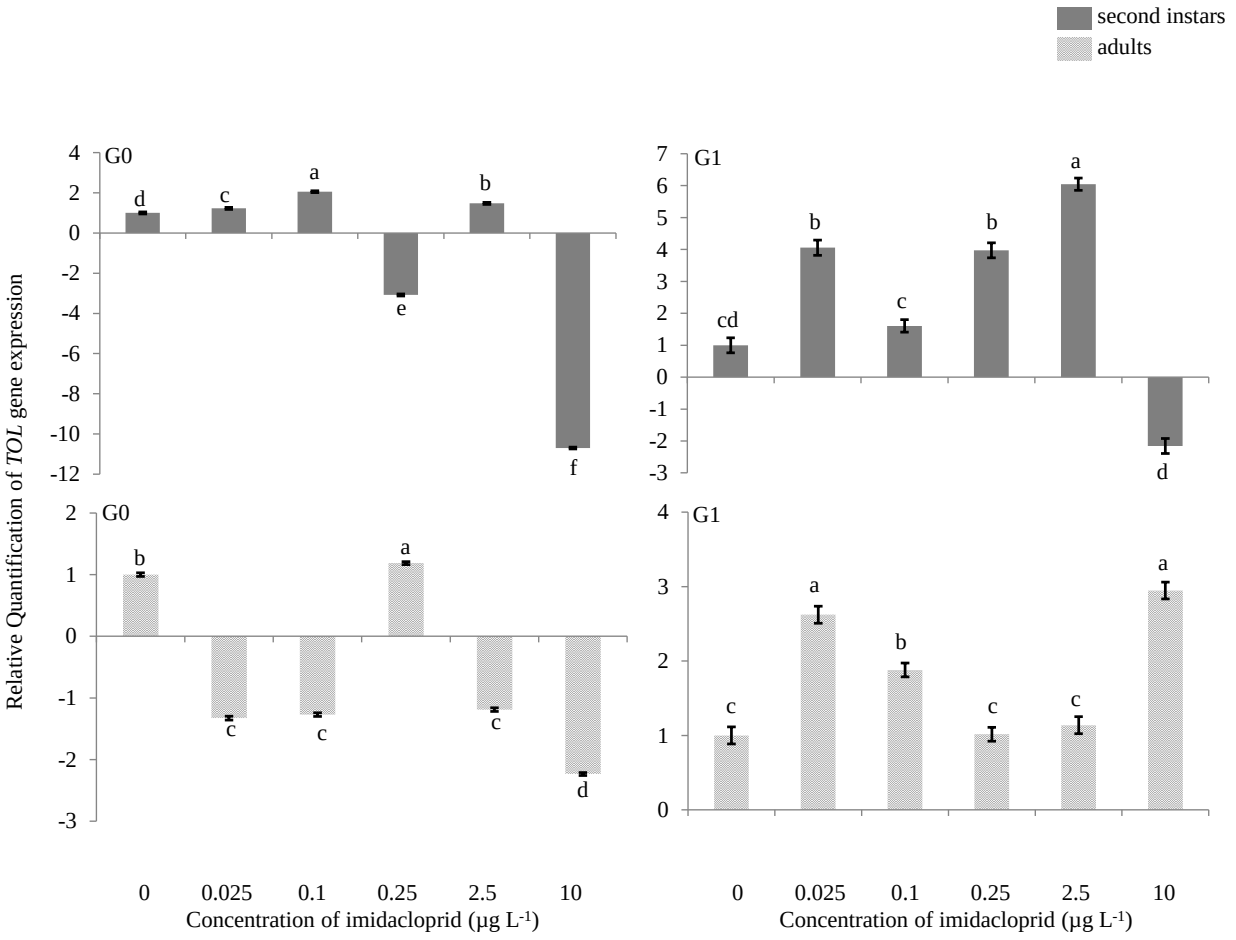

Figure 3.4. Take-out like (*TOL*) gene regulation during imidacloprid-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing second instars and adults from initial (G0) and succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

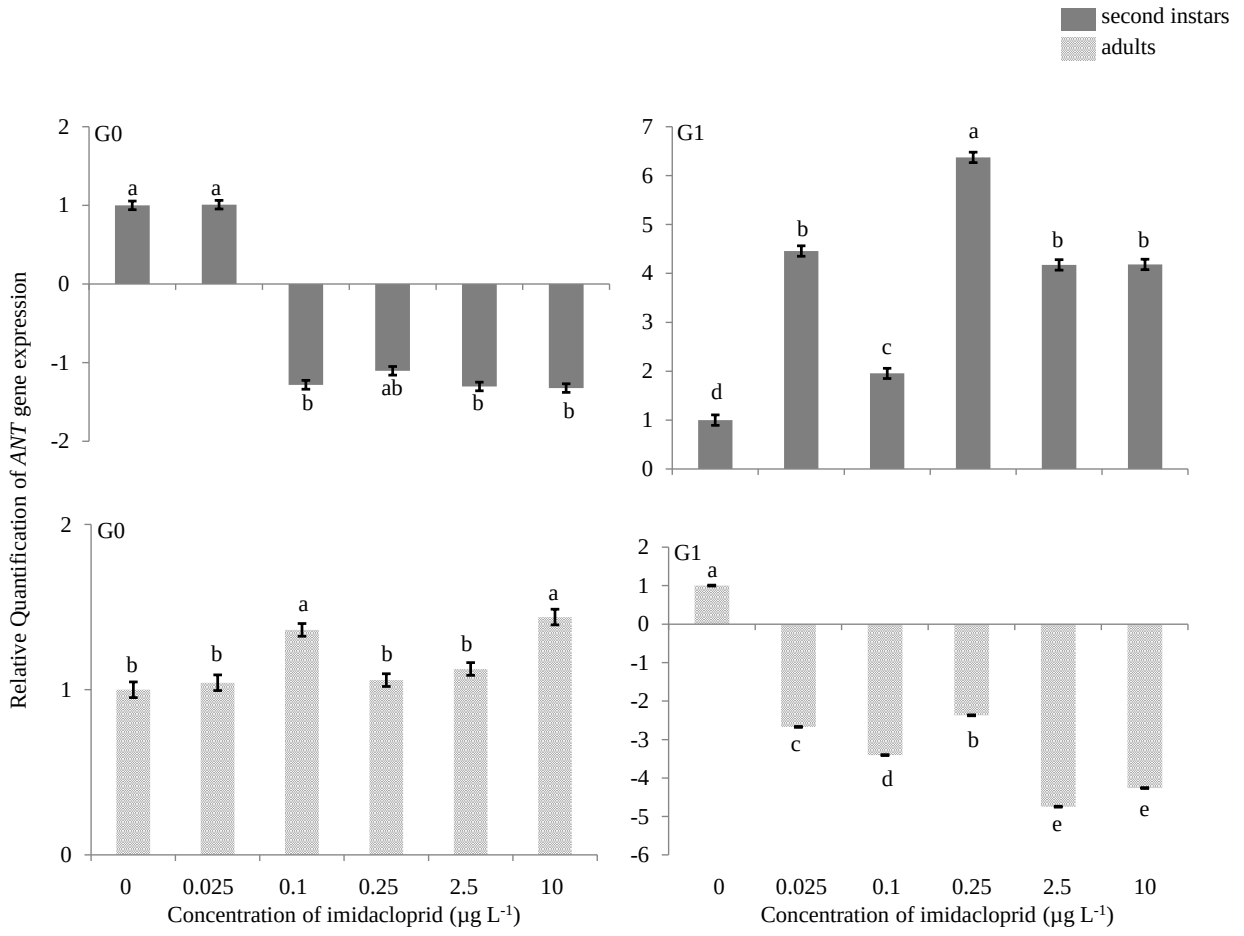

Figure 3.5. Adenosine nucleotide translocase (*ANT*) gene regulation during imidacloprid-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing second instars and adults from initial (G0) and succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

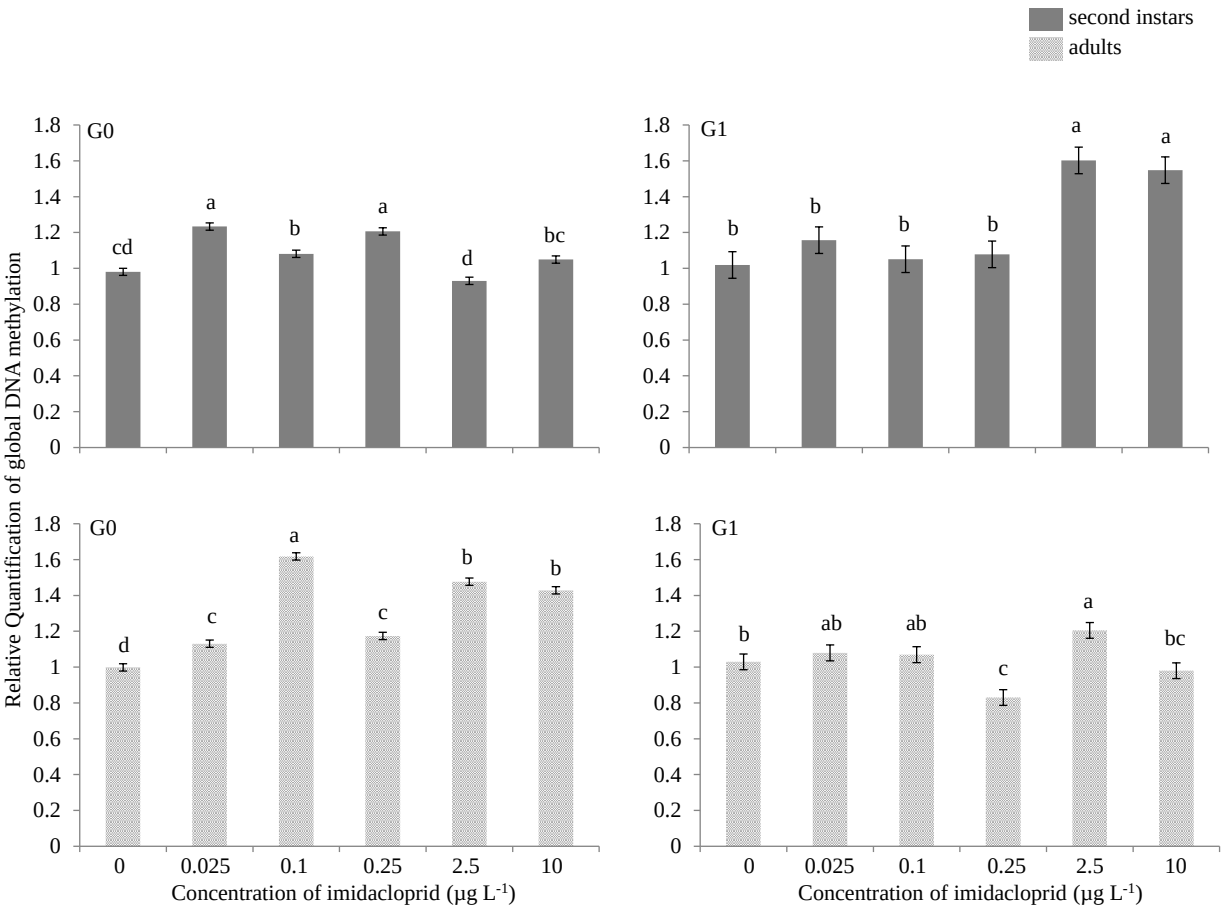

Figure 3.6. Global DNA methylation during imidacloprid-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing second instars and adults from initial (G0) and succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

No change in methylation was detected at 10 µg a.i. L^{-1}. In G0 adults, 10-60% hypermethylation was seen at various imidacloprid concentrations. In G1 second instars, significant hypermethylation in the order of 50-60% above the control was found only at 2.5 and 10 µg a.i. L^{-1}. In G1 adults, hypomethylation of 25% and 10% occurred at 0.25 and 10 µg a.i. L^{-1}, respectively, while 10% hypermethylation was observed at 2.5 µg a.i. L^{-1}. Other concentrations did not show any change in global DNA methylation relative to control (Figure 3.6).

3.5 Discussion

I used *M. persicae* and imidacloprid on potato to study gene regulation during insecticide-induced hormesis. When first instars were continuously exposed for two generations to sublethal concentrations of imidacloprid previously shown to induce stimulated reproduction (Chapter 2), intra- and inter-generational differences in up- or down-regulation of genes were found. Several stress, dispersal, and developmental genes were affected and, in multiple instances, there was evidence of up-regulation of genes in one life stage or generation that was countered by down-regulation of the same genes in subsequent life stages or generations.

Mild heat stress induced hormetic responses, such as increased longevity and thermo-tolerance, have been positively associated with levels of Hsp70 expression in bacterial cell lines (Mosser et al. 2000), the insects *C. tentans* (Yin et al. 2006), *Drosophila melanogaster* (Hercus et al. 2003), *Bemisia tabaci* (Gennadius) (Cui et al. 2008), and in the nematode *Caenorhabditis elegans* (Maupas) (Olsen et al. 2006). On the other hand, Hsp70 expression has been associated with reduced fecundity/reproduction in *L. huidobrensis* (Huang et al. 2007), *D. melanogaster* (Hercus et al. 2003), *T. vaporariorum* (Westwood), and *B. tabaci* (Gennadius) (Cui et al. 2008). This is possibly attributable to accumulation of Hsp and associated traits (thermotolerance) at a cost of impaired fecundity.

When *Hsp60* was analyzed in this study, continuous exposure of *M. persicae* to sublethal concentrations of imidacloprid previously shown to stimulate reproduction had no effect or resulted in down-regulation of the gene in second instars and adults of the initial generation, followed by up-regulation in second instars of the succeeding generation. This is not surprising, as Hsp responses to mild stress have been shown to vary as a function of life stage and generation depending on the organism and type of stressor. For example, heat shock induces Hsp expression more rapidly than concentrations of copper, in fathead minnow, *Pimephales promelas* Rafinesuqe, (Sanders et al. 1995). Chronic exposure to cadmium did not induce Hsp expression in gill tissue of sea mussels, *Mytilus edulis* (L.), but the gene was expressed when coupled with a heat shock (Veldhuizentsoerkan et al. 1991). Down-regulation of Hsp4 was found in *C. elegans* only after a second treatment of sublethal concentrations of mercury (Helmcke and Aschner 2010).

Down-regulation of Hsp expression in insects not well understood (Mahroof et al. 2005). Alternation of up- and down-regulation occurred across generations and between nymphs and adults within a generation in the present study. For example, up-regulation of Hsp expression in G1 second instars was followed by down-regulation in adults. Similar alternations were found in Hsp70 expression in egg, larva, pupa and adult of *T. castaneum* exposed to mild heat stress (Mahroof et al. 2005). Stimulated fecundity in *M. persicae* exposed to hormetic concentrations of imidacloprid could be due to down-regulation of Hsp gene in G0 and G1 (Chapter 2).

