Characterization of detoxification enzymes in Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae)

by

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Abstract

Japanese beetle, *Popillia japonica* Newman, is a highly polyphagous scarab with a host range of over 300 plant species spreading over 79 families. It is a cosmopolitan pest with agricultural, landscape and horticultural importance, over 460 million USD is spent every year to control both the larva and adult stage. Currently, it is unclear how this polyphagous scarab is able to utilize an array of host plants with very dissimilar chemistry. Thus, the goal of our research is to understand the biochemical defense of this generalist herbivore that confers on it the ability to utilize diverse host plants. This study examines the roles of the widely studied multiple enzymes systems cytochrome P450 (P450), glutathione S-transferase (GST) and carboxylesterase (CoE) in the physiology and host plant utilization by Japanese beetle.

In the first objective (Chapter 2) we studied the induction of P450, GST and CoE activities in midguts of adult Japanese beetle after it had consumed single-plant or mixed-plant diets of preferred (rose, Virginia creeper, crape myrtle and sassafras), or non-preferred hosts(boxelder, riverbirch and red oak). Non-preferred hosts were only sparingly fed upon yet induced higher activities of P450, GST and CoE than did feeding on preferred hosts. Similarly, beetles tended to have greater enzyme activities after feeding on a mixture of plants compared to a single plant type. Consequences of higher induced activities of detoxification enzymes by nonpreferred hosts on the physiology and feeding ecology of Japanese beetles are discussed.

In Chapter 3 (i.e. objective 2), we studied the connection between geranium (quisqualic acid) intoxication and the activities of P450, GST and CoE in the midgut of adult Japanese beetles. Beetles fed varying quantities of toxic petals of geranium flowers or agar plugs

containing quisqualic acid were assayed for midgut enzyme activities. Contrary to expectation, activities of P450, GST and CoE were induced in Japanese beetles that consumed both geranium petals and quisqualic acid. Peak enzyme activity occurred after 24 h of feeding but peak paralysis (intoxication) occurred after just 12 h. Our study suggests that consumption toxic geranium and quisqualic acid do induce of active detoxification enzymes, however this induction does not completely protect Japanese beetle from being paralyzed by geranium and quisqualic acid.

In the last objective (Chapter 4), we characterized the expression profiles of P450, GST and CoE in the immature life stages of Japanese beetles using multiple model substrates and also examined the influence of feeding duration and starvation on the activities of P450, GST and CoE in the midgut of adult Japanese beetle using a preferred host plant Virginia creeper. Our results show that activities of these enzymes are developmentally expressed in the different life stages of Japanese beetles, with enzymes from each life stage having varying affinity to the substrate and reaction speed. Furthermore, a feeding bout of 24 h was enough to induce optimal activities of P450, GST and CoE in the midgut of adult beetles. The consequences of the observed pattern of enzyme activities in the physiology and ecology of the Japanese beetle are discussed.

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CHAPTER 1

Introduction and Literature Review

1.1 Herbivore adaptation to xenobiotics

Phytophagous insects utilize plants to satisfy their nutrient requirements for fitness and survival. However plants have evolved different defensive tactics; morphological, behavioral, or biochemical in nature, to deter assaults from herbivores (Schoonhoven et al., 2005). It is widely acknowledged that herbivorous insects are challenged by toxic chemicals, known as secondary plant metabolites, from the their plant hosts (Dowd et al., 1983). Chemical defenses of plants are one of the main selection pressures that has led to the diversity in insect herbivores based on the range of plants they eat as food (Fraenkel, 1959). This paradigm is further evidenced by the fact that certain plant alleleochemicals are expressed in distinct families of plant (Fraenkel: 1959,1969; Schoonhoven et al., 2005). The feeding regimes of insect herbivores is a continuum that spans from monophagy; specialization on few plant species within a genera, to polyphagy; generalism on diverse plants species spreading across different plant families. In the middle of this continuum are the oligophagous herbivores that feed and oviposit on plants of closely related genera within a plant family (Krieger et al. 1971; Ahmad 1983; Bernays, 1999).

Plant primary chemical defenses are allelochemicals produced that negatively affect non-adapted herbivores by reducing digestibility of food and induces toxicity (Frankael, 1959; Krieger et al., 1971, Schoonhoven et al., 2005). The consequences of the deleterious effect of plant allelochemicals on insect herbivores include: 1) reduced growth and feeding, as observed when *Heloithis zea* consumes xanthotoxin containing diet (Neal and Berenbaum, 1989).,2) reduced fecundity and survival - generalist Japanese beetles lay fewer eggs and don't live for long when conditioned to toxic geranium plants (Potter and Held, 1999). Other negative effects of plant chemical defense on non-adapted herbivores results in oviposition preferences and exposure to predation, (Fordyce and Agrawal 2001; Schoonhoven et al., 2005) which limit the success and survival of herbivores. Interestingly, herbivores have coevolved by developing strategies to overcome the hurdle of plant toxins present in their diets. Sequestration and increased rates of excretion are methods by which insects disencumber plant toxins in their diets.

However, studies have shown that insects greatly rely on biochemical means of metabolizing these plant toxins in their diets through the use of detoxification enzymes (Dowd et al., 1983; Karban and Agrawal, 2002).

Cytochrome P450, glutathione *S* transferase (GST) and carboxlesterase (CoE) are superfamilies of detoxification enzymes widely studied for their roles in metabolism of insect xenobiotics (Berenbaum, 1991; Yu, 1982; 1984; Snyder et al., 1993; Francis et al., 2005; Xie et al., 2010). These enzymes act on endogenous substrates, generally toxic lipophilic plant toxins or insecticides, to increase solubility for easier elimination out of the insect's system (Feyereisen, 2005; Yu, 1996; Singh et al., 2001). These enzymes are involved in Phase I (P450, COE), Phase II (GST), or Phase III (ATP-binding cassette) stages of xenobiotic metabolism (Yu, 2008). Phase I reactions include oxidation, hydrolysis and reduction and they generally convert xenobiotic substrate into more polar products which can be excreted or subjected to phase II reactions. The phase II reactions are conjugation reactions involving endogenous compounds like amino acids, glutathione and others, thereby increasing the water solubility of the xenobiotic, making it easily excreted. Phase III reactions involves membrane-bound efflux pumps that transports the metabolites and toxic xenobiotics outside the cell cytoplasm (Broehan et al., 2013). Elevated activities of these enzymes involved in detoxification are linked with increased host plant utilization and insecticide resistance (Snyder and Glendenning, 1996; Yu, 1989; Yu et al., 2003; Zhang et al., 2007).

Insect herbivores usually specialize on host plant(s) on which they are metabolically-equipped to breakdown host plant defense (Feyereisen, 2005). The host range of insect herbivores is reflection of the plasticity of host plant defense adaptation. Therefore, studying the activities of detoxification enzymes in insect herbivores in relation to host plant feeding can reveal further insight of their roles in insect herbivory and can potentially lead to identification of target site of for incorporation of management tactics.

1.2 Previous studies on detoxification enzymes (P450, GST and COE) in other herbivorous insects: P450s and plant allelochemicals

P450s (i.e., cytochrome P450, mixed function oxidases) represents a large superfamily of enzymes encoded by CYP genes. Individual P450 protein is the end product of a single CYP

gene. This group of 45-55kDa proteins have a spectrophotometric absorbance peak close to 450 nm when complexed with carbon monoxide, which is the reason for the inclusion of '450' in their name. They commonly function as a monooxygenase where they transfer an atom of oxygen from molecular oxygen to a substrate and reduce the other oxygen atom into water (the reason they are also mixed function oxidase). However they catalyze other numerous reactions such as aromatic and aliphatic epoxidation, dealkylation and desulfuration (Rose and Hodgson, 2004). These hemoproteins are present in bacteria, fungi, plants, insects and vertebrates, and have varying degrees of substrate specificity. Mammals were the source of early studies on this group of enzymatic proteins. There are about 57 and 100 P450 genes in the genomes of human and insect respectively. P450 enzymes, like other detoxification enyzmes exist in multiple isoforms that exhibit varying overlapping substrate specificity (Feyereisen, 2005).

The role of P450 in detoxification of plant allelochemicals was first reported by Brattsten et al., (1977). In that work, larvae of *Spodotera eridania* that had fed had four times the P450 activity compared to unfed larvae. Fed larva also showed lower susceptibility to the insecticide nicotine, relating mechanism of insecticide resistance to plant utilization. Sixth instar larvae of *Peridroma saucia* fed with peppermint leaves had a nine fold increase in microsomal aldrin epoxidase activity relative to larvae fed with bean leaves. The former set of larvae had better tolerance to Malathion and methomyl (Berry et al., 1980). Toxic plant chemicals known to affect herbivores' behavior and feeding ability, interact with detoxification enzymes activities. The tobacco hornworm (*Maduca sexta*) is naturally adapted to solanaceous group of plants with relative high amount of nicotine due to its ability of metabolic detoxification of toxic nicotine (Synder and Glending, 1996). Naïve hornworms are easily poisoned by nicotine-containing diets but after approximately 36 hours, they resumed feeding. The initial halt of feeding and later resumption is directly linked to P450 induction. When a P450 inhibitor; pipernoyl butoxide (which had no direct effect on feeding) was incorporated into *Maduca sexta* diet, it reduced consumption of nicotine-containing diets; indicating the need for P450 induction for continued consumption (Synder and Glending, 1996). These studies revealed that host plant induced activity of detoxification enzymes could have implication on insect management with insecticides.

The isoforms of P450 or other detoxification enzymes and their level of induction varies with respect to specific body tissue, the inducing compound (host plant allelochemicals and pesticides), duration of exposure and also the insect life stage (Feyereisen, 2005; Ranson and Hemmingway, 2005). Synder et al., 1995 reported that CYP4M1 and CYP4M3 are induced to different levels in the fat body and midgut of tobacco hornworm, level of induction of peaked at wandering phase in fat body while midgut expression was highest during feeding bouts. Brattsten (1980) found out that P450 activity in the sixth instar of *Spodoptera eridania* fed with semi synthetic diets varied in the midgut and fat body depending on the concentration of plant secondary metabolite. The study by Brattsten (1980) showed that aldrin epoxidation and *p*-chloro N-methylaniline (PCNMA) N-demethylation activities were higher in the midgut than in the fat body but not methoxyresorufin *O*-demthylation. The inductive ability of host plant allelochemicals also changes with the age of the plant and the age of the insect. Yu (1982) reported that matured corn-plant leaves induced the greatest activity of P450 in *Spodoptera frugiperda* midgut which also increased the tolerance of fall armyworm to organophosphate insecticides.

1.3 Insect glutathione S-transferase (GST)

Glutathione-S transferases (GSTs) are a superfamily of intracellular enzymes that are famous for their roles in the metabolism of xenobiotics in plants and animals. They catalyze various reactions on both exogenous and endogenous substrates; typically, catalyzing the conjugation of reduced glutathione (GSH) - a tripeptide, and electron-deficient substrates (Yu, 1996). GSTs are capable of undergoing numerous reactions such as S-alkylation of GSH by alkyl halides and related compounds, substitution of alkyl halogen or nitro groups by the addition of GSH to different epoxides and unsaturated compounds including aldehydes, ketones, lactones, nitriles and nitro compounds. A diversity of plant allelochemicals can act as substrates for GSTs includes indole-3-carbinol, flavone and benzyl isothiocynate (Yu, 1996 for more review).

Different plant allelochemicals induce different GST isoenzymes.

Studies on the fall armyworm (Yu, 1982; 1984; 1992) showed that different tested plants induced GST activity toward DCNB (1,2- Dichloro-4-nitrobenzene) in various degrees, though some plants like bermudagrass, corn, potato, cucumber, carrot and broccoli had no effect on

DCNB activity while Parsnip caused the highest induction of GST activity. Xanthotoxin was later discovered to be the active inducer of GSTs in Parsnip leaves. This same compound has also been reported to induce P450 and GSTs in *Trichoplusia ni* and in the black swallowtail, *Papilio polyxenes* (Lee 1991). Host switch in in the western root worm *Diabrotica virgifera virgifera* from corn ear to squash was accompanied with 3-fold in GST activity (Muehleisen et al., 1989). The induction of GSTs also have consequence in the tolerance of insecticides by insects; Yu (1982) observed that cowpea-fed larvae of fall army worm were more tolerance to Diazinon, methamidophos and methyl parathion compared to larvae fed soybean. Clark (1989) reported that the activities of GST in tobacco budworm, *Heliothis virscens*, is decreased when it feeds on wild tomatoes leaves, showing the presence of insect GST repressors in their diets. Yu (1996), suggested that there are no qualitative difference in the GSTs induced when fall armyworm where fed with cowpea and soybean and also claimed that the inducibility of GSTs varied between specialists and generalists. The activities of GST studied in certain insects tissues helped in understanding the roles played by GSTs. Discovering that the sigma GSTs are mainly expressed in the indirect flight muscles of houseflies in association with troponin and drosophila reveals that these GSTs are more structural rather than catalytic (Ranson and Hemmingway, 2005). The use of tissue homogenate in GST activity is a reflection of the gross GST isoenzymes activity toward a particular test substrate. The midgut and fat body of insects have been highly used as the source of GSTs that are involved in the metabolism of xenobiotics because they are important sites for detoxification of xenobiotics. Yu (1996) proposed that "polyphagous species have evolved multiple glutathione S-transferases which may help detoxify diverse toxic allelochemicals found in their host plants" based on his work on three lepidopterous polyphagous insects and two monophagous species (Yu, 1989).

1.4 Insect carboxylesterase

Esterases are a superfamily of enzymes involved in the hydrolysis of ester-containing compounds via the addition of water to yield an acid and alcohol. Esterases are widely distributed in tissues and subcellular fractions where they act upon both endogenous substrates and xenobiotics. They are also involved in physiological and behavioral process in insects such as degradation of pheromones and some semiochemicals (Montella et al., 2012). Due to the large diversity in the chemical nature of these esterases, they have been classified, based on their

reaction with the phosphate trimester (paraoxon) in organophosphates (OP) into three groups (Aldridge, 1953). A-esterases are not inhibited but hydrolyze OPs, B-esterases are inhibited by OPs, and C-esterases, which are indifferent to OPs, do not hydrolyze them. However, this classification has been found not to be completely satisfactory because of the overlapping substrate specificity of these enzymes (Yu, 2008).

Carboxylesterases (CoEs) are B-esterases and well documented for their role in degrading OPs, carbamates, pyrethroids and juvenoids in insects. A common example is the hydrolysis of malathion into α-and β-monoacids and ethanol (Yu, 2008). Other B-esterases include amidases, cholinesterases, monoacylglycerol lipases and arylamidases. Most of B-esterases have a similar and conserved catalytic site, which is an active serine residue which act as a nucleophile during hydrolysis of susbstrate. Montella et al., (2012) suggested the monitoring the activity of these enzymes is crucial in pest management especially the adaptability of insects towards xenobiotics.

The hydrolysis mechanism of carboxylesterase is a two-step process that involve the nucleophilic attack of the carbonyl carbon of the ester bond (in the substrate) by the serine hydroxyl of the enzyme to release an alcohol and the addition of a molecule of water to the enzyme-substrate complex to yield an acid and a free active enzyme. The enzyme-substrate after the first step complex could be phosphorylated, carbamylated or acylated depending on the nature of ester bond present in the substrate. Elevated CoE activities associated with insecticide resistance to OPs and carbamates, has been reported in more than 30 species of insects of both medical and agricultural importance (Hemingway and Karunarate, 1998).

Though detoxification enzymes usually reduce the toxicity of their substrates, it is worthy of mention that in some cases reactive intermediates can be formed that are much more toxic than the parent compound; bio-activation. A classic example is seen in organophosphate insecticides and the carcinogenic metabolites of aryl hydrocarbons (Feyereisen 2005). This is the principle that is exploited in pro-insecticides that are used for controlling insects (Neuman 1985). Detailed reviews on the importance of these enzymes are well discussed in: Yu (1996), Feyereisen (2005), and Montella et al., (2012). These comprehensive reviews also address the naming and classification of the different families of these enzymes in insects.

Most of the research done with insect GSTs and other detoxification enzymes are in the order of Lepidoptera and Diptera with little attention given to the beetles, which are the most specious order of insects. The Japanese beetles is highly successful insect with wide host plant with diverse allelochemicals and it is more than important to investigate the role played by GSTs in the adaptability of this insect to its broad host range.

1.5 Japanese beetle, a pest of economic importance

Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae: Rutelinae), is an introduced pest with broad range of plant hosts with turf, horticultural and agricultural values. Over 460 million USD is spent every year to control both the larva and adult stage. An estimated $234 million of loss per year, $78 million for control and $156 million to replace damaged turf, is associated with just the larval stage (USDA/APHIS 2007). The adults inflict economic damage to foliage, flowers and fruits of crops like maize, soybeans, apples and other crops. Fleming (1972), estimated the host range of the prolific polyphagous Japanese beetle to be over 300 species (79 families) of plants.

1.5.1 Morphological description of Japanese beetle

The adult beetle is normally 8-11 mm in length and 5-7 mm wide with the female usually larger than the male. The body is bright metallic green with elytra, coppery brown, that do not fully cover the abdomen exposing a row of five tufts of abdominal hairs on both sides of the abdomen. The dorsal side of the body is flattened and without pubescence or scales while the ventral surface has short grey hairs. The sexes are differentiated by the first tarsal segment, which is shorter and stouter in the male compared to that of the female. The tibia spur of the male is while that of the female is rounded (Fleming, 1972).

Females deposit white eggs, spheroid or ellipsoid in shape, 5.1-7.6 cm beneath the soil. As the embryo develops, the eggs enlarge and become spherical (Held and Vittum, 2012). The grubs are C-shaped, scaraeiform larvae, approximately 1.5 mm in length as neonates. Fully developed first, second and third instar grubs are 10.5 mm, 18.5 mm and 32 mm in length, respectively. Japanese beetle grubs have a characteristic V-shaped arrangement of short spines on the medial sides of the last abdominal segment, differentiating them from other larval scarabs (Fleming, 1972). Prior to pupation, a pre-pupal stage do occur in Japanese beetle which retain the

body form of the third instar larva but it does not feed and does not have a C-shape configuration. It ejects all accumulated gut contents losing it gray appearance and remains almost inactive (Fleming, 1972). The pupa is bare, and lies within the cast skin left by the larva. At full size, it is about 14 mm long and 7 mm wide, partially resembling the adult though the wings, legs and antennae are held tightly to the body (Fleming, 1972).