Down-regulation of Hsp90 and Hsp70 was reported in rat brains exposed to various concentrations of DDT (Shutoh et al. 2009) and bronchial cancer in smokers with chronic obstructive pulmonary disease (Cappello et al. 2006). In these studies, down-regulation of Hsp expression was suggested to be a recovery response to regain homeostasis (Cappello et al. 2006,

Shutoh et al. 2009). Similarly, down-regulation of *Hsp60* expression of G0 *M. persicae* nymphs and adults and G1 adults in the present study could have been due to the insect undergoing a recovery process following the mild pesticide exposure. Down-regulation of heat shock protein is often seen during chronic exposure to mild levels of stress (Cappello et al. 2006, Shutoh et al. 2009). When *C. tentanus* was exposed to a 35^{o} C heat shock, recovery was characterized by reduced Hsp70 expression (Karouna-Renier and Zehr 1999).

Up-regulation of *Hsp60* follows an accumulation of unfolded (damaged) proteins resulting from stress or injury in an organism (Parsell and Lindquist 1993). *Hsp60* up-regulation was observed in *M. persicae* G1 nymphs to higher sublethal concentrations of imidacloprid (0.25 - 10 µg a.i. L^{-1}). Due to overcompensation following insecticide exposure in G0, the insect probably conditioned to higher insecticide concentrations in G1 by elevating expression of *Hsp60*. Alternatively, accumulation of damaged proteins triggered the up-regulation of the gene.

As noted above, accumulation of Hsp often results in decreased fecundity (Huang et al. 2007) but, in fact, fecundity and reproduction is controlled by JH (Verma 1981, Dawson et al. 1987, Riddiford 2003, Goodman and Granger 2005). Also, JH regulates metamorphosis (Hartfelder 2000, Riddiford 2003, Goodman and Granger 2005) and is important in assessing nutritional information (Noriega 2004, Nouzova et al. 2011) in insects. In aphids, the *FPPS* I gene is an important enzyme regulator and its down-regulation increases JH titer in females (Keeling et al. 2004). It catalyzes formation of farnesyl diphosphate (FPP), a precursor needed in biosynthesis of JH, alarm pheromones and sex pheromones (Dawson et al. 1987, Vandermoten et al. 2009). JH is known to stimulate reproduction in *M.* persicae and elevated JH titers in maternal aphids inhibit wing development and promote development of apterous forms (Tamaki 1973, Verma 1981). In pea aphid, *Acrythosiphon pisum*, the mother that perceives stress cues

such as crowding transmits the information to unborn progeny (Brisson 2010). It is possible, therefore, that G0 *M. persicae* adults perceived the stress of sublethal imidacloprid exposure and passed the signal to G1 nymphs, as indicated by the strong up-regulation of the *FPPS* I gene. This was countered (compensated) in G1 adults, as observed by down-regulation of the *FPPS* I gene, again coinciding with the hormetic (stress) response.

It was previously shown that stimulated fecundity in *M. persicae* exposed to hormetic concentrations of imidacloprid resulted in increased JH III titers (Yu et al. 2010). This suggests that increased *M. persicae* fecundity and JH titers should be correlated with down-regulation of *FPPS* I gene, since down-regulation of this gene results in increased JH titers. In the present study, down-regulation of *FPPS* I observed in G0 adults exposed to 0.025 µg a.i. L^{-1} and in G1 adults exposed to 0.025 and 0.1 µg a.i. L^{-1} was associated with higher aphid fecundity (Chapter 2). However, significant down-regulation of *FPPS* was also seen at imidacloprid concentrations that did not result in stimulated reproduction, suggesting that other genes, also needed for JH production, were not affected by the insecticide treatment. G1 nymphs might have overcompensated for the *FPPS* I down-regulation in G0 nymphs and adults, as observed by the up-regulation of *FPPS* I, but again down-regulated this gene in G1 adults, associated with stimulated fecundity (Chapter 2). A detailed study on regulation of more genes in the JH biosynthesis pathway in the context of mild stress and other transcriptional factors involved in adaptive cellular stress response pathways could affirm the genetic underpinnings of hormetic reproductive responses in *M. persicae*.

Aphids sense stress such as overcrowding and onset of winter using chemoreceptors and other cues (Dixon 1998, Braendle et al. 2006). They attempt to evade the situation by producing reproductive alates or parthenogenetic alates, likely reflecting adaptations to colonize new plant

hosts during such conditions. Dispersal-related genes such as *OSD*, *TOL* and *ANT* are more highly expressed (2-5 fold) in alates than apterous aphids (Ghanim et al. 2006). In the present experiment *OSD*, *TOL* and *ANT* gene expression varied in apterous individuals exposed continuously to sublethal hormetic concentrations of imidacloprid. Complete wing development was not observed, but at higher concentrations small wing pads appeared in some insects (data not shown, Chapter 2).

Up-regulation of the *OSD* gene in G0 second instar *M. persicae* exposed to certain imidacloprid concentrations was followed by down-regulation of the gene in G0 adults and greater down-regulation in G1 second instars. *OSD* gene regulation is inversely related to aphid fecundity (Bos et al. 2010). Immature G0 aphids stressed at certain imidacloprid concentrations (up-regulation of *OSD* gene) may have conditioned future adults for increased reproduction. In G0 adults, *OSD* gene down-regulation occurred at all concentrations, possibly indicating compensation to the stress, coinciding with increased reproduction at those same concentrations (Chapter 2). Likewise, *OSD* gene down-regulation in G1 second instars occurred at concentrations of imidacloprid identical to those previously shown to elicit a hormetic reproductive response in *M. persicae*. Down-regulation of this gene in second generation nymphs is probably due to overcompensation hormesis where initial disruption is followed by overcompensation during continuous exposure to sublethal concentrations of the insecticide stressor. The up-regulation of *OSD* in G1 adults exposed could be attributed to the energy stress or depletion of resources, and probably not susceptibility of the insect to those low concentrations.

Trends similar to *OSD* gene regulation were observed with *TOL*. This gene is associated with chemoreception (Jacobs et al. 2005, Weil et al. 2009, Fan et al. 2011) and circadian control

of feeding behavior. *TOL* can be induced by starvation (Fujikawa et al. 2006, Weil et al. 2009), juvenile hormone binding proteins in response to fluctuating JH titers (Bohbot and Vogt 2005), during courtship and mating, or when regulating antennal responses to food, hosts, or pheromones (Dauwalder et al. 2002). The complexity of this gene and its involvement in multiple functions does not readily permit direct correlation with the hormetic responses previously observed (Chapter 2). In G0 second instars, the 10-fold down-regulation at 10 µg imidacloprid L^{-1} may be explained by the intensity of stress at this dose, which was the highest used in the experiments. In G0 adults, less down regulation of *TOL* occurred at 10 µg imidacloprid L^{-1}, and at other concentrations of the degree of up- or down-regulation of the gene was insignificant. However, up-regulation of *TOL* occurred at all concentrations in G1 second instars.

The *ANT* gene regulates mitochondrial proteins that function as carriers of important metabolites involved in a number of mitochondrial processes, mainly catalyzing exchange of ADP for ATP across inner mitochondrial membranes (Zhang et al. 1999). The unresponsiveness of the *ANT* gene in G0 suggests that exposure to hormetic concentrations of imidacloprid results in no additional energy expenditures for the aphids. However, as with the *TOL*, *Hsp60* and *FPPS* genes, significant up-regulation of *ANT* occurred in G1 nymphs, indicating extra energy was expended. This suggests that even though no extra energy was expended in G0 adults, the insect was probably still stressed, had a hormetic response in form of stimulated reproduction and overcompensation, as *ANT* gene up-regulation, in G1 nymph resulted. Down-regulation of the *ANT* gene in G1 adults probably reflects the cessation of energy requirements and or other regulatory process (up-regulation in second instar) during the development phase in G0 nymphs.

Overall, I interpret the up- or down-regulation of *Hsp60*, *FPPS* I, *OSD*, *TOL* and *ANT* genes in G0 to be a priming response (Costantini et al. 2010) in preparation for further adverse conditions, *vis a vis* insecticide exposure. It is unclear, however, if insecticide-induced hormesis in insects is an epigenetic process, resulting in heritable changes in gene expression (Glastad et al. 2011, Gressel 2011). DNA methylation is an important epigenetic mechanism for regulation of gene expression. In insects DNA methylation is thought to play a role in developmental responsiveness to environmental factors and may provide critical contributions to insect developmental and phenotypic variation (Bass and Field 2011, Glastad et al. 2011, Gressel 2011). Increases in methylation, termed hypermethylation, typically reduce DNA transcription and usually result in inactivation of genes, although this is not always the case (Suzuki and Bird 2008). Concurrent amplification and methylation of an esterase gene in greenbug, *Shizaphis graminum* (Rondani), suggested heritability rendering increased resistance to organophosphorus insecticides (Ono et al. 1999). Similarly, in *M. persicae*, the E4 gene is important in expression/amplification of insecticide-detoxifying esterases, and reduced DNA methylation in this insect coincides with a loss of E4 gene expression (Hick et al. 1996, Field et al. 2004). In the present study, methylation occurred initially in G0 exposed to low doses of imidacloprid but not in G1, possibly indicating a heritable adaptation to those concentrations of imidacloprid. In second instars of the succeeding generation, only exposure to higher concentrations of imidacloprid increased methylation, possibly indicating adaptation to lower concentrations. This suggests that trans-generationally the insect would be able to cope with higher levels of stress (higher concentrations). G1 adults were probably adapted to lower concentrations, with the exception of the response to 0.25 µg L^{-1}, which resulted in hypomethylation. This could possibly be due to DNA instability or new synthesis of certain genes at that particular concentration.

Imidacloprid resistance in insects via mutations has been reported in the past (Elbert and Nauen 1996, Wen et al. 2009, Bass and Field 2011) and insecticide-resistance is associated with amplification and methylation of esterase genes (Ono et al. 1999, Field 2000, Field et al. 2004). I speculate that insecticide-induced hormesis might serve as a precursor to insecticide tolerance and ultimately resistance (Gressel 2011). Further investigation is needed in this area to fully comprehend how insect response to low levels of stress in the form of hormesis relates to methylation changes across generations.

4 EFFECT OF SUBLETHAL CONCENTRATIONS OF PRECOCENE-I ON FECUNDITY OF GREEN PEACH APHID AND GENE REGULATION DURING ITS EXPOSURE

4.1 ABSTRACT

Low dose imidacloprid exposed *Myzus persicae* produced more progeny than controls in several instances. Studies in the past also have noted concurrent JH III titers increase, suggesting overcompensation due to low dose imidacloprid exposure lead to allocation of resources toward reproduction. Precocene, an anti-JH agent, could negate the JH III titer production. Here, the hypothesis that blocking JH production, eventually leading to no stimulation in fecundity, of *M. persicae* exposed one-time topically to sublethal concentrations of precocene was tested. Also, stress response (*Hsp60*), dispersal (*OSD*, *TOL* and *ANT*) and developmental (*FPPS* I) genes regulation were studied over three time points (G0 adult, G1 nymph and G1 adult stages) during precocene-induced hormesis with emphasis on *FPPS* I, JH precursor gene. Hormetic stimulation (1.5 to 2 times over controls) was noted in fecundity of *M. persicae* exposed to sublethal concentrations of precocene. However, no direct relation between *FPPS* (or other selected genes) gene regulation and fecundity was established. This reiterates that trigger in adaptive cellular response pathways leads to a hormetic response. Key feature of this study was the magnitude of regulation that ranged from 2- to 300-fold compared to 2- to 10-fold noted in imidacloprid (a xenobiotic) study and this was probably due to the regulatory nature of precocene.

4.2 INTRODUCTION

Stressor-induced high-dose inhibition coupled with low-dose stimulation is termed hormesis (Calabrese 2008, Mattson 2008). It has been observed in a plethora of organisms responding to a wide range of chemical, physical, and biological stressors. Insecticide-induced hormesis has been studied in detail (Cohen 2006, Cutler 2013), as has hormesis due to pathogens that affect insects (Wojda et al. 2009, Leroy et al. 2012, Ramanaidu and Cutler 2013). It is generally manifest in insects as increased reproductive output in response to low doses of insecticide or stress. Little work has been done on insect hormesis with compounds that interfere with development and reproduction such as juvenile hormone (JH). This is of interest because JH is known to directly affect both of these processes in aphids (Hamnett and Pratt 1983, Staal 1986, Mittler 1991, Peric-Mataruga et al. 2006), and insects in general (Hartfelder 2000, Riddiford 2003).

Yu et al. (2010) showed that during imidacloprid-induced hormetic responses, JH titers increased similarly to fecundity. This poses the question: does a compound that inhibits JH still induces hormetic responses in insects in the form of stimulated reproduction? Compactin, fluoromevalonate, imidazoles, and precocene are insect growth regulators (IGR) that are anti-juvenile hormone agents that affect either the mevalonate pathway in JH biosynthesis, or the corpora allata (CA) directly, the organ that produces JH (Staal 1986). Precocenes (precocene I and precocene II) have anti-allatal properties and induce precocious metamorphosis in insects, including green peach aphid, *Myzus persicae* (Sulzer) (Bowers et al. 1976, Hales 1976). However, due to their JH agonist activity at high doses, vertebrate toxicity, higher degradation rates and ineffectiveness on all life stages of different insect orders, precocenes are seldom used as insecticides (Staal 1986). Recent studies that suggested the use of precocene as a positive

control for testing IGRs on soil microorganisms also reported higher chronic toxicity than fenoxycarb, methoprene, and tebufenozide (Campiche et al. 2006). There have been few studies into hormetic, sublethal and chronic effects of these types of growth and development regulating compounds on insects.

In this study, I tested the hypothesis that topical exposure to low concentrations of precocene-I (precocene), an anti-JH agent, can induce a hormetic response in *M. persicae*, in the form of stimulated reproduction. Probably due to the occurrence of completely developed CA in later instars, this anti-JH agent typically is most effective against insect in mid- to late-instars (Staal 1986). I therefore used third instar *M. persicae* to initiate the experiments. To gain insight into potential molecular changes during precocene-induced hormesis, gene regulatory responses of certain dispersal-related (*OSD*, *TOL* and *ANT*), stress-related (*Hsp60*) and a JH precursor (*FPPS* I) genes at these concentrations were analyzed (Chapter 3).