1.5.2 History and distribution

The Japanese beetle is native to the main islands of Japan-Kysuhu, Shikoku and southern Honshu. It was a pest of minor economic importance in Japan probably due to the abundance of natural enemies and poor environment for larva development. The pest was first noted in the United States in Southern New Jersey, mid-August 1916. In the eastern USA, the beetles flourished due to the absence of native natural enemies, an abundance of grass suitable for the root-feeding larva, and numerous species of adult food plants. The pest population had since increased dispersing into new areas especially with aid of human movement. As at 1998, this beetle is well established in all states east of the Mississippi River except Florida, as well as part of the Wisconsin, Minnesota, Iowa and Nebraska and into Southern Ontario and Quebec, Canada (Potter and Held, 2002).

1.5.3 Seasonal biology

Japanese beetle is univoltine in most of the its range in the USA but in northern parts of the range like southern Ontario, northern New England, some populations takes two years to complete a generation (Vittum et al., 1999). Depending on the region, adults emerge between mid-May in the southern states (Georgia, Alabama) to mid-June in Northeast (New York, New England). Though the males usually emerge first (few days earlier than the females), the sex ratio of adults is usually 1:1 (Potter and Held, 2002). Unmated emerging females usually mate before feeding and then begin oviposition. Adults commence feeding on host plants preferably on the ones which conspecifics have already aggregated on. Females often re-mate and alternate between feeding and oviposition in the soil. The average number of eggs laid by a female in an adult life span of 4-6 weeks is 40-60 eggs, which are laid singly in the top layer of the soil. If the soil around the feeding vicinity is not suitable for oviposition, the beetles will move to oviposition sites with adequate soil moisture (Fleming 1972). Japanese beetles cause injury by their feeding activities of the both the larval and adult stage. Though Japanese larva are

polyphagous, they are usually restricted of feeding on the roots of a plant (grasses inclusive) - the plants can be of different species. While feeding, the grubs destroy roots and root hairs resulting in wilting and thinning of turfs. Adult feeding results in heavy defoliation and consequently the loss of the aesthetics of ornamentals. The adults also reduce the market values of fruits (Potter and Held, 2002).

1.5.4 Management and control

Because adults attack above ground plants parts and larvae feeds on roots, different tactics must be employed to control populations and damage. Monitoring Japanese beetle grubs, which are often aggregated, can be done with aid of sod cutter, or a golf course cup cutter (Potter, 1998). The grubs tends to be more at patches with perennial grasses that are highly attractive and also soil moisture, texture and sunlight also play important roles in the distribution and population of the grubs. The economic threshold for Japanese beetle larvae management varies spatially and also depends on the nature of the ecosystem, species of turfgrass and the agronomic practices practiced. (Potter and Held, 2002). During the flight season, adult Japanese beetles are best monitored using traps and phonological information. Ladd et al., (1981) reported that the combination of a food lure (phenethyl propionate, eugenol and geraniol) and japonilure, the synthetic sex pheromone of Japanese beetles had a greater capture than each of the lures used singly. The use of trap is also plagued by the fact it had to be emptied daily to remain efficient and it never really reduced Japanese's beetle damage to neighboring fields.

1.6 Previous investigation on detoxification enzymes in Japanese beetle

The only reported study on detoxification enzymes in Japanese beetle is Ahmad (1983). Ahmad (1983) evaluated the level of P450 induction in the adult and larval stages of Japanese beetle using PCNMA N-demethylation activity. P450 activity was highest in the midgut tissues compared to other tissues like fat body, malpighian tubules. This work also suggested that in the different life stages of this insect, the activity of P450 is peak during the polyphagous-feeding adult followed by the feeding early third instar larval and lowest in the diapause pupal stage. After comparing the P450 activity induced by feeding on preferred and non-preferred hosts, Ahmad (1983) proposed that induction of P450 is not related to host preference but likely in response to plant allelochemicals.

Recent advances in the knowledge of detoxification enzymes in insects have shown these enzymes exist in multiple forms with varying degree of substrate specificity which could also overlap, hence it is been suggested that one substrate is not adequate to evaluate the overall activity of detoxification enzymes (Feyereisen, 2005). More so, other studies (Xie et al., 2009, Yu 1984) have shown insects rely on more than one type of detoxification enzyme to metabolize plant allelochemicals in their diets. This study examines the roles of the widely studied multiple enzymes systems (P450, GST and CoE) in the physiology and host plant utilization by Japanese beetle; using multiple chemical substrates to evaluate enzyme activities.

1.7 Research goal and specific objectives

This research seeks to unravel the biochemical defense of the generalist insect herbivore; Japanese beetle, that confers on it the ability to utilize diverse host plants of agricultural, horticultural and landscape importance to man. The current (short-term) goal of my study is to determine which of the enzyme groups (P450, GST and/or CoE) are induced in response to different groups of plant allelochemicals and in the long term, the genes encoding for the induced enzyme(s) will be identified, allowing for the development of novel control tactics like RNA interference. I hypothesized that both adult and larval stages of Japanese beetles rely on multiple enzymes to utilize diverse host plants, and the respective activities of each enzyme will be influenced by the nature of the host plant or allelochemical. Furthermore, I also expect the titers of these enzymes in Japanese beetles are temporally (life stages) and spatially influenced (tissue specific), especially in the adult. In order to achieve the current goal of this research, the followings are the specific objectives (including hypotheses and rationales):

1. **Study the effect of diet mixing and host plant preference on activities of detoxification in adult Japanese beetle.**

Hypothesis: Increasing the role of diets or feeding on non-preferred host plants will induce higher activities of one more detoxification enzymes (P450, GST and CoE) in adult Japanese beetle.

Rationale: Classic reviews (Fleming, 1972; Potter and Held, 2002; Held, 2004) showed that Japanese beetle is a successful generalist herbivore that utilizes diverse host plants of varying degree of preference. This experiment focuses on the effect of diet mixing and feeding on host

plant of varying preference on the titers of three superfamilies of detoxification enzymes (P450, GST and CoE). This experiment will reveal if Japanese beetle increase the use of the same set of enzyme(s) for monophagy during polyphagy.

2. **Geranium (quisqualic acid) intoxication and detoxification enzymes in Japanese beetle**

Hypothesis*:* feeding on geranium does not induce activity of detoxification enzymes which is why beetles become paralyzed.

Rationale: this experiment explores the paralysis caused by feeding on petals of zonal geranium and the effect on the activities of detoxification enzymes in adult Japanese beetles.

3. **Influences of: life stage, tissue, gender, and time of feeding on the activities of P450, GST and CoE in Japanese beetle**.

Hypothesis: the expression and activity of P450, GST and CoE varies across different tissues in the body of Japanese body.

Rationale: Ahmad (1983) compared the tissue specific activity of P450 and noted greater expression in the gut and fat body of adults and 3rd instar grubs. This work will determine activities of three different enzymes in adults, eggs, 1st, 2nd, and 3rd instar larvae. Tissue specific expression and sex specific expression will be determined for adults. The results should provide insight into the role played by this enzymes and perhaps reveal the localization of genes encoding these enzymes.

An understanding of the biochemical defense system via the use of detoxification enzymes in the insect will be central to the development of a sustainable control strategy that can limit the economic loss caused by this insect. The findings and outcomes of this study will as serve as a foundation for further studies on the molecular genetics and biology of the putative genes that confers allelochemical resistance and tolerance in Japanese beetle.

Chapter 2:

Multiple Detoxification enzymes facilitate Polyphagy in adult Japanese beetles, *Popillia japonica* Newman

ABSTRACT

Induction of cytochrome P450, glutathione S transferase (GST) and carboxylesterase (CoE) activity was measured in guts of the scarab *Popillia japonica* Newman, after it had consumed single-plant or mixed-plant diets of preferred (rose, Virginia creeper, crape myrtle and sassafras) or non-preferred hosts. The goal of this study was to clarify how detoxification enzymes facilitate the species' broad host range. Non-preferred hosts (boxelder, riverbirch and red oak) were only sparingly fed upon but induced higher activities of P450, GST and CoE than did feeding on preferred hosts. Similarly, beetles tended to have greater enzyme activities after feeding on a mixture of plants compared to a single plant type. The metabolic cost of elevated detoxification enzymes on non-preferred hosts, or when switching between hosts, may explain, in part, the existence of marked feeding preferences in this highly polyphagous pest. Consequences of higher induced activities of detoxification enzymes by non-preferred hosts on the physiology and feeding ecology of Japanese beetles is discussed.

2.1 Introduction

Plant chemical defenses can reduce growth, fecundity, or survival of non-adapted herbivores (Fraenkel, 1959; Krieger et al., 1971; Schoonhoven et al. 2005). Adapted herbivores are able to cope with plant toxins through various adaptations; e.g., behavioral avoidance, sequestration, increased rates of excretion, or detoxification enzymes for biochemical metabolism (Dowd et al., 1983; Karban and Agrawal, 2002). Cytochrome P450, glutathione S transferase (GST) and carboxylesterase (CoE) are superfamilies of detoxification enzymes involved in insects' metabolism of plant secondary compounds (Feyereisen, 2005; Yu, 2008). These enzymes, which are involved in phase I (P450, CoE) and phase II (GST) stages of xenobiotic metabolism in insects (Yu, 2008), generally function by increasing the solubility of toxic lipophilic substrates to hasten excretion. Elevated activities of detoxification enzymes have been linked with increased host plant utilization and insecticide resistance (Yu, 1989; Snyder and Glendenning, 1996; Yu et al., 2003, Zhu et al., 2010). Studying the activity and plasticity of detoxification enzymes in insect herbivores can clarify their roles in determining dietary range, and potentially also yield novel target sites for management tactics (e.g., Mao et al. 2007).

Adult Japanese beetles are dietary generalists that can feed on >300 plant species in 79 plant families (Fleming, 1972; Potter and Held 2002) varying widely in secondary chemistry. Polyphagous insects must have adaptations to cope with a broader range of plant secondary compounds than encountered by insect herbivores having a more restricted diet (Bernays and Chapman, 1994; Schoonhoven et al., 2005; Feyereisen, 2005). Dietary range and host preferences of adult Japanese beetles have been summarized based on historical field observations (Fleming, 1972; Held, 2004) and some feeding trials (e.g., Ladd 1987, 1989; Keathley and Potter 2008), but relatively little is known about the extent to which induction of detoxification enzymes facilitates their polyphagy.

Ahmad (1983) compared a gut P450 activity in adult *P. japonica* fed a mixed-plant diet or foliage from a single preferred host species. His trials, which used one substrate (p-chloro-N-methylaniline), showed elevated midgut enzymatic activity in beetles associated with the mixed diet, indicative of induction by plant secondary chemicals. Ahmad's (1983) result results are supportive of the hypothesis (Krieger et al., 1971; Mullin, 1986; Yu, 1989;

et al., 2003) that polyphagous species, because of their encountering of more chemically diverse plant toxins, tend to have higher activity of detoxification enzymes than do specialists.

More recent studies (reviews, Feyereisen 2005; Ranson and Hemingway, 2005) suggest that polyphagous insects have greater diversities of genes encoding for different isoenzymes of detoxifcation enzymes with varying substrate specificity. Furthermore, insect herbivores irrespective of the host breath often deploy more than one detoxification enzyme system (Xie et al., 2010; Yang et al., 2001). Activities of GST and CoE, which may also facilitate metabolism of secondary plant chemicals and insecticides (Lee, 1991; Yu, 1996; Xie et al., 2010), have not been previously studied in P. japonica. Biochemical characterization remains a crucial first step in the study of detoxification enzymes, especially for insect species (i.e. Japanese beetles) whose genome has not been sequenced (Feyerisen, 2005). This study sought to biochemically characterize and quantify activities of P450, GST and CoE detoxification enzymes in the midgut of adult *P. japonica* using multiple substrates in order to clarify their role in the beetles' ability to feed on such a wide host range. Induction of detoxification enzymes in beetles after feeding on preferred or non-preferred host plants, and on single- or mixed-plant diets was also characterized.

2.2 Materials and methods

2.2.1. Source of insects and host plants

Adult beetles were collected from Town Creek Park in Auburn, AL, using traps (Pherocon® JB trap, Trécé, Adair, OK) baited with a food lure (2-phenyl-ethyl propionate, eugenol, and gereniol; 3:7:3 ratio). To ensure a diversity of allelochemical profiles, multiple host plants from different orders and families varying in relative palatability to *P. japonica* were used (Fleming, 1972, Table 1). Foliage or flower petals were collected from plantings on the Auburn University campus < 6 h before being used in each experiment. Leaves and flowers offered to the beetles were collected from at least four plants per species, and were examined visually to be disease free.

Table 2.1. Japanese beetle's host plants of varying preference evaluated for induction of detoxification enzymes

Species	Common name	Family	Plant part offered
Preferred Hosts			
Sassafras albidum	Sassafras	Lauraceae	Foliage
Lagerstroemia indica 'Dynamite'	Crape myrtle	Lythraceae	Foliage
Rosa x radazz 'knockout'	Rose	Rosaceae	Petal
Parthenocissus tricuspidata	Virginia creeper	Vitaceae	Foliage
Non-preferred Hosts			
Quercus falcata	Southern red oak	Fagaceae	Foliage
Betula nigra	River birch	Betulaceae	Foliage
Acer negundo	Boxelder	Sapindaceae	Foliage

2.2.2 Reagents and supplies

Except where mentioned, all reagents and supplies were purchased from VWR Scientific and were of high grade and quality.

2.2.3 Feeding assays

Only active, apparently healthy beetles free of any morphological defects were used in experiments. For each trial, 25 beetles, a mixture of males and females, were held individually in 100 × 15 mm sterile polystyrene petri dishes with foliage or petals and allowed to feed for 24 h. Each dish contained a moistened filter paper (90 mm diameter) to prevent desiccation of the plant material. The feeding assays under each experiment were replicated four times. A 24 h

feeding bout is sufficient for induction of P450, GST and CoE (Ahmad 1983, *unpublished data*). Starved beetles (no food provided) were used as controls because field-collected insects are mixed age and have unknown dietary histories. Starvation, which we reasoned would provide insight about baseline levels of each enzyme system, has been used to condition *P. japonica* before feeding assays (e.g., Held and Potter, 2004; Keathley and Potter, 2008; Ranger et al., 2010). Fed and starved beetles were maintained at 14:10 (L: D) photoperiod and 25–27 °C). Leaves or petals were provided without limit to each beetle during the trial.

The first experiment tested the effect of feeding on preferred and non-preferred host plants on beetles' enzymatic activity levels. Treatments included three preferred host plants, crape myrtle, rose, and sassafras, and three non-preferred but palatable host plants, red oak, boxelder, and river birch (Table 1). The second experiment compared activities of detoxification enzymes under short-term feeding bouts on single or multiple host plants. Virginia creeper, rose and sassafras, three preferred host plants (Table 2), were used for this experiment. Beetles restricted to feeding on a single plant species were provisioned with that plant material for 24 h. Beetles receiving the mixed-plant diet were provisioned with plant tissue from a different host species every 12 h and 8 h for two- and three-plant mixtures, respectively, of the same plant materials used in the single species diets (Table 2). Each experiment was replicated four times on different days.

2.2.4 Quantifying consumption of host plants

Leaves or petals were weighed and scanned individually prior to use and the mass per unit leaf area was determined. After feeding, the remnant tissue was re-scanned, missing area was computed with digital imaging software (Image J, http://imagej.net), and the amount consumed (mg) by each beetle was determined. Beetles that did not feed were excluded before dissection. Prior observation showed that that foliage and petals have no significant water and weight loss, as used in this study.

Table 2.2. Feeding regimes for Japanese beetle single and mixed diet experiment

Diet treatment	Host plant species
Monophagy	
	Virginia creeper
	Rose
	Crape myrtle
Polyphagy	
	Virginia creeper and Rose (V-R)
	Crape myrtle and Rose (C-R)
	Virginia creeper and Crape myrtle (V-C)
	Virginia creeper, Crape myrtle and Rose (V-C-R)

2.2.5 Dissection for removal of midguts

After feeding, beetles were pinned on an ice-chilled waxed-bottom petri dish covered with Parafilm to prevent contamination and dissected under a binocular microscope. Guts were removed using sterilized dissecting scissors and forceps. The guts from beetles fed the same diet were immediately transferred into 1.5 ml Eppendorf tubes placed on dry ice. After dissection, the gut tissues were stored in -80 °C freezer until further processing.

2.2.6 Extraction of microsomes and cytosols

This procedure was adapted from the method of Lee and Scott (1989). Beetle guts were homogenized in 10 ml of ice-cold homogenization buffer (0.1 M phosphate buffer, pH 7.5; 10% glycerol, 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF and 1 mM PTU). The homogenate was filtered through a double-layered cheesecloth into a 15 ml centrifuge tube. The filtrate was centrifuged at 10,000 g for 30 min at 4 °C (Beckman Allegra 25R, equipped with a type TA-14-50 rotor). The pellet (nuclear debris, mitochondria and other materials) was discarded and the supernatant was transferred into a 30ml PC Oak Ridge centrifuge tube (Thermo Scientific, Asheville, NC) and spun in an ultra-centrifuge (Sorvall, Discovery 90SE, equipped with a TI 865

rotor) at 100,000 g for 1 h at 4 °C. The resulting supernatant (cytosolic fraction) was removed and used as the source of enzymes for the GST and CoE activity assays. Pellets (microsomes), the source of P450 enzyme, were dissolved in a re-suspension buffer (0.1 M phosphate buffer, pH 7.5; 20% glycerol, 1mM EDTA, 0.1 mM DTT and 1 mM PMSF). All steps were performed on ice. The microsomes and cytosols (source of GST and CoE) were temporarily stored at -80 °C.

2.2.7 Protein content determination

Protein concentrations present in the microsome and cytosol fractions of each experimental group were determined by the Bradford (1976) method using Bio-Rad dye reagent and bovine serum albumin as the standard. A minimum of triple determinations was done for each sample using a UV-VIS spectrophotometer (Du 640, Beckman Coulter, Brea, CA).