4.3 METHODS AND MATERIALS

4.3.1 Plant and insect maintenance

M. persicae cultures and potato plants were maintained as described in Chapter 2.

4.3.2 Chemicals

Precocene (7-methoxy-2,2-dimethyl-3-chromene, Sigma-Aldrich, ON, Canada) was suspended in acetone (Fisher Chemical, ON, Canada) to obtain a 1000 mg L^{-1} stock solution. Only the dilutions contained olive oil (1:19). Preliminary experiments were conducted using serial dilutions and fecundity responses to obtain a no observable adverse effect concentration that was 3 mg precocene L^{-1}. Precocene concentrations of 0.01, 0.03, 0.1, 0.30, 1.0, 3.0, and 10 mg L^{-1}

were used in topical exposure experiments. Controls in these experiments consisted of acetone and olive oil only. Fresh stock solutions of test concentrations were prepared for each bioassay.

4.3.3 Topical exposure

4.3.3.1 Fecundity

Five third instars each were placed in clean glass Petri plates (9 cm) and sprayed in a Potter tower (Burkard Scientific, Uxbridge, United Kingdom) at 78 kPa with 5 ml of control or insecticide solution. After each treatment, aphids were transferred to plastic Petri plates (5.5 cm diameter) lined with Whatman No. 1 filter paper containing two untreated potato leaf discs (1.8 cm diameter). Leaf discs were replaced every second day with freshly excised leaf discs. Fecundity was recorded every second day. For each exposure scenario, the experiment was a randomized complete block design, with precocene concentration being the main factor of interest. Each bioassay had seven precocene concentrations, and for each there were five Petri dishes, each containing five aphids. Each bioassay was considered an experimental block, and each bioassay was conducted three times. Repeated measures analyses were conducted using Proc Mixed in SAS (SAS 2008), with the error terms assumed to be normal with constant variance but not to be independent. Autoregression (AR (1)) represented the appropriate type of dependence for covariance structure. Residuals were used to verify the assumptions of normal error distribution and constant variance. Data were log-transformed as needed to meet the assumptions. If means were significantly different, they were separated using a LSD test (α = 0.05). Backtransformed means are reported as required.

4.3.3.2 Gene expression analyses

The experimental design for treating aphids was the same as described in Chapter 3, but was initiated with 10 third instars. On day 4, 9, and 13 (representing G0 adult, G1 second instar and adult stages, respectively) five aphids per Petri plates were collected totaling 25 aphids per concentration for genetic analyses. *Myzus persicae* genes selected for gene expression analysis were identical to those described in Chapter 3: *Hsp60*, *FPPS* I, *ANT*, *OSD* and *TOL*. Methods of analyzing expression of these genes and statistical analysis are described in Chapter 3. A linear scale was used in figures where positive values represent up-regulation and negative values represent down-regulation of the gene.

4.4 RESULTS

4.4.1 Fecundity

Topical exposure to precocene had a significant effect on *M. persicae* reproduction (G0, $F_{7, 334}$ = 13.15, P = 0.0001; G1, $F_{7, 334}$ = 5.84, P = 0.0001; total, $F_{7, 110}$ = 4.48, P = 0.0002), but response varied among bioassay replicates (blocks) (G0, $F_{2, 334}$ = 4.02, P = 0.0188; G1, $F_{2, 334}$ = 5.34, P = 0.0052; total, $F_{2, 110}$ = 10.68, P = 0.0001). Aphid fecundity was stimulated at precocene concentrations of 0.03 and 10 mg L^{-1} in G0, with 1.5-2 times more progeny being produced compared to controls, but fecundity for all other treatments and time points was similar to or less than the controls (Table 4.1). Reduced aphid fecundity of 50% or more at 0.01 and 0.03 mg L^{-1} of precocene over controls was found in G1 ($P < 0.05$), whereas other concentrations that experienced reduced fecundity in G0 recovered to control levels in G1. At 0.3, 1 and 3 mg L^{-1} of precocene, there was an increase (14-32%) in fecundity over controls in G1 that was statistically insignificant, probably reflecting the variance in bioassay replicates. Overall, the total number of aphids produced in two generations was not significantly different from that in controls except at

0.01 mg L^{-1} of precocene where about 33% fewer aphids were found (Table 4.1). Certain deformities were also noted in G0 adults (adultoids) resulting from precocene exposure (Figure 4.1).

4.4.2 Gene expression analyses

Topical exposure to sublethal concentrations of precocene resulted in up- and down-regulation of the genes analyzed (Table 4.2).

FPPS I gene. Down-regulation of the *FPPS* I gene was seen in G0 adults at all precocene concentrations except 0.03 and 3 mg L^{-1}. This ranged from a 4-fold decrease at 1.0 mg L^{-1} of precocene, to a 31-fold decrease in *FPPS* expression at 0.1 mg L^{-1} precocene (Figure 4.2). In G1 second instars, expression of the gene did not change at any concentration of precocene. There was a 4.3-fold up-regulation of *FPPS* I gene expression in G1 adults at 0.1 mg L^{-1} of precocene, but other concentrations did not result in changes in gene expression.

Hsp60 gene. Following topical treatment of third instars with precocene, there was a 3- to 36-fold up-regulation of *Hsp60* gene expression in G0 *M. persicae* adults, and 2- to 4-fold down-regulation in G1 second instars (Figure 4.3). Two-fold up-regulation was observed at 0.1 mg L^{-1} of precocene and 5- and 11-fold down-regulation at 3 and 10 mg L^{-1} precocene in G1 adults. *Hsp60* gene expression did not change in G1 adults exposed to 0.01, 0.03, 0.3 and 1 mg L^{-1} of precocene.

OSD gene. Three- to 42-fold up-regulation of the *OSD* gene was observed in G0 adults when exposed to various concentrations of precocene, but exposure to 1 mg L^{-1} did not change the expression of this gene (Figure 4.4). In G1 second instars, 3- to 23-fold down-regulation was observed across all concentrations of precocene. In G1 adults, 2- to 38-fold up-regulation was

Table 4.1. Least-squares means of two-generational[a] fecundity following topical exposure of *Myzus persicae* to sublethal concentrations of precocene.

Conc. (mg L^{-1})	**Generation[a,b]**		**Total**
	G0	**G1**	
0	4.47b	3.99a	44.39ab
0.01	3.02c	1.49c	29.78c
0.03	6.89a	2.14bc	50.78a
0.1	2.75c	3.31ab	37.51bc
0.3	3.64bc	5.25a	51.32a
1	1.78d	4.55a	38.22bc
3*	3.77bc	4.78a	50.50a
10	7.93a	3.49ab	55.30a
30[c]	0	0	0
SEM	0.11	0.13	4.52

[a] Third instars were topically treated and thereafter reared on untreated potato leaf discs. Fecundity of each resulting adult was recorded every 2 days until it died. In the succeeding generation, 5 randomly selected 24 h old nymphs were tracked and fecundity of the resulting adults was recorded every 2 days until they died. G0 is initial generation, G1 is progeny of G0.

[b] Progeny per adult data were log transformed before analysis. Backtransformed means are presented. Values followed by different letters are significantly different (LSD, $\alpha = 0.05$). SEM values are not backtransformed

[c] G0 nymphs did not survive to adulthood when treated with 30 mg L^{-1}. This concentration was not included in the analysis (Figure 4.1).

* denotes NOAEC as determined in preliminary experiments by one way ANOVA.

Table 4.2. *P*-values for a two generation (G) experiment examining gene regulation during precocene-induced hormesis in second instar (N) and adult (A) *Myzus persicae*.

Source of Variation	**Generation-time point**		
	G0-A	**G1-N**	**G1-A**
Hsp60	0.0001	0.0001	0.0001
FPPS I	0.0001	0.0001	0.0001
OSD	0.0001	0.0001	0.0001
TOL	0.0001	0.0001	0.0001
ANT	0.0001	0.4259	0.0001

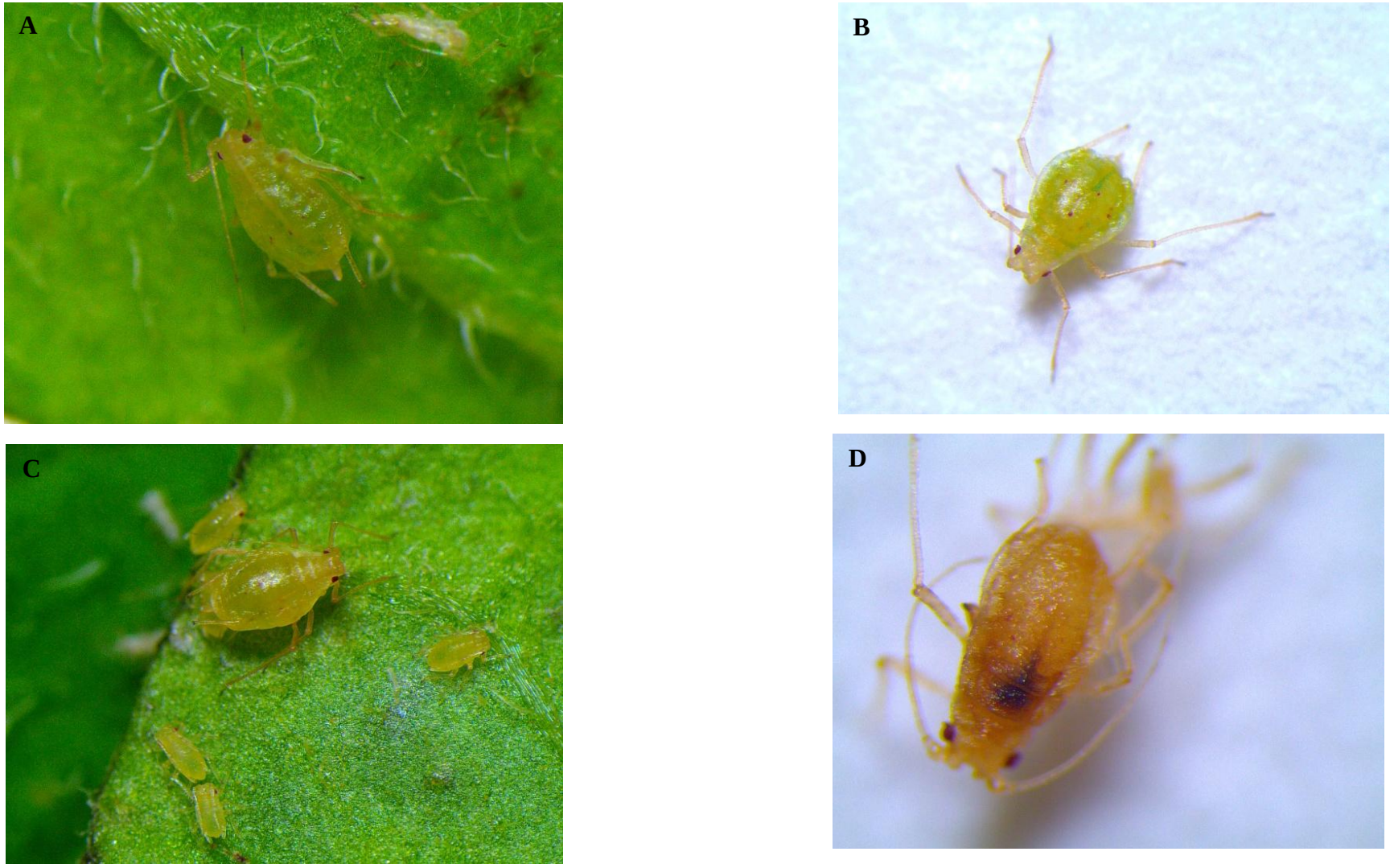

Figure 4.1. Some noted deformities in *Myzus persicae* resulting from one-time topical exposure to sublethal concentrations of precocene. A: Control; B: Miniature adultoid, resulting from 0.03 mg L^{-1} precocene exposed third instar, with thickened rims (lateral thickenings), long legs and green streak (reserved resources). C: Bulged adultoid, resulting from 10 mg L^{-1} precocene exposed third instar and D: Dead adultoid, resulting from 30 mg L^{-1} precocene exposed third instar.

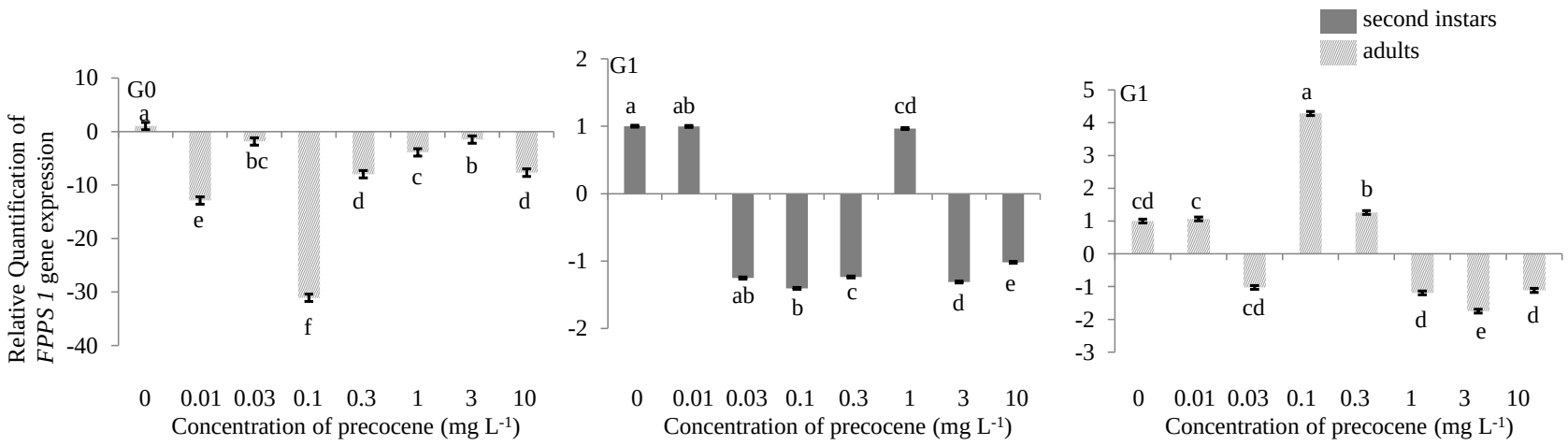

Figure 4.2. Farnesyl diphosphate synthase (*FPPS*) I gene regulation during precocene-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing adults from initial (G0) and; second instars and adults from succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

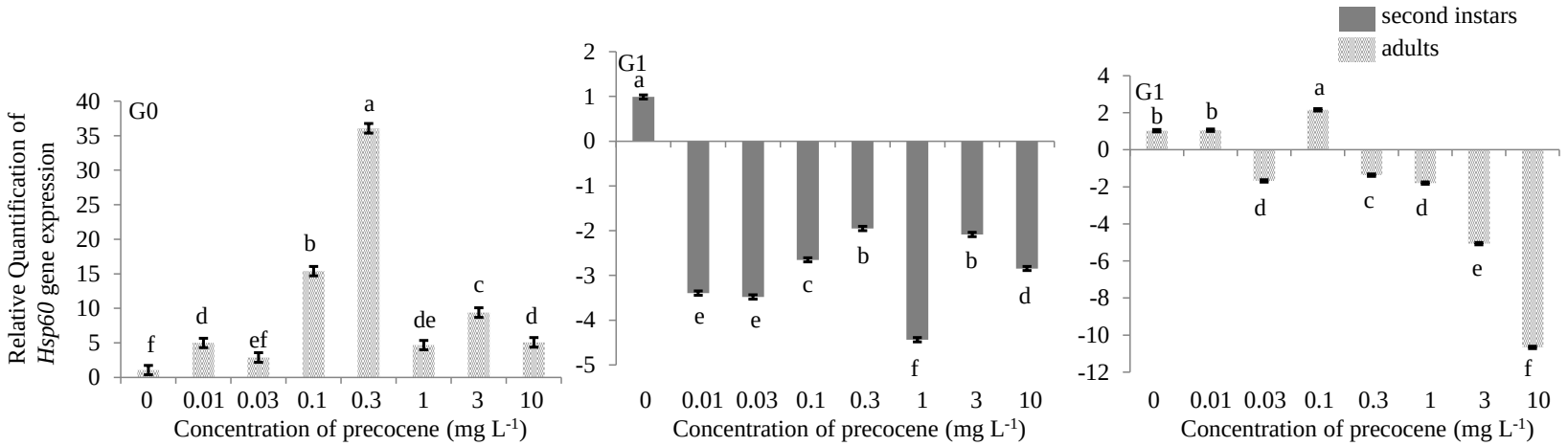

Figure 4.3. Heat shock protein (*Hsp*) *60* gene regulation during precocene-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing adults from initial (G0) and; second instars and adults from succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

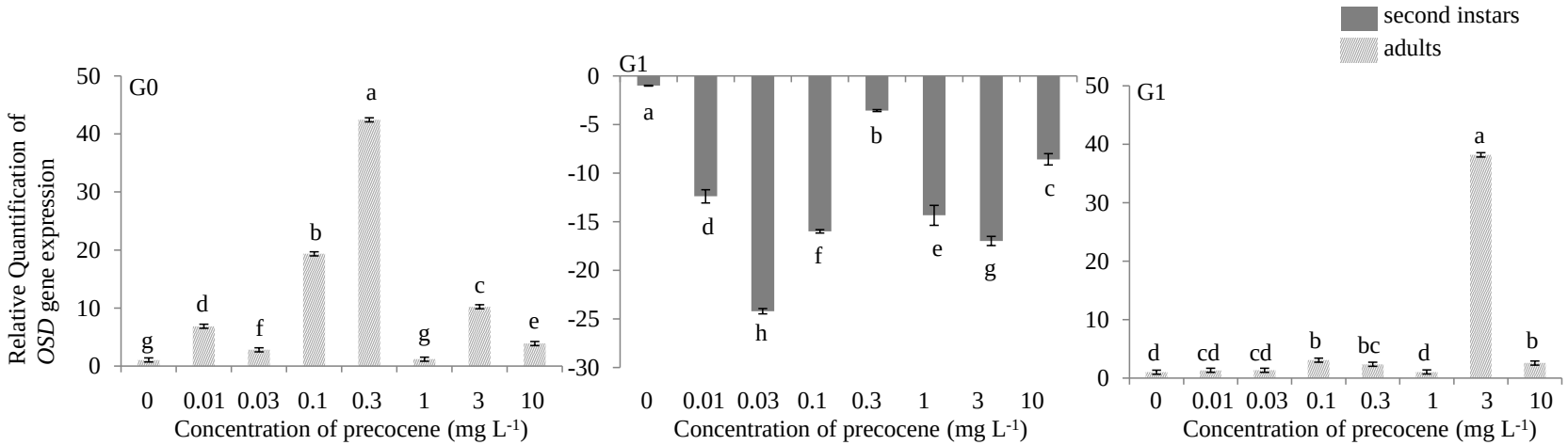

Figure 4.4. Olfactory Segment-D (*OSD*) gene regulation during precocene-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing adults from initial (G0) and; second instars and adults from succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

observed at 0.1, 0.3, 3 and 10 mg L^{-1} of precocene while other concentrations did not change the expression of the gene.

TOL gene. Up-regulation of the *TOL* gene was seen in G0 adults at all precocene concentrations except 1 and 10 mg L^{-1}. This ranged from a 12-fold increase at 0.03 mg L^{-1} of precocene, to a 204-fold increase in *TOL* expression at 0.3 mg L^{-1}precocene (Figure 4.5). In G1 second instars, there was down-regulation of 4- to 45-fold at 0.01, 0.03, 0.1 and 0.3 mg L^{-1} of precocene, and 2-fold up-regulation at 1, 3 and 10 mg L^{-1} of precocene. In G1 adults, 2-, 3- and 69-fold up-regulation was observed at 0.01, 0.3 and 3 mg L^{-1} of precocene, respectively, down-regulation was observed at 0.1 and 10 mg L^{-1}, and no change at 0.03 mg L^{-1} of precocene.

ANT gene. In G0 adults, *ANT* gene was up-regulated by precocene by 3- to 294-fold (Figure 4.6) but 1 mg L^{-1} of precocene did not change the expression of this gene. In G1 second instars, no significant change in expression was observed at any concentration. In G1 adults, a 26- and 2-fold up-regulation occurred at 0.1 and 0.3 mg L^{-1} of precocene, down-regulation at 1, 3 and 10 mg L^{-1} of precocene, and no change in *TOL* gene expression occurred at 0.03 mg L^{-1} of precocene.

4.5 DISCUSSION

When third instar *M. persicae* were topically treated with sublethal concentrations of precocene, G0 adults responded with stimulated fecundity at certain concentrations. Stimulated reproduction was not observed at these concentrations in G1 adults, possibly reflecting a transgenerational form of overcompensation hormesis and the aphid's reestablishment of homeostasis. A similar result was observed when first instar *M. persicae* were one-time topically treated with 0.6 µg L^{-1} imidacloprid where 2-fold increased reproduction over controls was

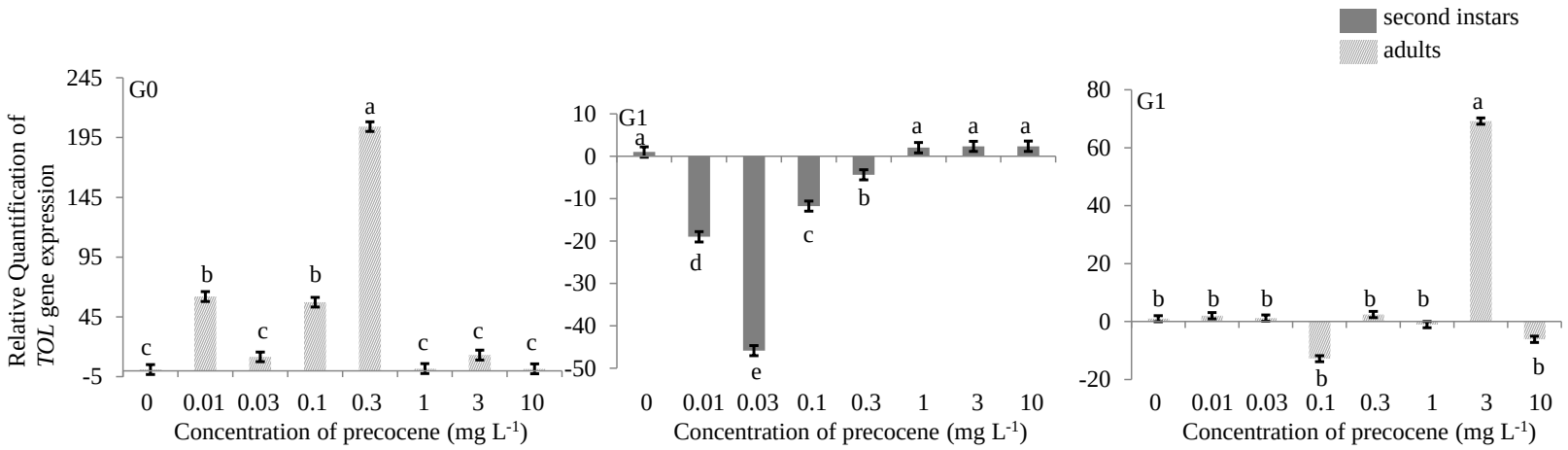

Figure 4.5. Take-out like (*TOL*) gene regulation during precocene-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing adults from initial (G0) and; second instars and adults from succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

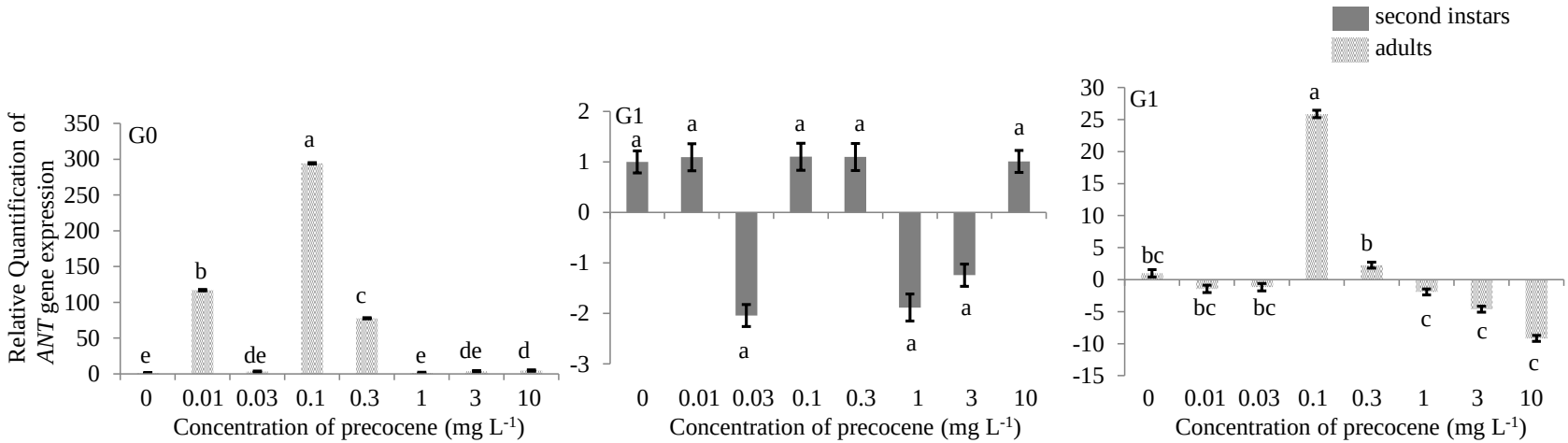

Figure 4.6. Adenosine nucleotide translocase (*ANT*) gene regulation during precocene-induced hormesis in green peach aphid, *Myzus persicae*. Test aphids representing adults from initial (G0) and; second instars and adults from succeeding (G1) generations were used for down-stream analyses. Error bars represent standard error of mean. For a given time point, bars with different letters above them are significantly different ($P \leq 0.05$, LSD test).

initially observed, followed by reduced reproduction at the same concentrations in the subsequent generation (Chapter 2). Other studies have found that exposure of insects to low doses of compounds that interfere with development can stimulate reproduction. When diamondback moth, *Plutella xylostella* (L.), larvae were exposed to a LC10 concentration of hexaflumuron, an chitin synthesis inhibitor, the gross reproduction rate of adults that subsequently developed increased (Mahmoudvand et al. 2011). When third instar *Eurygaster ntegriceps* Put. was exposed to precocene, percent egg hatch increased (Amiri et al. 2010). Similarly, when the rotifer *Brachionus calyciflorus* Pallas was exposed to precocene, stimulation in reproduction occurred (Lv et al. 2012).

When *M. persicae* was exposed to sublethal concentrations of imidacloprid, concurrent stimulation in fecundity and increased JH III titers was observed (Yu et al. 2010). Although the mechanism of vitellogenin (Vg) regulation by JH III is unclear, JH is essential for Vg synthesis (Hartfelder 2000), serving as a possible explanation for stimulation in fecundity in *M. persicae* exposed to hormetic concentrations of imidacloprid (Yu et al. 2010). Precocene antagonizes JH production but at sublethal concentrations, increased reproduction occurred in the present study. There are two possible explanations for this. First, Vg may still be produced by the insects even if JH is blocked. In *Tribolium castaneum* (Herbst),Vg synthesis still occurred with blocked transduction of JH and nutritional signals as long as expression of genes coding for insulin-like peptides, insulin receptor, serine/threonine-specific protein kinase and Fork head transcription factor (FoxO) were not hindered (Sheng et al. 2011). A similar mechanism has yet to be confirmed in viviparous *M. persicae,* but similar production of Vg in the absence of JH could occur. Low doses of precocene could trigger a hormetic response through adaptive cellular stress response pathways such as the insulin pathway (FoxO pathway) or heat shock factor

pathway (Son et al. 2010), phenotypically manifesting as increased reproduction .

Second, although I did not measure JH III titers, a several fold down-regulation of *FPPS* I was observed at almost all concentrations. The *FPPS* gene was previously thought to exclusively influence JH biosynthesis, but also has been related to terpenoid-pheromone production in some insects (Keeling et al. 2004, Taban et al. 2009). The pheromone (E) β-farnesene (EBF) is used by *M. persicae* (Lewis et al. 2008, Vandermoten et al. 2008) and its regulation *de novo* is governed by JH (Tillman et al. 2004, Vandermoten et al. 2012). One could speculate that down-regulation of the *FPPS* gene, which results in decreases in EBF (Lewis et al. 2008, Vandermoten et al. 2008), could increase *M. persicae* fecundity because the converse was true in *Aphis gossypii* Glover, where synthetic EBF-stimulated first instars produced significantly lower numbers of nymphs as adults (Su et al. 2006). A similar result was obtained with ca. 60% reduction in fecundity when *M. persicae* were reared on EBF synthase emitting plants (de Vos et al. 2010). On the other hand, EBF emission did not change the expression of the *FPPS* gene nor reduce reproduction in EBF-habituated *M. persicae* probably due to habituation (desensitization of genes) of *M. persicae* to EBF (de Vos et al. 2010).