2.2.8 P450 activity assays

P450 *O*-deethylation of 7-ethoxycoumarin to 7-hydroxycoumarin (ECOD) and oxidative demethylation of ethyoxyresorufin to resorufin (EROD) activity was measured by a spectroflurometer (Lee and Scott, 1992; Guo et al., 2014). A sample (30–50 µl) of the microsomal fraction and 40 µl of ECOD buffer (50mM Tris-base, 150mM KCl and 1mM EDTA, pH 7.8) containing 4nM of 7-ethoxycoumarin (7-EC) was added to each well of a 96-well plate (Black flat-bottom BD Falcon; Thermo Scientific, Asheville, NC). The reaction was initiated by adding 0.1 µM NADPH (freshly prepared) to each well. The reaction was incubated for 30 min at 30 °C. The fluorescence of NADPH was quenched by adding 0.3 µM oxidized glutathione and a 0.5 unit of glutathione reductase (Sigma-Aldrich, St. Louis, MO) to each well. The reaction was stopped after 10 min at 25 °C by adding 120 µl of stop solution (50% acetonitrile and 50% 0.05 M, pH10 Trizma-base buffer). The amount of 7-hydroxycourmarin produced was measured using a microplate reader (Cyataion 3: Biotek, City, State) at a wavelength of 390 nm and 465 nm for excitation and emission respectively. The control was the reaction between NADPH and 7EC without microsomal fraction. A product standard curve was also generated by using seven different dilutions of 100 µM of 7-hydroxycoumarin under the aforementioned conditions.

P450 EROD activity was determined by measuring fluorescence intensity from reactions involving 30–50 µl of microsomal fraction and 0.3 *p*Mol of ethoxyresorufin. Each reaction was

brought up to 100 µl with EROD buffer (0.1 M phosphate buffer) in a single well of a 96-well plate (Black flat-bottom, BD Falcon). The reaction was started by adding 10 µl of 0.01M NADPH to each well. The mixture was shaken gently and incubated for 30 min at 30 °C after which fluorescence intensity was read at wavelengths of 530 nm and 580 nm for excitation and emission respectively. The control reaction is that between NADPH and the substrate with no enzyme source. A product standard curve was designed to know the amount of the product (resorufin) generated in the reaction using different concentrations. Both ECOD and EROD P450 activity was expressed as *p*moles of product formed per min per mg protein.

2.2.9 GST activity assays

GST activity was determined as the ability of cytosolic fractions to catalyze the conjugation of glutathione (GSH) and chroloro-2, 4-dintrobenzene (CDNB), a method modified from Habig et al., (1976). The reaction was carried out in a transparent 96-well plate. Phosphate buffer (0.1M pH 6.5) containing EDTA (1mM), PMSF (1mM) and DTT (1mM) was used. For each reaction, freshly prepared GSH (dissolved in buffer) was mixed with 5 µl of the enzyme source in a final volume of 200 µl and a final GSH concentration of 2.5 mM. Reactions were incubated for 3 min at 25 °C, after which 10 µl of 30 mM CDNB (dissolved in alcohol and freshly prepared) was added. The control reaction is the non-enzymatic reaction between GSH and CDNB under the same conditions. The mixture was shaken slightly and the change in absorbance was measured for 5 min using a plate reader (Cyataion 3: Biotek) set at 340nm. GST activity was expressed as micromoles CDNB conjugated per min per mg protein using the extinction co-efficient of 5.3 $mM^{-1}cm^{-1}$ for S-(2,4-dintrophenyl) glutathione.

2.2.10 Carboxylesterase activity assays

This was based on the hydrolysis of α- and β-naphthylacetate (α- and β-NA) to α- and β-naphthol by modified methods of Van Asperen (1962), and Zhang et al., (2007). The reaction was carried out in a transparent flat bottom 96-well plate. The working concentration of the substrates (α- and β-naphthylacetate) was 0.3 mM (containing 0.3 mM eserine), diluted from their respective 0.03 M stocks. Each assay reaction contained: 60 µL phosphate buffer (0.04M, pH7.0), 50 µL of the supernatant (enzyme source) and 80 µL of 0.3 mM of substrate solutions. The reaction (slightly shaken) was incubated at 37 °C for 30 min and stopped by adding 20 µL of stop solution (two parts of 1% Fast Blue BB and five parts of 5% sodium dodecyl sulfate). Color

was allowed to develop for 15 min at room temperature, and the absorbance was measured at 600 nm for the hydrolysis of α-NA, and at 550 nm for β-NA using microplate reader (Cytation 3: Bio-Tek). The products' (α- and β naphthol) standard curve was generated by measuring optical absorbance at same wavelength for enzymatic reactions, at different concentrations. To each well was added 0, 1, 5, 10, 20 and 30 µL of 32mM of α- and β naphthol dissolved in acetone and also 20 µL of stop solution. CoE α- and β-naphthylacetate.

2.2.11 Data analyses

One-way ANOVA was used to compare the average mass of tissue eaten by beetles from the single plant and mixed-plant dietary regimes, and for the resulting activities of each enzyme (P450, GST and CoE). Pre-planned orthogonal contrasts were used to test overall group differences between beetles' consumption of single diet and mixed diet and also enzymatic activities (P450, GST and CoE).

The relative amount of host plant foliage or petal tissue consumed and induced enzymatic activities (P450, GST and CoE) in beetles after feeding on preferred and non-preferred hosts were compared by analysis of variance (ANOVA) with means for each host plant separated by Tukey's HSD (SAS JMP version Pro 11, SAS Corp.). Pre-planned orthogonal contrasts were used to test overall group difference between beetles' consumption of preferred versus non-preferred host plants, and also enzymatic activities (P450, GST and CoE).

2.3. Results

2.3.1 Feeding and enzyme activities on preferred and non-preferred hosts plants

Consumption of plant material was consistent with previous rankings (Fleming, 1972; Held, 2004) of the plant species' relative preference to Japanese beetles. The beetles consumed significantly more mass of preferred host plant material (226 ± 41 mg) than that of the less-preferred hosts (27.2 ± 2.3 mg) (orthogonal contrasts; $t = 13.61$ $P < 0.001$). There were no significant differences in consumption among non-preferred hosts (Table 3). However, among preferred host plants, crape myrtle had highest average amount consumed, followed by rose petals and sassafras foliage ($F_{(5,15)} = 288.4$, $P < 0.005$). P450 activity level was not necessarily

correlated with consumption. For example, P450 EROD and ECOD activities were similar for crape myrtle (preferred) and river birch (less preferred) despite a 130-fold difference in consumption (Figure 1).

Beetles that were provided food (preferred or non-preferred host plants) had greater induced activities of P450 EROD ($t = 4.61$, $P < 0.001$) and ECOD ($t = 4.1$, $P < 0.001$) activities when compared with starved beetles (orthogonal contrasts, Table 3). Non-preferred host plants, as a group, induced about 1.5-fold greater P450 ECOD activity compared to induction by preferred hosts ($t = 5.04$, $P < 0.001$; Table 4). There was variation in induced P450 activities (ECOD and EROD) for beetles fed particular preferred or non-preferred hosts; e.g., beetles that consumed crape myrtle (preferred) had similar P450 ECOD activities to those fed boxelder or river birch (non-preferred). Crape myrtle induced P450 EROD activity greater than the other preferred hosts, and P450 EROD activity after feeding on sassafras or rose was no different than in starved beetles (Figure 1). There was greater P450 EROD activity ($t = 5.83$, $P<0.001$) in midguts from beetles fed preferred hosts compared to less-preferred hosts (Table 4).

Feeding in general or consumption of preferred hosts specifically did not induce higher GST activity relative to starved beetles (Table 4). Consumption of non-preferred hosts, however, yielded greater GST activity in the midgut compared to starved beetles or beetles fed preferred hosts ($t = 4.52$, $P \leq 0.001$). Beetles' GST activity was similar among all hosts except red oak and boxelder despite significant variation in feeding amount (Table 3, Figure 2). Feeding on foliage of boxelder, a non-preferred plant, induced the highest level of GST activity.

Fed beetles, regardless of host, had greater α-NA and β-NA CoE activities ($P<0.001$) than did starved beetles (Table 4). Beetles that consumed non-preferred hosts had higher α-NA and β-NA CoE activity ($P <0.05$) compared to beetles fed preferred hosts. Red oak, crape myrtle, and river birch induced the highest α-NA CoE activity (Figure 3), whereas red oak, river birch, and sassafras induced greatest β-NA CoE activity. Beetles consumed greater amounts of crape myrtle than of any of the other hosts that induce high CoE activity (Table 2.3).

Table 2.3. Average amount (mg) of preferred and non-preferred host plants consumed by Japanese beetle

Host plants	Consumption ± S.E. (mg)
Preferred Hosts	
Sassafras	103±15[c]
Crape Myrtle	413±21[a]
Rose	164±7.2[b]
Non-preferred Hosts	
Boxelder	30±5.5[d]
Red Oak	22.9±4.2[d]
Riverbirch	30.8±1.5[d]
1-Way ANOVA	
$F_{6, 46}$	288.4**
Orthogonal comparison	
Preferred Hosts group mean	226±41
Non-Preferred Hosts group mean	27.2±2.3
t (Preferred Hosts vs Preferred Hosts)c	13.61***

‡Data shows means ± SE of mass of host plant consumed in 4 independent experiments by adult Japanese beetles. Means within each column not accompanied with the same superscript are significantly different (one-way ANOVA; means separated by Tukey's HSD). [c]Student's t-test (1 df) for orthogonal comparison between groups. *** P<0.0001,

Table 2.4. Orthogonal contrasts for P450 , GST and CoE activities for Japanese beetles fed with preferred and non-preferred hosts

	P450 activity		GST activity	CoE activity	
[a]Groups	ECOD	EROD	CDNB	α-NA	β-NA
Starved (S)	102.2±18	43.8±7	47.13±2.5	35.19±4.3	63.56±3.2
Fed group mean	171.7±9.2	87.65±6.2	59.5±4.7	63.7±3.4	105.3±6.6
PH group mean	139.5±9.5	95.68±10	46.03±5	58.66±4.3	92.9±7.2
NPH group mean	203.9±13	79.63±6	73±6.9	68.75±5.1	117.6±10.5
[b]Orthogonal comparisons					
S vs F	4.1***	4.61***	1.5[ns]	3.06***	3.54***
PH vs NPH	5.04***	4.28***	4.52***	2.07*	2.17*
S vs PH	2.06*	5.83***	0.13[ns]	2.13*	2.55*
S vs NPH	5.62***	2.8**	3.06**	3.6***	4.08***

[a]Groups mean ± standard error for P450, GST or CoE activities, **PH**- beetles fed preferred host plants, **NPH**- beetles fed non-preferred host plants. **F**- beetles fed PH or NPH plants

[b]student's *t*-test for orthogonal comparison between groups. *** P<0.0001, **P<0.001, *P<0.05

[ns]-no significant difference between compared groups. P450 ECOD and EROD activity expressed in pmol/min/mg protein, GST activity expressed in µmol/min/mg protein and CoE α and β -NA expressed in nmol/min/mg protein.

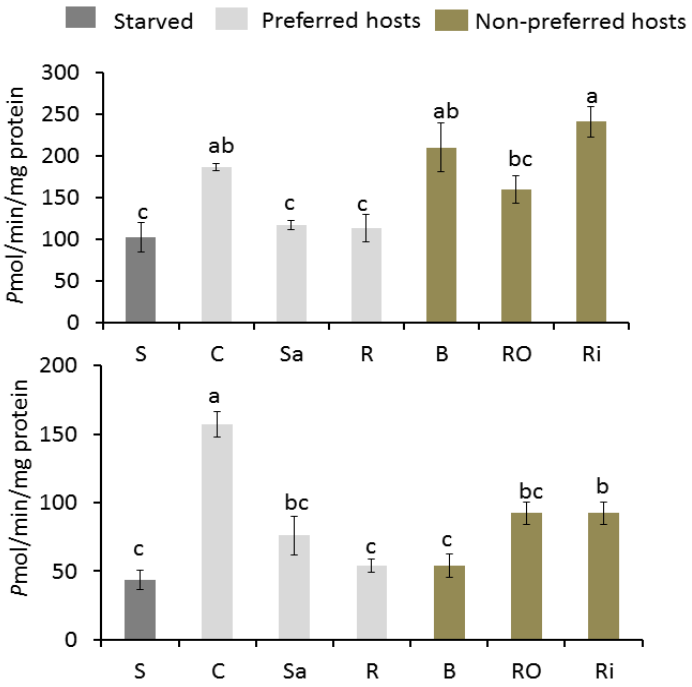

Figure 2.1. P450 ECOD (upper) ($F_{(6,46)}$=11.67) and EROD (lower) ($F_{(6,46)}$=26.72) activity of adult Japanese beetles fed preferred and non-preferred hosts. Letters above bars represent a means separation by Tukey's HSD (P<0.05). Letters on x axis denote host plant treatments as follows: **S**-starved beetles, **C**-crape myrtle, **Sa**-sassafras, **R**-rose, **B**-boxelder, **RO**-red oak, and **Ri**-river birch. Number of replication=4, 25 beetles per diet treatment

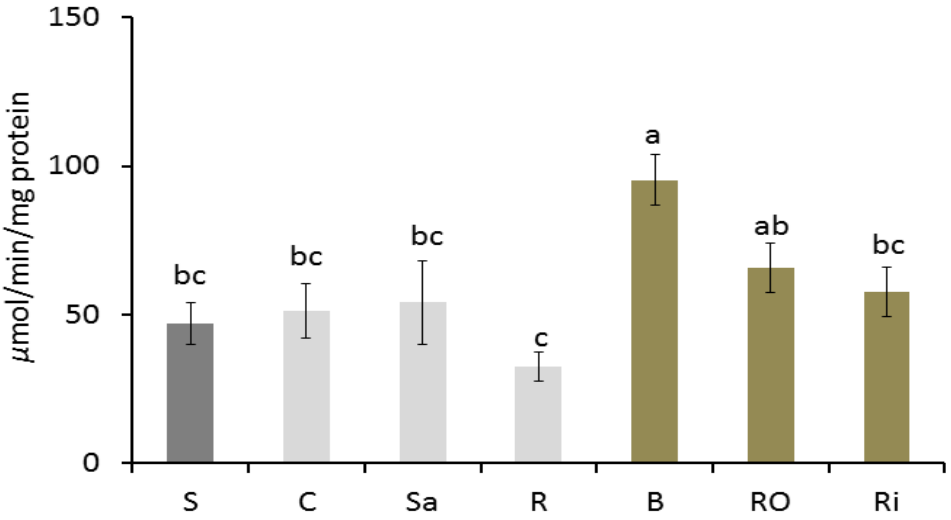

Figure 2.2. GST CDNB activity of adult Japanese fed preferred and non-preferred hosts. One way ANOVA, ($F_{(6,46)}$=7.1), letters above bars represent a means separation by Tukey's HSD (P<0.05). Letters on x axis denote host plant treatments as follows: S-starved, C-crape myrtle, Sa-sassafras, R-rose, B-boxelder, RO-red oak, and Ri-river birch. Number of replication=4, 25 beetles per diet treatment.

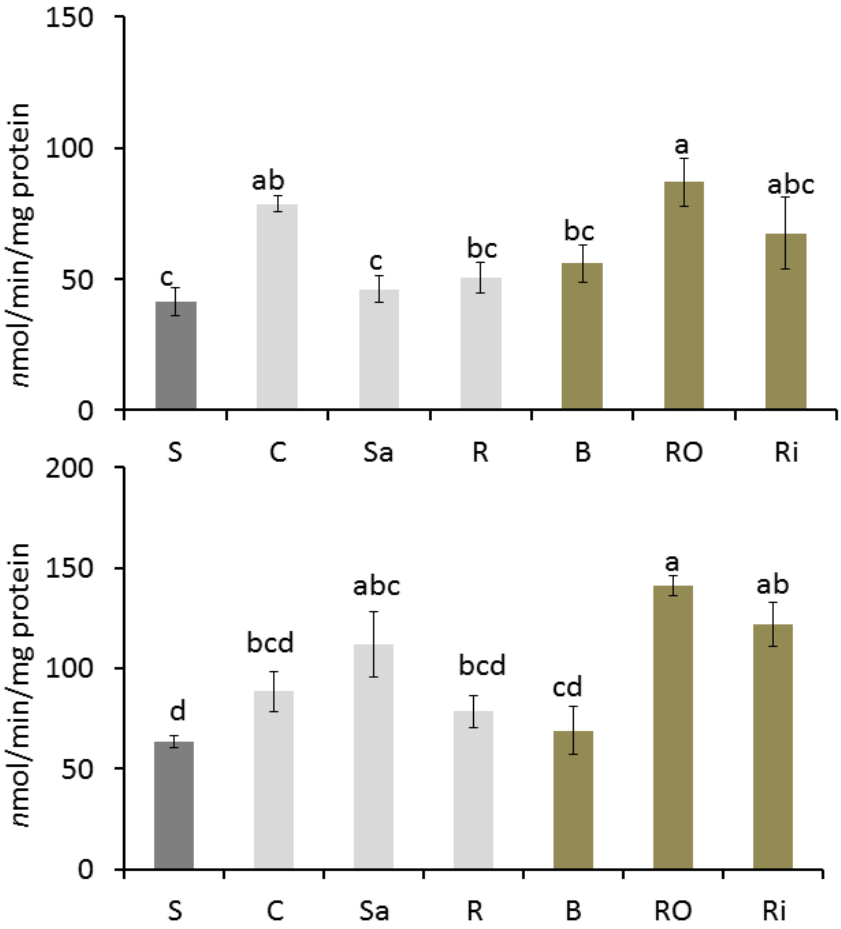

Figure 2.3. CoE α-NA (upper) ($F_{(6,46)}$=6.31) and β-NA (lower) ($F_{(6,46)}$=8.48) activity of adult Japanese beetles fed preferred and non-preferred hosts. Letters above bars represent a means separation by Tukey's HSD (P<0.05). Letters on x axis denote host plant treatments as follows: S-starved beetles, c-crape myrtle, Sa-sassafras, R-rose, B-boxelder, RO-red oak, and Ri-river birch. Number of replication=4, 25 beetles per diet treatment

2.3.2 Feeding and enzyme activities under mixed-plant and single plant diet regimes

There was a non-significant trend for beetles to consume more plant tissue under the mixed-plant feeding regime than when restricted to a single-plant diet (orthogonal contrasts; t = 0.13, P = 0.09; Table 5). Among specific diet treatments, feeding on only crape myrtle or on a mixed diet of Virginia creeper and crape myrtle resulted in the highest consumption. Diet mixing induced greater GST (t = 6.29, P < 0.001) and CoE α-NA activity (t = 3.14, P < 0.001) compared to single species diets. There was a trend for greater P450 ECOD (t = 1.8, P < 0.08)

and β-NA CoE (t = 1.91 P = 0.06) activities for beetle in the mixed-plant feeding regime (Table 6). However, consumption of monophagous diets induced greater P450 EROD activity (t = 9.12 $P < 0.001$) compared to consumption of multiple plant species. Enzyme activities in beetles that consumed the three-species mixture (V-C-R) were never greater than in beetles that consumed diets with just two species.