Up-regulation of Hsp genes, to maintain homeostasis, is common in organisms subjected to heat stress, heavy metals, infection, and chemical toxicants (Parsell and Lindquist 1993, Schoffl et al. 1998, Karouna-Renier and Zehr 1999, Son et al. 2010), and has also been associated with hormetic responses. For example, Hsp70 expression was associated with increased longevity and thermo-tolerance in bacterial cell lines (Mosser et al. 2000), and the insects *Cydia pomonella* (Yin et al. 2006), *Drosophila melanogaster* (Hercus et al. 2003), and *Liriomyza huidobrensis* (Huang et al. 2007) as a result of mild heat stress-induced hormesis. However, increases in Hsp do not seem to coincide with increased reproduction in insects

(Hercus et al. 2003, Huang et al. 2007). Similarly, in the present study, when *M. persicae* nymphs were exposed to sublethal concentrations of precocene, up-regulation of *Hsp60* was observed at all concentrations, but reproduction decreased, increased, or was unaffected with no clear dose-response relationship with reproduction. Stimulated fecundity over controls at certain concentrations where *Hsp60* up-regulation occurred was probably because of other genes compensating for the stress induced by sublethal concentrations of precocene.

The up-regulation of *Hsp60* in G0 adults was followed by down-regulation of the gene in G1 nymphs, suggesting a recovery process following initial adaptation to the chemical stressor (Karouna-Renier and Zehr 1999, Mahroof et al. 2005). In G1 adults, no response was observed at lower concentrations, while at higher concentrations aphids attempted to recover from the stress. Similarly, precocene exposure resulted in Vg decline, which returned to normal levels after a few days (Amiri et al. 2010). In the present experiments, the concentration of precocene was low and the insect compensated for this tolerable level of stress. The G1 nymphs had no change in expression of the gene that later had a counter effect in G1 adults at one or two concentrations as suggested by the temporary influence of precocene on offspring development (Kambhampati et al. 1984).

Aphids respond to stress by up-regulating *OSD*, *TOL* and *ANT* genes, along with 31 other genes that are predominantly expressed in alates (Chapter 3, (Ghanim et al. 2006)). *OSD* and *TOL* genes were up-regulated several fold in G0 adults when third instar *M. persicae* were topically treated with sublethal concentrations of precocene. Both the genes responded similarly except the magnitude of *TOL* gene up-regulation was 5 times higher than *OSD* gene expression at certain concentrations. Induction of *OSD* was previously shown to correspond to reduced fecundity (Bos et al. 2010, de Vos et al. 2010). However, the reproductive responses in this

experiment did not exhibit this relationship. A combination of other unanalyzed gene responses could be responsible, or the regulation of *OSD* and *TOL* genes might simply have no influence on *M. persicae* reproductive outputs. When Colorado potato beetle, *Leptinotarsa decemlineata* (Say) were treated with sublethal dose of precocene, a change in form and number of sensillae of the antenna was observed suggesting a chemo-sensory disruption (Farazmand and Chaika 2011). *OSD* and *TOL* genes might be involved in such functions in *M. persicae*. Compensation in the form of down-regulation of both the genes was observed in G1 second instars at lower concentrations. At higher concentrations of precocene, statistically insignificant up-regulation of *TOL* gene expression was observed in G1 nymphs due to prevailing stress. In order to attain homeostasis, in G1 adults there was up-regulation of *OSD* and *TOL* gene expression at higher concentrations, with no change in expression of these genes at lower concentrations. *ANT* gene regulation was similar to *OSD* and *TOL* gene expression in G0 adults suggesting allocation of resources to the lower concentrations. Reduction in fecundity at certain concentration was probably due to diversion of resources towards up-regulation of the *ANT* gene. When the insect recovered from (adapted to) short duration stress, no extra-energy was need as suggested by unchanged *ANT* gene regulation in G1 nymphs.

In conclusion, the hypothesis that precocene, an anti-JH agent, would not stimulate the reproductive responses in *M. persicae*, was rejected. Few sublethal concentrations of precocene induced stimulation in reproduction to a tune of 1.5 to 2-fold higher than controls. There were no clear linkages between exposure of sublethal concentrations of precocene, phenotypic response (increased fecundity), and expression of genes examined in this study. There were differences in phenotypic and molecular responses at different sublethal concentrations of precocene, and these varied depending on life-stage and generation. Yu et al. (2010) have shown

that imidacloprid-induced hormesis in *M. persicae* resulted in concurrent stimulatory responses of fecundity and JH III. But when *M. persicae* were topically exposed to precocene, stimulation in fecundity was observed. This suggests that regulation of the *FPPS* gene (JH III) and Vg synthesis are not correlated. Imidacloprid and other stressors have yielded hormetic responses with short term exposure similar to precocene but unlike imidacloprid-induced hormesis (Chapter 2 and 3), precocene-induced reproductive responses were not very apparent and the amplitude of up- or down-regulation of most genes was noticeably higher. This is probably is due to the growth regulating nature (hormonal) of precocene rather than xenobiotics, such as imidacloprid. This study suggests that trade-offs among life history traits coupled with the rate at which an insect copes with short-term stress to attain homeostasis i.e., a generation or two was sufficient. To understand the connections between phenotypic responses at the molecular level, additional genes (e.g., those associated with the mevalonate pathway, insulin pathway), biochemical endpoints such as measuring JH III titers, and Vg titers should be considered.

5 GENERAL DISCUSSION

In toxicology, threshold and linear non-threshold models have traditionally governed the study of dose-response in theory and practice (Cox 1987). Hormetic models that are biphasic in nature and emphasize mechanistic, physiological and evolutionary understandings have largely been ignored (Calabrese 2005). Hormetic models have gained prominence in the past two decades and have been tested across numerous stressor-response-responder combinations. Relatively few insecticide-induced hormesis models have been used to understand the fundamentals of this phenomenon at various levels of biological organization particularly its biochemical underpinnings (Smirnoff 1983, Mukherjee et al. 1993, Yu et al. 2010, Celorio-Mancera et al. 2011), and the consequences of such responses at the individual and population levels (Kuenen 1958, Doutt and Smith 1971, Chelliah and Heinrichs 1980, Smirnoff 1983, Lowery and Sears 1986, Morse and Zareh 1991, Mukherjee et al. 1993, Walthall and Stark 1997, Morse 1998, Kramarz and Stark 2003, Zanuncio et al. 2003, Cutler et al. 2005, Zalizniak and Nugegoda 2006, Cutler et al. 2009, Guedes et al. 2010, Yu et al. 2010, Guo et al. 2012, Cutler 2013). However, many aspects pertaining to the hormetic response, including life-stage sensitivity, effects of different routes of exposure and duration of exposure, effects across generations, consequences on biological fitness, and gene-level responses remain poorly studied (Cutler 2013).

In an attempt to address a few of these aspects, the first objective of my thesis investigated transgenerational effects on reproductive responses of *M. persicae* exposed to sublethal concentrations of imidacloprid. I explored various exposure scenarios over multiple generations to examine the temporal nature and biological consequences of the hormetic dose-response (Chapter 2). A temporal shift in the peak hormetic concentration was evident between the first and second generation that later was compromised in third and fourth generations in a

continuous exposure scenario (Chapter 2). This probably was due to exhaustion of potential resources used to deal with the stress, which were over-exploited in the first two generations. Doubling of fecundity in the first and second generations followed by a steep decline in later generations at the same concentrations clearly underscores the occurrence of transgenerational trade-offs. Cutler et al. (2009) demonstrated this change over time using *M. persicae* adults when only second generation fecundity (50% higher than controls) was hormetic following exposure to low doses of imidacloprid.

The groundwork for this objective started with identifying tradeoffs within a generation. Following exposure to a stressor, insecticide-induced hormesis will often stimulate longevity but this comes at the expense of reduced fecundity (Hercus et al. 2003), or vice versa. In other words, hormesis results in tradeoffs involved in allocation of resources (energetic) among life history traits (Forbes 2000, Jager et al. 2012). When I used fecundity, longevity and length of the adult as endpoints, tradeoffs were not apparent but, interestingly, similar trends were found in fecundity and longevity suggesting simultaneous stimulation among life history traits i.e., multiple-trait tradeoffs (Agrawal et al. 2010). Similar results were reported in other insect-insecticide models (Cutler et al. 2009). With limited resources, multiple-trait tradeoffs in an organism could place its immune system in jeopardy (Sheldon and Verhulst 1996, Schulenburg et al. 2009). However, I suggest that a similar scenario might not prevail in my test subjects because of two reasons. Temporally, tradeoffs were apparent (reproductive responses) in continuous exposure scenario suggesting that fecundity of one generation should be compared to fecundity of the next and not longevity or other endpoints. Hence, when tradeoffs are evident in fecundity transgenerationally, the immune system or other detrimental systems may not be compromised. Secondly, the greenhouse results (r_i increase) that indicated adaptation to a

stressor (imidacloprid) probably could be extrapolated to any given stressor because hormesis is a general phenomenon that results from triggering adaptive pathways (Costantini et al. 2010, Son et al. 2010).

As adults, the aphids probably were able to recover from transient over-corrections due to sublethal concentrations of imidacloprid (Cutler et al. 2009) and hence fecundity of parental generation was not hormetic. *M. persicae* nymphs pass through four molts before developing into adults and probably these molts enable it to entrain via overcompensation to certain sublethal concentrations of imidacloprid (Chapter 2). It was evident in a one-time exposure scenario where first instars exposed briefly (2 days) to the lowest concentration produced fewer offspring than the control as the adults probably had less energetic resources remaining to allocate to reproduction after handling the tolerable stress i.e., able to deal with those transient over-corrections at the cost of reproduction. Below hormetic concentrations there could be ultra-low doses that are detrimental to the exposed organism and this is termed hypersensitivity (Sykes et al. 2006). The occurrence of hypersensitivity (i.e., reduced fecundity) that I observed with short-term exposure has previously been observed in insects (Luckey 1968) and plants (Belz and Piepho 2012). This was seen in my greenhouse experiment where treatment of plants with 0.25 µg imidacloprid L^{-1} resulted in almost two-fold increase in r_i and 4.5-fold more total aphids compared to controls while certain concentrations below this decreased the number of aphids (Chapter 2). When an organism exclusively allocates resources to combat the stressor it might survive the situation only until such time as a build-up of damage due to minimal dedication towards maintenance of basic metabolic functions, passes a threshold, i.e., slow-death (McNamara and Buchanan 2005). In my experiments this was constantly evident at higher concentrations (e.g., 25 µg imidacloprid L^{-1}) in the brief-exposure scenarios. When maintenance

is not compromised, this thriving capacity could result in/invested towards higher fecundity at concentrations just below the highest concentration (e.g., 10 µg imidacloprid L^{-1}). Similar findings were reported where mortality in an organism increased after the removal of the stressor (McNamara and Buchanan 2005).

Models that fit hormetic data (Cedergreen et al. 2005) provided a good fit for reproductive responses in the continuous exposure scenario (first two generations) and r_i from the greenhouse experiment (Chapter 2). However, the modeled data masks the "dips" which I assume to be due to a toxic/hypersensitive concentration (resulted in lower fecundity than controls) in the hormetic zone. Similar limitations were previously noted in statistical models that describe hormesis (Belz and Piepho 2012). I consistently found pre-hormetic (hypersensitivity) toxicity in several of my experiments (Chapters 2 and 4). Visually, these might fit a "triphasic" model (N-shaped) as opposed to biphasic (Sykes et al. 2006). The occurrence and mechanisms of this phenomenon should be explored further, along with refinement of models that better take into account such pre-hormetic toxicity.

The second objective of my thesis focused on gene regulation during imidacloprid-induced hormesis. Initially gene-level responses (up- or down-regulation) were assessed as an endpoint to hormesis. Surprisingly, besides mirroring the fecundity responses in few instances (Chapter 2), tradeoffs were observed transgenerationally when gene regulation during imidacloprid-induced hormesis was analyzed at four time points (second instar nymphs and adults of first and second generations) (Chapter 3). Several stress, dispersal, and developmental genes were affected in multiple instances, and there was evidence of gene up-regulation countered by down-regulation in subsequent life stages or generations. Alternation of up- and down-regulation of Hsp gene occurred across generations and between nymphs and adults within

a generation. Down-regulation of Hsp expression is probably a recovery response to regain homeostasis (Cappello et al. 2006, Shutoh et al. 2009). Down-regulation of *Hsp60* expression observed at several time points could have been due to the organism undergoing a recovery process following mild stress exposure (Cappello et al. 2006, Shutoh et al. 2009).

Dispersal-related genes such as olfactory segment-D (*OSD*), take-out like (*TOL*) and adenosine nucleotide transferase (*ANT*) are more highly expressed (2-5 fold) in alates than apterous aphids (Ghanim et al. 2006). I found that *OSD*, *TOL* and *ANT* gene expression varied in apterous individuals exposed continuously to sublethal hormetic concentrations of imidacloprid and no wing development was observed. A series of up- and down-regulations depicts initial disruption followed by compensation/overcompensation finally leading to new-normal or adapted state. Similar trends were observed in *TOL* gene regulation (Chapter 3) but no correlation to fecundity was obtained.

ANT gene was unresponsive initially (G0) when stress was administered suggesting that exposure to hormetic concentrations of imidacloprid results in no additional energy expenditures for the aphids. However, as with *TOL* and *Hsp60* genes, significant up-regulation of *ANT* occurred in G1 nymphs, indicating extra energy was expended. Down-regulation of the *ANT* gene in G1 adults probably depicts the exhaustion of energy during the development process. In insects, increased JH titers could be due to down-regulation of farnesyl diphosphate synthase (*FPPS*)-I gene (Keeling et al. 2004) and vice versa (Belles et al. 2005). Significant down-regulation of *FPPS* was seen at imidacloprid concentrations that did or did not result in stimulated reproduction, suggesting no direct correlation of this gene with fecundity. However, tight regulatory responses that were hormetic in nature were observed among the time points selected.

Activation (up-regulation) or inactivation (down-regulation) of genes in a response to external cue is regulated by DNA methylation. Increases in methylation, termed hypermethylation, typically reduce DNA transcription and usually result in inactivation of genes, although this is not always the case (Suzuki and Bird 2008). It is an important epigenetic mechanism for regulation of gene expression is thought to play a role in developmental responsiveness to environmental factors and may provide critical contributions to insect developmental and phenotypic variation (Bass and Field 2011, Glastad et al. 2011, Gressel 2011). In *M. persicae*, reduced DNA methylation coincides with a loss of E4 gene expression (Hick et al. 1996, Field et al. 2004). Imidacloprid resistance in insects via mutations has been reported in the past (Elbert and Nauen 1996, Wen et al. 2009, Bass and Field 2011) and insecticide-resistance is associated with amplification and methylation of esterase genes (Ono et al. 1999, Field 2000, Field et al. 2004). Although further investigation is needed, my data on global methylation suggests that insecticide-induced hormesis might serve as a precursor to insecticide tolerance and ultimately resistance (Gressel 2011) as inheritance of adaptive traits was evident (Chapter 2). Global DNA methylation occurred initially but not in later generations suggesting possible inheritance of adaptive traits and transgenerationally the insect could be able to cope with higher levels of stress (higher concentrations).

The third objective of my thesis addressed an interesting question. When *M. persicae* were exposed to sublethal concentrations of imidacloprid, concurrent stimulation in fecundity and JH III titers was observed (Yu et al. 2010) and JH is essential for vitellogenin (Vg) synthesis (Hartfelder 2000). Blocking JH might affect its involvement in hormetic responses but this hypothesis was rejected because precocene, which antagonizes JH production (i.e., no stimulation in reproduction) at sublethal concentrations, increased reproduction in *M. persicae* at

certain concentrations. In *Tribolium castaneum* (Herbst), Vg synthesis still occurred with blocked transduction of JH as long as the insulin pathway (Fork head transcription factor (FoxO)) was not hindered (Sheng et al. 2011). This suggests that low doses of precocene could trigger a hormetic response through adaptive cellular stress response pathways such as the insulin pathway (FoxO pathway) (Son et al. 2010), phenotypically manifesting as increased reproduction (Chapter 4). Although tight regulatory responses were noted in other genes (*OSD*, *TOL*, *ANT* and *Hsp60*) in *M. persicae* exposed to low doses of precocene, no clear connection to fecundity was observed. Compared to gene regulation during imidacloprid-induced hormesis, the magnitude of gene regulation was several-fold higher during precocene-induced hormesis. Not every gene analyzed responded similarly to imidacloprid-induced regulation. Irrespective of the stressor used, *ANT* and *FPPS* I had similar trends but not the magnitude of regulation. This again reiterates the probable involvement of adaptive cellular response pathways but not the regulation pathway of reproduction as in an unexposed/untreated insect. The several-fold change in gene expression could be due the regulatory mode of action of the insecticide (precocene) or the response as a result of brief-exposure. But, the phenotypic responses (fecundity) surprisingly had similar patterns (N-shaped).

CONCLUSION

In this thesis I have found that insecticide-induced hormesis is real and reproducible. When certain criteria, such as the use of a sensitive stage of the organism, precise route and duration of exposure, inclusion of stressor concentrations 10-20 fold below the traditional threshold, are met, the response is dramatic. As understood in the past, the nature of a hormetic curve is biphasic, but not smooth. Also, depending on the duration of exposure, the curve could appear to be triphasic (N-shaped) in nature. I acknowledge the occurrence of hypersensitive and hormetic

concentrations below the traditional threshold that makes modeling hormetic data quite challenging. Not all concentrations below the threshold being hormetic coupled with the modest response of hormetic concentrations de-emphasize its crucial role as a precursor in pest resurgence.

Inclusion of temporal aspect into hormetic studies assists one to understand the underlying principles of overcompensation stimulation hormesis. The effect is not limited to phenotypic data but can be traced into gene-level (up- or down-regulation) responses and more evidently in global DNA methylation data where inheritance was clearly traced. However, direct linkage between gene-level and phenotypic responses were not identified suggesting that hormesis is triggered by adaptive cellular stress response pathways but not the normal regulatory pathways.

PROSPECTS FOR FUTURE WORK

The inclusion of biochemical-level endpoints such as measuring the JH titers or vitellogenin titers on par with stimulated fecundity coupled with *FPPS* gene regulation could have "connected the dots" and should be addressed for two reasons. First, fecundity is directly proportional to amount of vitellogenin that is regulated by JH (Belles et al. 2005). *FPPS* gene regulation could target JH or (E) β Farnesene. If down-regulation of *FPPS* corresponds to increased JH (Keeling et al. 2004), one can deduce its correlation with stimulation in fecundity. But if the opposite occurs, down-regulation corresponding to decreased JH, no correlation can be deduced. Second, if no connection between the processes always exists (Sheng et al. 2011), one can affirm that hormetic response occurs due to trigger of adaptive cellular stress response pathways (Son et al. 2010).

Again, although I analyzed Hsp 60 gene responses with respect to insecticide-induced hormesis, which indirectly might have emphasized the involvement of heat shock factor pathway, no clear connection with fecundity was derived. Instead, if adaptive stress response pathways (gene regulation and related biochemistry) were studied, a better understanding of the "cause-effect" relationship of the responses observed at higher hierarchical levels (individual or population) may have been possible. For example, analyzing genes involved in the insulin pathway and their regulation during insecticide-induced hormesis is always a better route to choose because it is recently learnt that those genes might influence JH or vitellogenin in certain conditions (Sheng et al. 2011).

Phenotypically, reproduction in insects serves as an excellent endpoint in such studies but it should be coupled with other traits. For example, if aphids were to be used as test subjects, longevity, length of the antennae, length of the cornicles, and weight could be used. Tradeoffs (intra- and transgenerational) might be evident when such combinations are addressed in a single study. Bagging the insects in greenhouse experiments did not have an effect on aphid movement as most of the aphids were found in the top 5-10 cm of the plants. But, hormesis experiments should be attempted in field conditions thus accounting for possible interactions (natural enemies, abiotic factors) and elimination of artificial factors (bagging). Xenohormesis (inter species/genera communications) on natural enemies and by-stander/spectator effects (intra specific communications) might add-up to make insecticide-induced hormesis evident and highlight its role in pest resurgence. Insecticide-induced hormesis might be defined as "seemingly innocuous" because of its modest responses yet dangerous consequences.

Integrated pest management tactics assist in judicial use of pesticides and probably reduce the occurrence of hormetic concentrations. Considering the duration for a pest to make

an insecticide ineffective and or resistant, hormesis studies should be employed in pre-trials to avoid or lengthen the duration. Although complete elimination of pesticides holds the key to most of these problems (such as pest resurgence), the immediate and effective control tactic (chemical control) is needed in current day agriculture for maximum profits. Rotation and or inclusion of new insecticides with unique modes of action find its demand in current circumstances.

6 REFERENCES

Agrawal, A. A., J. K. Conner, and S. Rasmann. 2010. Tradeoffs and negative correlations in evolutionary ecology . In M. A. Bell, W.F. Eanes, D.J. Futuyma, and J.S. Levinton (ed.), Evolution After Darwin: the First 150 Years. Sinauer Associates, Sunderland, MA.

Amiri, A., A. R. Bandani, and S. Ravan. 2010. Effect of an anti-juvenile hormone agent (Precocene I) on Sunn pest, *Eurygaster integriceps* (Hemiptera: Scutelleridae) development and reproduction. Afr. J. Biotechnol. 9: 5859-5868.

Bai, D., S. C. R. Lummis, W. Leicht, et al. 1991. Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neuron. Pestic. Sci. 33: 197-204.

Bass, C., and L. M. Field. 2011. Gene amplification and insecticide resistance. Pest Manag. Sci. 67: 886-890.

Belles, X., D. Martin, and M. D. Piulachs. 2005. The mevalonate pathway and the synthesis of juvenile hormone in insects. Annu. Rev. Entomol. 50: 181-199.

Belz, R. G., and H. P. Piepho. 2012. Modeling effective dosages in hormetic dose-response studies. PLoS One 7: e33432.

Belz, R. G., N. Cedergreen, and H. Sorensen. 2008. Hormesis in mixtures – Can it be predicted? Sci. Tot. Environ. 404: 77-87.

Bird, A. 2007. Perceptions of epigenetics. Nature 447: 396-398.

Blackman, R. L. 1974. Life-cycle variation in *Myzus persicae* (Sulz.) (Hom., Aphidiadae) in different parts of the world, in relation to genotype and environment. Bull. Entomol. Res. 63: 595-607.

Blackman, R. L., and V. F. Eastop. 1995. Aphids on the world's crops: An Identification and Information Guide, John Wiley & Sons New York, USA.

Blackman, R. L., and V. F. Eastop. 2000. Aphids on the world's crops: An Identification and Information Guide, 2nd ed. John Wiley & Sons, Chichester, UK.

Bohbot, J., and R. G. Vogt. 2005. Antennal expressed genes of the yellow fever mosquito (*Aedes aegypti* L.); characterization of odorant-binding protein 10 and takeout. Insect Biochem. Mol. Biol. 35: 961-979.

Bonasio, R., S. Tu, and D. Reinberg. 2010. Molecular signals of epigenetic states. Science 330: 612-616.

Bos, J. I. B., D. Prince, M. Pitino, et al. 2010. A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). PLoS Genet. 6.

Bowers, W. S. 1976. Discovery of insect anti-allatotropins, pp. 394-408. In L. Gilbert (ed.), The juvenile hormones. Plenum Press, New York.

Bowers, W. S., T. Ohta, J. S. Cleere, et al. 1976. Discovery of insect anti-juvenile hormones in plants. Science 193: 542-547.

Braendle, C., G. K. Davis, J. A. Brisson, et al. 2006. Wing dimorphism in aphids. Heredity 97: 192-199.

Brain, P., and R. Cousens. 1989. An equation to describe dose responses where there is stimulation of growth at low-doses. Weed Res. 29: 93-96.

Brisson, J. A. 2010. Aphid wing dimorphisms: linking environmental and genetic control of trait variation. Phil. Trans. R. Soc. B 365: 605-616.

Calabrese, E. J. 2005. Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. Environ. Pollut. 138: 379-411.

Calabrese, E. J. 2008. Hormesis: Why it is important to toxicology and toxicologists? Environ. Toxicol. Chem. 27: 1451-1474.

Calabrese, E. J. 2010. Hormesis is central to toxicology, pharmacology and risk assessment. Hum. Exp. Toxicol. 29: 249-261.

Calabrese, E. J., and L. A. Baldwin. 2002. Defining hormesis. Hum. Exp. Toxicol. 21: 91-97.

Calabrese, E. J., and L. A. Baldwin. 2003. Toxicology rethinks its central belief. Nature 421: 691-692.

Calabrese, E. J., I. Iavicoli, and V. Calabrese. 2013. Hormesis: Its impact on medicine and health. Hum. Exp. Toxicol. 32: 120-152.

Calabrese, E. J., K. A. Bachmann, A. J. Bailer, et al. 2007. Biological stress response terminology: Integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. Toxicol. Appl. Pharmacol. 222: 122-128.

Campiche, S., K. Becker-Van Slooten, C. Ridreau, et al. 2006. Effects of insect growth regulators on the nontarget soil arthropod *Folsomia candida* (Collembola). Ecotoxi. Environ. Safe. 63: 216-225.

Campos, B., B. Pina, M. Fernandez-Sanjuan, et al. 2012. Enhanced offspring production in *Daphnia magna* clones exposed to serotonin reuptake inhibitors and 4-nonylphenol. Stage- and food-dependent effects. Aquat. Toxicol. 109: 100-110.

Cappello, F., A. Di Stefano, S. David, et al. 2006. HSP60 and HSP10 down-regulation predicts bronchial epithelial carcinogenesis in smokers with chronic obstructive pulmonary disease. Cancer 107: 2417-2424.

Carelli, G., and I. Iavicoli. 2002. Defining hormesis: The necessary tool to clarify experimentally the low dose-response relationship. Hum. Exp. Toxicol. 21: 103-104.

Casida, J. E., and G. B. Quistad. 2004. Why insecticides are more toxic to insects than people: the unique toxicology of insects. J. Pestic. Sci. 29: 81-86.

Cedergreen, N., C. Ritz, and J. C. Streibig. 2005. Improved empirical models describing hormesis. Environ. Toxicol. Chem. 24: 3166-3172.

Celorio-Mancera, M. L., S.-J. Ahn, H. Vogel, et al. 2011. Transcriptional responses underlying the hormetic and detrimental effects of the plant secondary metabolite gossypol on the generalist herbivore *Helicoverpa armigera*. BMC Genomics 12.

Chelliah, S., and E. A. Heinrichs. 1980. Factors affecting insecticide-induced resurgence of the brown plant hopper, *Nilaparvata lugens*, on rice. Environ. Entomol. 9: 773-777.

Chelliah, S., L. T. Fabellar, and E. A. Heinrichs. 1980. Effect of sub-lethal doses of three insecticides on the reproductive rate of the brown planthopper, *Nilaparvata lugens*, on rice. Environ. Entomol. 9: 778-780.

Cohen, E. 2006. Pesticide-mediated homeostatic modulation in arthropods. Pest. Biochem. Physiol. 85: 21-27.

Cooper, J., and H. Dobson. 2007. The benefits of pesticides to mankind and the environment. Crop Prot. 26: 1337-1348.

Costantini, D., N. B. Metcalfe, and P. Monaghan. 2010. Ecological processes in a hormetic framework. Ecol. Lett. 13: 1435-1447.

Costantini, D., P. Monaghan, and N. B. Metcalfe. 2012. Early life experience primes resistance to oxidative stress. J. Exp. Biol. 215: 2820-2826.

Cox, C. 1987. Threshold dose-response models in toxicology. Biometrics 43: 511-523.

Cui, X., F. Wan, M. Xie, et al. 2008. Effects of heat shock on survival and reproduction of two whitefly species, *Trialeurodes vaporariorum* and *Bemisia tabaci* biotype B. J. Insect Sci. 8: 1-10.

Cusson, M., C. Beliveau, S. E. Sen, et al. 2006. Characterization and tissue-specific expression of two lepidopteran farnesyl diphosphate synthase homologs: Implications for the biosynthesis of ethyl-substituted juvenile hormones. Proteins: Struct., Funct., Bioinf. 65: 742-758.

Cutler, G. C. 2013. Insects, insecticides and hormesis: Evidence and considerations for study. Dose-Response 11: 154-177.

Cutler, G. C., C. D. Scott-Dupree, J. H. Tolman, et al. 2005. Acute and sublethal toxicity of novaluron, a novel chitin synthesis inhibitor, to *Leptinotarsa decemlineata* (Coleoptera : Chrysomelidae). Pest Manag. Sci. 61: 1060-1068.

Cutler, G. C., K. Ramanaidu, T. Astatkie, et al. 2009. Green peach aphid, *Myzus persicae* (Hemiptera: Aphididae), reproduction during exposure to sublethal concentrations of imidacloprid and azadirachtin. Pest Manag. Sci. 65: 205-209.

Dauwalder, B., S. Tsujimoto, J. Moss, et al. 2002. The *Drosophila* takeout gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. Genes Dev. 16: 2879-2892.

Davis, J. M., and D. Svendsgaard. 1990. U-shaped dose-response curves: Their occurrence and implications for risk assessment pp. 71-83, J. Tox. Environ. Health.