Feeding on rose petals induced the lowest activities of P450 (Figure 4), GST (Figure 5), and α-NA CoE (Figure 6) relative to other single species diets. Mixing crape myrtle or Virginia creeper with rose increased GST activity, and the three-species mixed diet increased activities of all enzyme groups except β-NA CoE. Beetles consumed significantly more foliage of crape myrtle than of Virginia creeper in single species diets and GST activity was similarly greater for beetles that consumed crape myrtle (Figure 5). Activities of CoE and P450 were similar on single-plant diets of crape myrtle or Virginia creeper. Mixing crape myrtle and Virginia creeper did not significantly change activity of CoE, but did produce significantly greater P450 ECOD activity relative to those plants consumed individually. Mixing crape myrtle and Virginia creeper did not change GST activity relative to crape myrtle alone but was greater than Virginia creeper as a single species diet.

Table 2.5. Average amount (mg) of host plants consumed by Japanese beetle under different feeding regime

Feeding regime	Consumption ± S.E. (mg)
Monophagy	
Virginia creeper	173±37[c]
Crape Myrtle	355±60[a]
Rose	168±11[c]
Polyphagy	
Virginia creeper & Crape Myrtle	328±65[a]
Virginia creeper & Rose	186±26[bc]
Crape Myrtle & Rose	285±29[abc]
Virginia creeper ,Crape Myrtle & Rose	301±57[ab]
1-Way ANOVA	
$F_{6,13}$	9.19**
Orthogonal comparison	
Monophagy group mean	232±34
Polyphagy group mean	255±25
t (Monophagy vs Polyphagy)[c]	0.13[ns]

‡Data shows means ± SE of mass of host plant consumed in four independent experiments by adult Japanese beetles. Means within each column not accompanied with the same superscript are significantly different (one-way ANOVA; means separated by Tukey's HSD).
[c]Student's t-test (1 df) for orthogonal comparison between groups. *** P<0.0001,
[ns]-no significant difference between compared groups

Table 2.6. Orthogonal contrasts for P450, GST, and CoE activities for Japanese beetles fed with single and mixed diets

	P450 activity		GST activity	CoE activity	
[a]Groups	ECOD	EROD	CDNB	α-NA	β-NA
Starved	143±17.3	35.7±3.6	33.5±1.4	54.7±4.4	56±3.4
Fed	165.6±10.3	98.9±7.7	44.4±2.3	86±3.93	93.6±4
Monophagy	147.7±14.5	198±16	34.9±2.9	73.8±4.9	84.1±4.9
Polyphagy	179±14.1	85.4±6	51.5±2.7	95.2±5.3	100.7±5.8
[b]orthogonal comparisons					
S vs F	1.47[ns]	8.13**	4.14**	3.8**	4.04**
M vs P	1.8*	9.12**	6.29**	3.14**	1.91ns
S vs M	0.28ns	10.2**	0.46[ns]	2.16*	2.93**
S vs P	2.24*	5.93**	6.57*	4.72**	4.49***

[a]Groups mean ± standard error for P450, GST or CoE activities

[b]student's *t*-test for orthogonal comparison between groups. *** P<0.0001, **P<0.001, *P<0.05

[ns] - no significant difference between compared groups. , **P-** beetles mixed diets, **M**- beetles fed single diets. S-starved beetles, F- beetles fed single or mixed diets plants. P450 ECOD and EROD activity expressed in pmol/min/mg protein, GST activity expressed in μmol/min/mg protein and CoE α and β - NA expressed in nmol/min/mg protein.

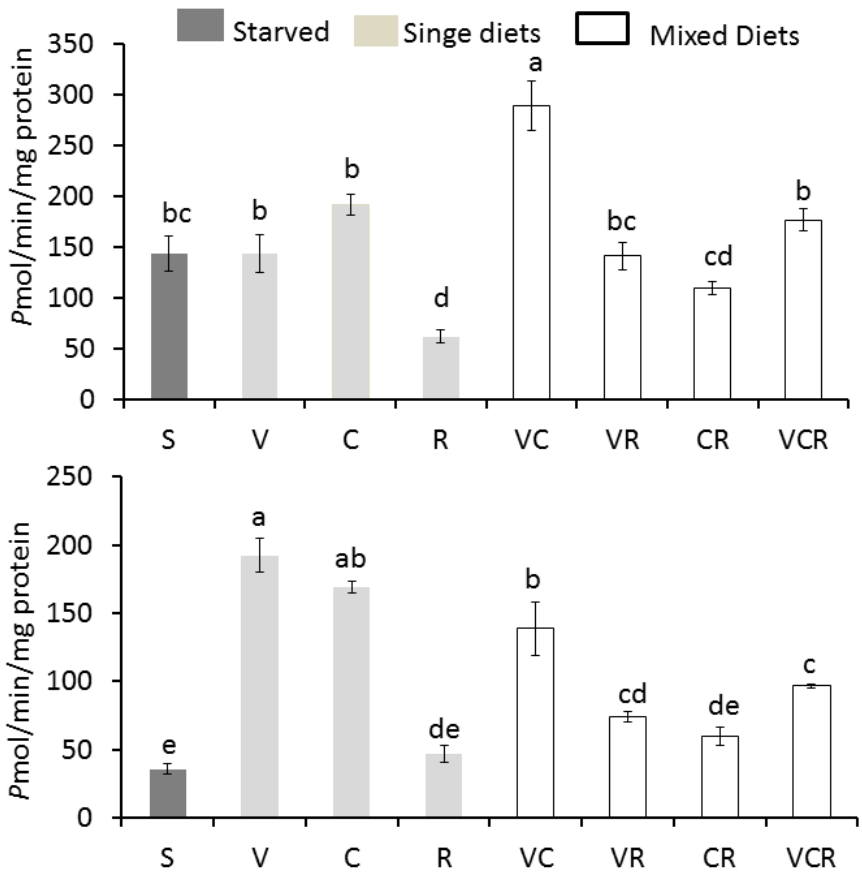

Figure 2.4. P450 ECOD (upper) ($F_{(7,53)}$=21.94) and EROD (lower) ANOVA($F_{(7,53)}$=56.7) Activity of adult Japanese beetles provided simulated monophagous and polyphagous diets. Letters above bars represent a means separation by Tukey's HSD (P<0.05). Letters on x axis denote host plants feeding regimes (Table 2) as follows: S-starved beetles; C-crape myrtle, V-Virginia creeper, R-rose. Number of replication=4, 25 beetles per diet treatment.

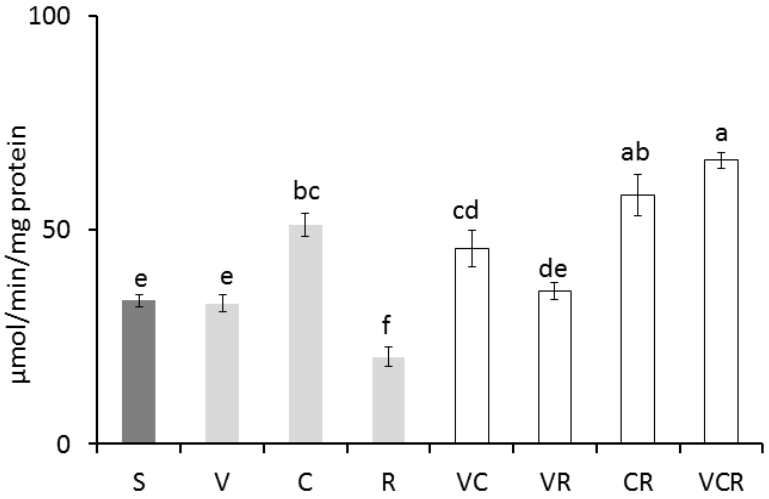

Figure 2.5. GST CDNB activity of adult Japanese beetles provided simulated monophagous and polyphagous diets. $F_{(7,53)}$=38.4 , letters above bars represent a means separation by Tukey's HSD (P<0.05). Letters on x axis denote host plants feeding regimes (Table 2) as follows: **S**-starved beetles; **C**-crape myrtle, **V**-Virginia creeper, **R**-rose. Number of replication=4, 25 beetles per diet treatment.