Dawson, G. W., D. C. Griffiths, N. F. Janes, et al. 1987. Identification of an aphid sex-pheromone. Nature 325: 614-616.

de Vos, M., W. Y. Cheng, H. E. Summers, et al. 2010. Alarm pheromone habituation in *Myzus persicae* has fitness consequences and causes extensive gene expression changes. Proc. Nat. Acad. Sci. USA 107: 14673-14678.

Dhadialla, T. S., A. Retnakaran, and G. Smagghe. 2005. Insect growth and development disrupting insecticides, pp. 55-155. In L. I. Gilbert, K. Iatrou and S. S. Gill (eds.), Comprehensive Molecular Insect Science, vol. 6. Pergamon press, New York, NY.

Dittrich, V., P. Streibert, and P. A. Bathe. 1974. An old case reopened: mite stimulation by insecticide residues. Environ. Entomol. 3: 534-540.

Dixon, A. F. G. 1998. Aphid ecology: an optimization approach, 2nd ed. Blackie & Sons, London.

Doutt, R. L., and R. F. Smith. 1971. The pesticide syndrome - diagnosis and suggested prophylaxis, pp. 3-15. *In* C. B. Huffaaker [ed.], Biol. control. Plenum Press, New York , NY.

Elbert, A., and R. Nauen. 1996. Bioassays for imidacloprid for resistance monitoring against whitefly *Bemisia tabaci*, pp. 731-738. In C. Brit. Crop Protect. (ed.), Proc. Brighton Crop, Prot. Conf. Pests Dis. , UK 2.

Elbert, A., R. Nauen, and W. Leicht. 1998. Imidacloprid, a novel chloronicotinyl insecticide: biological activity and agricultural importance., Springer, Berlin Heidelberg New York.

Elbert, A., B. Becker, J. Hartwig, et al. 1991. Imidacloprid-a new systemic insecticide. Pflanzenschutz-Nachr 44: 113-136.

Fan, J., F. Francis, Y. Liu, et al. 2011. An overview of odorant-binding protein functions in insect peripheral olfactory reception. Genet. Mol. Res. 10: 3056-3069.

Farazmand, H., and S. Y. Chaika. 2011. Effects of precocene I and II on the sensory system of antennae and mouthparts of Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Col.: Chrysomelidae). J. Entomol. Soc. Iran 31: 41-56.

Field, L. M. 2000. Methylation and expression of amplified esterase genes in the aphid *Myzus persicae* (Sulzer). Biochem. J. 349: 863-868.

Field, L. M., F. Lyko, M. Mandrioli, et al. 2004. DNA methylation in insects. Insect Mol. Biol. 13: 109-115.

Figueroa, C. C., N. Prunier-Leterme, C. Rispe, et al. 2007. Annotated expressed sequence tags and xenobiotic detoxification in the aphid *Myzus persicae* (Sulzer). Insect Sci. 14: 29-45.

Forbes, V. E. 2000. Is hormesis an evolutionary expectation? Funct. Ecol. 14: 12-24.

Frisbie, R. E. 2006. Relevance of pesticides in the management of arthropod pests, pp. 148. In J. N. All and M. F. Treacy (eds.), Use and management of insecticides, acaricides, and transgenic crops. The Entomological Society of America, Lanham, MD.

Fujikawa, K., K. Seno, and M. Ozaki. 2006. A novel Takeout-like protein expressed in the taste and olfactory organs of the blowfly, *Phormia regina*. Febs J. 273: 4311-4321.

Ghanim, M., A. Dombrovsky, B. Raccag, et al. 2006. A microarray approach identifies ANT, OS-D and takeout-like genes as differentially regulated in alate and apterous morphs of the green peach aphid *Myzus persicae* (Sulzer). Insect Biochem. Mol. Biol. 36: 857-868.

Glastad, K. M., B. G. Hunt, S. V. Yi, et al. 2011. DNA methylation in insects: on the brink of the epigenomic era. Insect Mol. Biol. 20: 553-565.

Goodman, W. G., and N. A. Granger. 2005. The juvenile hormones, pp. 319-408. In L. I. Gilbert, K. Iatrou and S. S. Gill (eds.), Comprehensive Molecular Insect Science, vol. 3. Elsevier, Boston.

Gressel, J. 2011. Low pesticide rates may hasten the evolution of resistance by increasing mutation frequencies. Pest Manag. Sci. 67: 253-257.

Guedes, N. M. P., J. Tolledo, A. S. Correa, et al. 2010. Stimulatory sublethal response of a generalist predator to permethrin: hormesis, hormoligosis, or homeostatic regulation? J. Appl. Entomol. 134: 142-148.

Guedes, R. N. C. 2009. Stimulatory sublethal response of a generalist predator to permethrin: Hormesis, hormoligosis, or homeostatic regulation? J. Econ. Entomol. 102: 170-176.

Guo, R. X., X. K. Ren, and H. Q. Ren. 2012. Effects of dimethoate on rotifer *Brachionus calyciflorus* using multigeneration toxicity tests. J. Environ. Sci. Health. [B] 47: 883-890.

Hales, D. F. 1976. Juvenile hormone and aphid polymorphism, pp. 105-115. *In* M. Luscher [ed.], Phase and caste determination in insects. Pergamon Press, Oxford.

Hamnett, A. F., and G. E. Pratt. 1983. The absolute-configuration of precocene-I dihydrodiols produced by metabolism of precocene-I by corpora allata of *Locusta migratoria*, *in vitro* Life Sci. 32: 2747-2753.

Hartfelder, K. 2000. Insect juvenile hormone: from "status quo" to high society. Braz. J. Med. Biol. Res. 33: 157-177.

Helmcke, K. J., and M. Aschner. 2010. Hormetic effect of methylmercury on *Caenorhabditis elegans*. Toxicol. Appl. Pharmacol. 248: 156-164.

Henschler, D. 2006. The origin of hormesis: historical background and driving forces. Hum. Exp. Toxicol. 25: 347-351.

Hercus, M. J., V. Loeschcke, and S. I. S. Rattan. 2003. Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress. Biogerontology 4: 149-156.

Hick, C. A., L. M. Field, and A. L. Devonshire. 1996. Changes in the methylation of amplified esterase DNA during loss and reselection of insecticide resistance in peach-potato aphids, *Myzus persicae*. Insect Biochem. Mol. Biol. 26: 41-47.

Huang, L.-H., B. Chen, and L. Kang. 2007. Impact of mild temperature hardening on thermo tolerance, fecundity, and Hsp gene expression in *Liriomyza huidobrensis*. J. Insect Physiol. 53: 1199-1205.

Ishaaya, I., and A. R. Horowitz. 1998. Insecticides with novel modes of action: An overview, pp. 289. In I. Ishaaya and D. Degheele (eds.), Insecticides with novel modes of action: Mechanism and application. Springer, Delhi.

Jacobs, S. P., A. P. Liggins, J. J. Zhou, et al. 2005. OS-D-like genes and their expression in aphids (Hemiptera : Aphididae). Insect Mol. Biol. 14: 423-432.

Jager, T., A. Barsi, and V. Ducrot. 2012. Hormesis on life-history traits: is there such thing as a free lunch? Ecotoxicol. 21: 1-8.

Janmaat, A., E. Borrow, J. Matteoni, et al. 2011. Response of a red clone of *Myzus persicae* (Hemiptera: Aphididae) to sublethal concentrations of imidacloprid in the laboratory and greenhouse. Pest Manag. Sci. 67: 719-724.

Jeschke, P., and R. Nauen. 2008. Neonicotinoids - from zero to hero in insecticide chemistry. Pest Manag. Sci. 64: 1084-1098.

Kambhampati, S., M. Mackauer, and K. K. Nair. 1984. Precocious metamorphosis and wing formation in the pea aphid, *Acyrthosiphon pisum*, induced by precocene analog 7-ethoxy-6-methoxy-2,2-dimethylchromene. Arch. Insect Biochem. 1: 147-154.

Karouna-Renier, N. K., and J. P. Zehr. 1999. Ecological implications of molecular biomarkers: assaying sub-lethal stress in the midge *Chironomus tentans* using heat shock protein 70 (HSP-70) expression. Hydrobiologia 401: 255-264.

Karunker, I., J. Benting, B. Lueke, et al. 2008. Over-expression of cytochrome P450 *CYP6CM1* is associated with high resistance to imidacloprid in the B and Q biotypes of Bemisia tabaci (Hemiptera: Aleyrodidae). Insect Biochem. Mol. Biol. 38: 634-644.

Keeling, C. I., G. J. Blomquist, and C. Tittiger. 2004. Coordinated gene expression for pheromone biosynthesis in the pine engraver beetle, *Ips pini* (Coleoptera : Scolytidae). Naturwissenschaften 91: 324-328.

Kennedy, J. S., M. F. Day, and V. F. Eastop. 1962. A conspectus of aphids as vectors of plant viruses, Commonwealth Institute of Entomology, London.

Kramarz, P., and J. D. Stark. 2003. Population level effects of cadmium and the insecticide imidacloprid to the parasitoid, *Aphidius ervi* after exposure through its host, the pea aphid, *Acyrthosiphon pisum* (Harris). Biol. Control 27: 310-314.

Kuenen, D. J. 1958. Influence of sublethal doses of DDT upon the multiplication rate of *Sitophilus granarius* (Coleoptera: Curculionidae). Entomol. Exp. Appl. 1: 147-152.

Le Bourg, E., K. Malod, and I. Massou. 2012. The NF-kappa B-like factor DIF could explain some positive effects of a mild stress on longevity, behavioral aging, and resistance to strong stresses in *Drosophila melanogaster*. Biogerontology 13: 445-455.

Leroy, M., T. Mosser, X. Maniere, et al. 2012. Pathogen-induced *Caenorhabditis elegans* developmental plasticity has a hormetic effect on the resistance to biotic and abiotic stresses. BMC Evol. Biol. 12.

Lewis, M. J., I. M. Prosser, A. Mohib, et al. 2008. Cloning and characterisation of a prenyltransferase from the aphid *Myzus persicae* with potential involvement in alarm pheromone biosynthesis. Insect Mol. Biol. 17: 437-443.

Liu, M. Y., and J. E. Casida. 1993. Relevance of [^{3}H] imidacloprid in the insect acetylcholine receptor. Pest. Biochem. Physiol. 46: 40-46.

Lowery, D. T., and M. K. Sears. 1986. Stimulation of reproduction of green peach aphid (Homoptera: Aphididae) by azinphos-methyl applied to potatoes. J. Econ. Entomol. 79: 1530 - 1533.

Luckey, T. D. 1968. Insecticide hormoligosis. J. Econ. Entomol. 61: 7-12.

Luckey, T. D. 2008. Sir Samurai T. D. Luckey, PhD. Dose-Response 6: 97-112.

Lv, L., Q. Jiang, X. Chen, et al. 2012. Effects of Juvenile Hormone and Precocene on the Reproduction of *Brachionus calyciflorus*. Int. Rev. Hydrobiol. 97: 435-444.

Magalhaes, L. C., R. N. C. Guedes, E. E. Oliveira, et al. 2002. Development and reproduction of the predator *Podisus distinctus* (Stal) (Heteroptera: Pentatomidae) exposed to sublethal doses of permethrin. Neotropical Entomol. 31: 445-448.

Mahmoudvand, M., H. Abbasipour, A. S. Garjan, et al. 2011. Sublethal effects of hexaflumuron on development and reproduction of the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). Insect Sci. 18: 689-696.

Mahroof, R., K. Y. Zhu, and B. Subramanyam. 2005. Changes in expression of heat shock proteins in *Tribolium castaneum* (Coleopter: Tenebrionidae) in relation to developmental stage, exposure time and temperature. Ann. Entomol. Soc. Am. 98: 100-107.

Mattson, M. P. 2008. Hormesis defined. Ageing Res. Rev. 7: 1-7.

McNamara, J. M., and K. L. Buchanan. 2005. Stress, resource allocation, and mortality. Behav. Ecol. 16: 1008-1017.

Mittler, T. E. 1991. Juvenile hormone and aphid polymorphism, pp. 453-474. In A. P. Gupta (ed.), Morphogenetic hormones of arthropods: roles in histogenesis, organogenesis and morphogenesis. Rutgers University Press, New Brunswick, New Jersey.

Mittler, T. E., J. Eisenbach, J. B. Searle, et al. 1979. Inhibition of kinoprene of photo-period induced male production in apterous and alate vivparae of the aphid *Myzus persicae*. J. Insect Physiol. 25: 219-226.

Morse, J. G. 1998. Agricultural implications of pesticide-induced hormesis of insects and mites. Hum. Exp. Toxicol. 17: 266-269.

Morse, J. G., and N. Zareh. 1991. Pesticide-induced hormoligosis of citrus thrips (Thysanoptera: Thripidae) fecundity. J. Econ. Entomol. 84: 1169-1174.

Mosser, D. D., A. W. Caron, L. Bourget, et al. 2000. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol. Cell. Biol. 20: 7146-7159.

Mukherjee, S. N., S. K. Rawal, S. S. Ghumare, et al. 1993. Hormetic concentrations of azadirachtin and isoesterase profiles in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Experientia. 49: 557-560.

Mullins, J. W. 1993. Imidacloprid - a new nitroguanidine insecticide. ACS symp. Series 254: 183-198.

National-Research-Council. 2000. The future role of pesticides in US agriculture. National Academy Press, Washington D. C.

National-Research-Council. 2006. Health Risks from Exposure to Low Levels of Ionizing Radiation: BEIR VII Phase 2, The National Academies Press.

Nauen, R. 1995. Behaviour modifying effects of low systemic concentrations of Imidacloprid on *Myzus persicae* with special reference to an antifeeding response. Pestic. Sci. 44: 145-153.

Nauen, R. 2006. Insecticide mode of action: Return of the ryanodine receptor. Pest Manag. Sci. 62: 690.

Nauen, R., A. Ebbinghaus-Kintscher, A. Elbert, et al. 2001. Acetylcholine receptors as sites for developing neonicotinoid insecticides, pp. 77-105. In I. Ishaaya (ed.), Biochmical sites of insecticide action and resistance Springer, New York.

Noriega, F. G. 2004. Nutritional regulation of JH synthesis: a mechanism to control reproductive maturation in mosquitoes? Insect Biochem. Mol. Biol. 34: 687-693.

Nouzova, M., M. J. Edwards, J. G. Mayoral, et al. 2011. A coordinated expression of biosynthetic enzymes controls the flux of juvenile hormone precursors in the corpora allata of mosquitoes. Insect Biochem. Mol. Biol. 41: 660-669.

Olsen, A., M. C. Vantipalli, and G. J. Lithgow. 2006. Lifespan extension of *Caenorhabditis elegans* following repeated mild hormetic heat treatments. Biogerontology 7: 221-230.

Ono, M., J. J. Swanson, L. M. Field, et al. 1999. Amplification and methylation of an esterase gene associated with insecticide-resistance in greenbugs, *Schizaphis graminum* (Rondani) (Homoptera : Aphididae). Insect Biochem. Mol. Biol. 29: 1065-1073.

Parsell, D. A., and S. Lindquist. 1993. The function of heat-shock proteins in stress tolerance - degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27: 437-496.

Parsons, P. A. 2000. Hormesis: an adaptive fitness response and an evolutionary expectation in stressed free-living populations, with particular reference to ionizing radiation. J. Appl. Toxicol. 20: 103-112.

Parsons, P. A. 2001. The hormetic zone: An ecological and evolutionary perspective based upon habitat characteristics and fitness selection. Q. Rev. Biol. 76: 459-467.

Peric-Mataruga, V., V. Nenadovic, and J. Ivanovic. 2006. Neurohormones in insect stress: a review. Arch. Biol. Sci. 58: 1-12.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29.

Pickering, A. M., L. Vojtovich, J. Tower, et al. 2013. Oxidative stress adaptation with acute, chronic, and repeated stress. Free Radic. Biol. Med. 55: 109-118.

Pockley, A. G. 2003. Heat shock proteins as regulators of the immune response. Lancet 362: 469-476.

Puinean, A. M., S. P. Foster, L. Oliphant, et al. 2010. Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insectcides in the aphid *Myzus persicae*. PLoS Genet. 6: 1-11.

Ramanaidu, K., and G. C. Cutler. 2013. Different toxic and hormetic responses of *Bombus impatiens* to *Beauveria bassiana*, *Bacillus subtilis* and spirotetramat. Pest Manag. Sci. 69: 949.

Ramsey, J. S., A. C. C. Wilson, M. de Vos, et al. 2007. Genomic resources for *Myzus persicae*: EST sequencing, SNP identification, and microarray design. BMC Genomics 8: 423.

Riddiford, L. M. 1994. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. Adv. Insect Physiol. 24: 213-274.

Riddiford, L. M. 2003. Juvenile hormone: The lock or the key to insect metamorphosis. Comp. Biochem. Phys. A. 134A: S3-S3.

Rikhy, R., M. Ramaswami, and K. S. Krishnan. 2003. A temperature-sensitive allele of *Drosophila* sesB reveals acute functions for the mitochondrial adenine nucleotide translocase in synaptic transmission and dynamin regulation. Genetics 165: 1243-1253.

Rodriguez, M., L. B. Snoek, J. A. G. Riksen, et al. 2012. Genetic variation for stress-response hormesis in *C. elegans* lifespan. Exp. Geront. 47: 581-587.

Salvucci, M., D. Stecher, and T. Henneberry. 2000. Heat shock proteins in whiteflies, an insect that accumulates sorbitol in response to heat stress. J. Therm. Biol. 25 363-371.

Sanders, B. M., J. Nguyen, L. S. Martin, et al. 1995. Induction and subcellular localization of two major stress proteins in response to copper in the fathead minnow *Pimephales promelas*. Comp. Biochem. Physiol. C 112: 335-343.

Sarov-Blat, L., W. V. So, L. Liu, et al. 2000. The *Drosophila* takeout gene is a novel molecular link between circadian rhythms and feeding behavior. Cell 101: 647-656.

SAS 2008. OnlineDoc computer program, version 9.2. By SAS, Cary, NC.

Schaible, R., F. Ringelhan, B. H. Kramer, et al. 2011. Environmental challenges improve resource utilization for asexual reproduction and maintenance in hydra. Exp. Geront. 46: 794-802.

Schoffl, F., R. Prandl, and A. Reindl. 1998. Regulation of the heat-shock response. Plant Physiol. 117: 1135-1141.

Schooley, D. A., K. J. Judy, B. J. Bergot, et al. 1973. Biosynthesis of juvenile hormones of *Manduca sexta*: labeling pattern from mevalonate, propionate and acetate. Proc. Natl. Acad. Sci. USA 70: 2921-2925.

Schulenburg, H., J. Kurtz, Y. Moret, et al. 2009. Introduction. Ecological immunology. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364: 3-14.

Schwartzberg, E. G., G. Kunert, S. A. Westerlund, et al. 2008. Juvenile hormone titres and winged offspring production do not correlate in the pea aphid, *Acyrthosiphon pisum*. J. Insect Physiol. 54: 1332-1336.

Scott, B. R., S. A. Belinsky, S. Leng, et al. 2009. Radiation-stimulated epigenetic reprogramming of adaptive-response genes in the lung: An evolutionary gift for mounting adaptive protection against lung cancer. Dose-Response 7: 104-131.

Sheldon, B. C., and S. Verhulst. 1996. Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11: 317-321.

Sheng, Z., J. Xu, H. Bai, et al. 2011. Juvenile hormone regulates vitellogenin gene expression through insulin-like peptide signaling pathway in the red flour beetle, *Tribolium castaneum*. J. Biol. Chem. 286: 41924-41936.

Shutoh, Y., M. Takeda, R. Ohtsuka, et al. 2009. Low dose effects of dichlorodiphenyltrichloroethane (DDT) on gene transcription and DNA methylation in the hypothalamus of young male rats: implication of hormesis-like effects. J. Toxicol. Sci. 34: 469-482.

Sibly, R. M., and P. Calow. 1986. Physiological ecology of animals - an evolutionary approach. Blackwell, Scientific publications, Oxford, UK.

Sinkkonen, A., M. Myyrä, O.-P. Penttinen, et al. 2011. Selective Toxicity at Low Doses: Experiments with Three Plant Species and Toxicants. Dose-Response 9: 130-143.

Sladan, S., K. Miroslav, S. Ivan, et al. 2012. Resistance of Colorado potato beetle (Coleoptera: Chrysomelidae) to neonicotinoids, pyrethroids and nereistoxins in Serbia. Romanian Biotechnol. Lett. 17: 7599-7609.

Smirnoff, W. A. 1983. Residual effects of *Bacillus thuringiensis* and chemical insecticide treatments on spruce budworm (*Chroistonuera fumiferana* Clemens). Crop Prot. 2: 225-230.

So, W. V., L. Sarov-Blat, C. K. Kotarski, et al. 2000. Takeout, a novel *Drosophila* gene under circadian clock transcriptional regulation. Mol. Cel. Biol. 20: 6935-6944.

Son, T. G., R. G. Cutler, M. P. Mattson, et al. 2010. Transcriptional mediators of cellular hormesis, pp. 69-93. In M. P. Mattson and E. J. Calebrese (eds.), Hormesis: Revolution in biology, toxicology and medicine. Springer, NewYork.

Southam, C. M., and J. Ehrlich. 1943. Effects of extract of western red-cedar heartwood on certain wood-decaying fungi in culture. Phytopathology 33: 517-524.

Staal, G. B. 1986. Anti juvenile-hormone agents. Annu. Rev. Entomol. 31: 391-429.

Stanley, K., and B. Fenton. 2000. A member of the Hsp60 gene family from the peach potato aphid, *Myzus persicae* (Sulzer.). Insect Mol. Biol. 9: 211-215.

Stark, J. D., and J. E. Banks. 2003. Population-level effects of pesticides and other toxicants on arthropods. Annu. Rev. Entomol. 48: 505-519.

Su, J., S. Zhu, Z. Zhang, et al. 2006. Effect of synthetic aphid alarm pheromone (E)-beta-farnesene on development and reproduction of *Aphis gossypii* (Homoptera : Aphididae). J. Econ. Entomol. 99: 1636-1640.

Suhett, A. L., C. E. W. Steinberg, J. M. Santangelo, et al. 2011. Natural dissolved humic substances increase the lifespan and promote transgenerational resistance to salt stress in the cladoceran *Moina macrocopa*. Environ. Sci. Pollut. Res. 18: 1004-1014.

Suzuki, M. M., and A. Bird. 2008. DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. 9: 465-476.

Sykes, P. J., A. A. Morley, and A. M. Hooker. 2006. The PKZ1 recombination mutation assay: A sensitive assay for low dose studies. Dose-Response 4: 91-105.

Taban, A. H., C. Tittiger, G. J. Blomquist, et al. 2009. Isolation and characterization of farnesyl diphosphate synthase from the cotton boll weevil, *Anthonomus grandis*. Arch. Insect Biochem. Physiol. 71: 88-104.

Tamaki, G. 1973. Insect developmental inhibitors - effect of reduction and delay caused by juvenile-hormone mimics on production of winged migrants of *Myzus persicae* (Hemiptera: Aphididae) on peach trees. Can. Entomol. 105: 761-765.

Thany, S. H. 2010. Neonicotinoid insecticides: Historical evolution and resistance mechanisms., pp. 683. In S. H. Thany (ed.), Insect niconitinic acetylcholine receptors. Springer, New York.

Tillman, J. A., F. Lu, L. M. Goddard, et al. 2004. Juvenile hormone regulates *de novo* isoprenoid aggregation pheromone biosynthesis in pine bark beetles, *Ips* spp., through transcriptional control of HMG-CoA reductase. J. Chem. Ecol. 30: 2459-2494.

Tomizawa, M., and J. E. Casida. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu. Rev. Entomol. 48: 339-364.

Vaiserman, A. M. 2012. Hormesis and epigenetics: Is there a link? Ageing Res. Rev. 10: 413-421.

Vandermoten, S., M. C. Mescher, F. Francis, et al. 2012. Aphid alarm pheromone: An overview of current knowledge on biosynthesis and functions. Insect Biochem. Mol. Biol. 42: 155-163.

Vandermoten, S., S. Santini, E. Haubruge, et al. 2009. Structural features conferring dual Geranyl/Farnesyl diphosphate synthase activity to an aphid prenyltransferase. Insect Biochem. Mol. Biol. 39: 707-716.

Vandermoten, S., B. Charloteaux, S. Santini, et al. 2008. Characterization of a novel aphid prenyltransferase displaying dual geranyl/farnesyl diphosphate synthase activity. Febs Lett. 582: 2471-2471.

Veldhuizentsoerkan, M. B., D. A. Holwerda, C. A. Vandermast, et al. 1991. Synthesis of stress proteins under normal and heat-shock conditions in gill tissue of sea mussels (*Mytilus edulis*) after chronic exposure to cadmium. Comp. Biochem. Physiol. C 100: 699-706.

Velini, E. D., E. Alves, M. C. Godoy, et al. 2008. Glyphosate applied at low doses can stimulate plant growth. Pest Manag Sci 64: 489-496.

Verma, K. 1981. Roles of juvenile hormone in the green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae). M.Sc, University of Bristish Columbia Vancouver.

Walthall, W. K., and J. D. Stark. 1997. Comparison of two population-level ecotoxicological endpoints: The intrinsic ($r_{(m)}$) and instantaneous ($r_{(i)}$) rates of increase. Environ. Toxicol. Chem. 16: 1068-1073.

Wang, X. Y., Z. Q. Yang, Z. R. Shen, et al. 2008. Sublethal effects of the selected insecticides on fecundity and wing dimorphism of green peach aphid (Hom: Aphididae). J. Appl. Entomol. 132: 135-142.

Wanner, K. W., L. G. Willis, D. A. Theilmann, et al. 2004. Analysis of the insect os-d-like gene family. J. Chem. Ecol. 30: 889-911.

Ware, G. W., and D. M. Whitacre. 2004. The pesticide book, 6th ed. Meisterpro Information Resources, Willoughby, OH.

Weil, T., J. Korb, and M. Rehli. 2009. Comparison of queen-specific gene expression in related lower termite species. Mol. Biol. Evol. 26: 1841-1850.

Weiner, S. A., and A. L. Toth. 2012. Epigenetics in social insects: a new direction for understanding the evolution of castes. Genet. Res. Int. 2012: 609810-609810.

Wen, Y., Z. Liu, H. Bao, et al. 2009. Imidacloprid resistance and its mechanisms in field populations of brown planthopper, *Nilaparvata lugens* Stal in China. Pest. Biochem. Physiol. 94: 36-42.

Williams, C. M. 1967. Third generation pesticides. Sci. Am. 217: 13-17.

Wojda, I., P. Kowalski, and T. Jakubowicz. 2009. Humoral immune response of *Galleria mellonella* larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions. J. Insect Physiol. 55: 525-531.

www.ucdavis.edu. http://studentfarm.ucdavis.edu/edumat/Parr_08b_Insects_ID_sheets.pdf.

Wyatt, G. R., and K. G. Davey. 1996. Cellular and molecular actions of juvenile hormone II. Roles of juvenile hormone in adult insects. Adv. Insect Physiol. 26: 1-155.

Yin, X., S. Wang, J. Tang, et al. 2006. Thermal conditioning of fifth-instar *Cydia pomonella* (Lepidoptera: Totricidae) affects HSP70 accumulation and insect mortality. Physiol. Entomol. 31: 241-247.

Yoshimi, T., K. Minowa, N. K. Karouna-Renier, et al. 2002. Activation of a stress-induced gene by insecticides in the midge, *Chironomus yoshimatsui*. J. Biochem. Mol. Toxicol. 16: 10-17.

Yu, Y., G. Shen, H. Zhu, et al. 2010. Imidacloprid-induced hormesis on the fecundity and juvenile hormone levels of the green peach aphid *Myzus persicae* (Sulzer). Pest. Biochem. Physiol. 98: 238-242.

Zalizniak, L., and D. Nugegoda. 2006. Effect of sublethal concentrations of chlorpyrifos on three successive generations of *Daphnia carinata*. Ecotoxicol. Environ. Saf. 64: 207-214.

Zanuncio, T. V., J. E. Serrao, J. C. Zanuncio, et al. 2003. Permethrin - induced hormesis on the predator *Supputius cincticeps* (Stal, 1960) (Heteroptera, Pentatomidae). Crop Prot. 22: 941-947.

Zhang, J., J. Huang, J. X. Hou, et al. 2012. Heat and starvation induced hormesis in longevity of *Oomyzus sokolowskii* (Kurdjumov) (Hymenoptera: Eulophidae) adult females. J. Therm. Biol. 37: 696-701.

Zhang, T.-Y., and M. J. Meaney. 2010. Epigenetics and the Environmental Regulation of the Genome and Its Function, pp. 439-466, Annu. Rev. Psychol., vol. 61.

Zhang, Y. L., and Z. Li. 2008. Two different farnesyl diphosphate synthase genes exist in the genome of the green peach aphid, *Myzus persicae*. Genome 51: 501-510.

Zhang, Y. Q., J. Roote, S. Brogna, et al. 1999. Stress sensitive B encodes an adenine nucleotide translocase in *Drosophila melanogaster*. Genetics 153: 891-903.