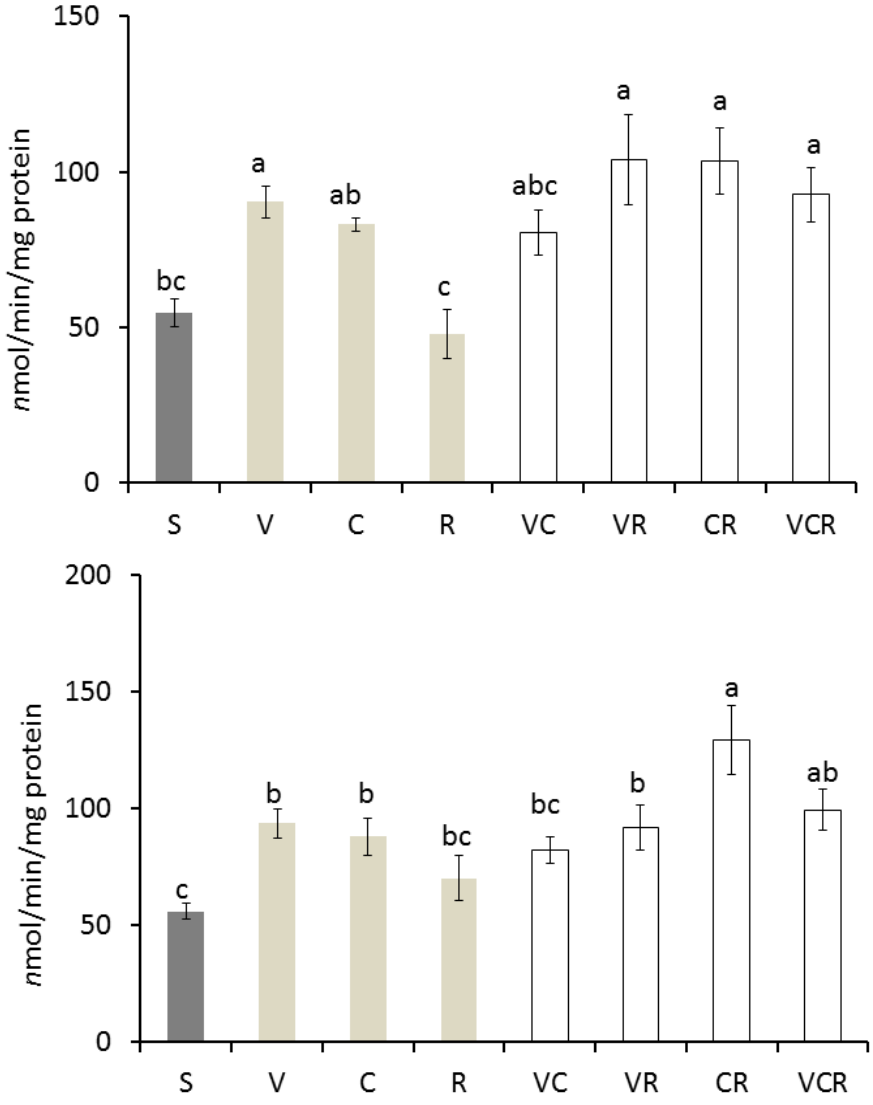

Figure 2.6. CoE α-NA (upper) ($F_{(7,53)}$=7.38) and β-NA (lower) ($F_{(7,53)}$=9.18) activity of adult Japanese provided simulated monophagous and polyphagous diets. Letters above bars represent a means separation by Tukey's HSD (P<0.05). Letters on x axis denote host plants feeding regimes (Table 2) as follows: **S**-starved beetles; **C**-crape myrtle, **V**-Virginia creeper, R-rose. Number of replication=4, 25 beetles per diet treatment.

2.4. Discussion and implications

This study is the first to compare activities of GST, P450, and CoE in midguts of adult *P. japonica* in response to feeding on single and mixed-plant diets. It extends earlier work (Ahmad 1983) that first demonstrated feeding-induced P450 *p*-chloro-*N*-methylanaline activity in the

beetle's midgut and fat body. We used two new substrates to confirm elevation of GST and CoE enzymes, too, in *P. japonica* midguts. Our findings are consistent with results of studies with other generalist feeders; e.g., whiteflies (Xie et al., 2010) and two-spotted spider mites (Gibric et al., 2011) that use multiple detoxification enzymes to facilitate polyphagy.

It was important to use different substrates to determine activity of different isoforms of P450 and CoE in Japanese beetle midguts. Consumption of preferred host plants by *P. japonica* tends to induce P450 proteins with better EROD activity compared to ECOD, however the reverse was found with non-preferred host plants. Similarly, the P450 EROD activity was high for beetles fed a single species diet and P450 ECOD activity was greater with mixed plant species diets. These results, consistent with previous studies in other insects (Xie et al., 2010; 2005; Zhang et al., 2005), suggest that detoxification enzymes in *P. japonica* most likely exist in multiple isoforms. It is not surprising that a diversity of enzyme systems and isoforms would be active in response to consumption of single species or mixtures of host plants. In addition to activation of multiple enzyme systems among generalist herbivores, future work may implicate a greater diversity of isoforms of specific enzymes as another enabler of polyphagy.

Various studies (e.g., Ladd 1987, 1989; Keathley and Potter, 2008; Held and Potter, 2004) have largely supported the historical rankings of Japanese beetle feeding preference (Fleming 1972) within its broad host range (>300 plant species). This study, too, found greater feeding on plant species ranked historically as preferred. Still, relatively little is known about the basis for host preference in *P. japonica*, although differences in plant secondary chemistry doubtless are important drivers. We tried to identify patterns in enzyme activity that may help to explain some of the physiological costs to Japanese beetles of feeding on particular plants. Although induction of detoxification enzymes across the full set of test plants was generally greater when beetles consumed non-preferred hosts, individual preferred or non-preferred hosts often grouped together by means separation. For example, P450 activity induced by feeding on crape myrtle grouped with at least one or more non-preferred hosts. The only consistent pattern was overall low enzyme activity when beetles consume rose petals, which are highly suitable as food (Held and Potter 2004). Enzyme induction is not a result of greater consumption alone, but likely reflects differences in types and concentration of secondary chemicals in the different

plant food. Incubation of Japanese beetle gut protein with a dose range of various phytochemicals might clarify how particular ones affect enzyme activity (Francis et al., 2005).

Although midgut enzyme activity did not necessarily correlate with patterns of preference, it may help explain differences in the beetles' longevity and fecundity (e.g., Held and Potter 2004, Keathley and Potter 2008) associated with feeding on particular hosts. If enzymatic detoxification is costly, associated reductions in fitness could be important drivers of dietary preference within the beetle's wide range of potentially acceptable hosts. For example, rose petals are highly palatable and support high fecundity (Held and Potter 2004), and feeding on them seems to entail relatively little metabolic expense toward detoxification (this study). Those factors may help to explain why *P. japonica* is such a bane to rosarians wherever the pest occurs.

Previous studies on dietary mixing in polyphagous insect herbivores have yielded contrasting results; e.g., favoring growth rate in a grasshopper (Bernays et al., 1994) but impairing growth of a whitefly (Bernays, 1999). Japanese beetles may benefit from dietary mixing with flowers because the relatively high sugar content of flower petals may help to meet the presumably high energetic demands of this bulky, day-flying scarab (Held and Potter 2004). Activity of GST, P450, and CoE in Japanese beetle midguts seems to increase with mixed diets (Ahmad 1983; this study) but mixing *per se* does not seem to be as important as the species represented in the mix. For example, if diet mixing alone was responsible, then the combination of three plant species should have produced the greatest enzyme activity but that was not found. Two-species mixed diets with rose generally had greater activity than consumption of rose petals alone. However, combinations of crape myrtle and Virginia creeper had increased activity, relative to their respective single species diets, in certain enzymes (i.e., P450 ECOD) but not others (CoE). It is evident that Japanese beetles rely on these assayed enzymes (P450, GST and CoE) and proteinases (Zavala et al., 2009) when encountering a diversity of host plants but studies using molecular techniques (e.g., RNA interference) would provide insight into the changes that accompany host switching and fitness traits like insecticide tolerance, survival, and fecundity. These enzymes are also potential target sites for the novel management tools for this economically-important insect (e.g., Mao et al. 2007).

The presence of detoxification enzymes in non-digestive insect tissues such as brain (Zhu et al., 2010) and fatbody (Ahmad, 1983; Lee, 1991; Heiko et al., 2014) of insect herbivores

suggests other physiological functions in Japanese beetle apart from the metabolism of host plant allelochemicals. Starved beetles had considerable P450 ECOD and GST CDNB activities. GST activity can be induced by oxidative stress, thereby metabolizing reactive oxygen species (ROS) in form of peroxidases (Singh et al., 2001; Ahmad and Pardini; 1988, Lee 1991). We have seen that prolonged (48 h) starvation of Japanese beetles also produces an increase in the activity of metabolic enzymes especially GST (unpublished data). Starvation is used sometimes in lab studies to generate hunger or allegedly to standardize the activity of detoxification enzymes in field collected beetles. Besides implications for handling insects in future studies, there are two key times in the beetle's ecology when this also may be relevant, when males mate-guard without feeding for extended periods (Kruse and Switzer, 2006), and when females are engaged in ovipositional bouts in the soil (Potter and Held 2002). During these times of starvation, enzymes like GST may protect the insects by metabolizing ROS that may accumulate. Tissue-specific expression studies and molecular tools could provide insight about other physiological functions of these enzymes in adult and larval forms of Japanese beetles.

CHAPTER 3:

Geranium/Quisqualic acid Intoxication and Detoxification Enzymes in Japanese beetle

Abstract

Japanese beetle (JB), is a generalist herbivore that feeds on >300 host plants that spreads across 72 plant families, but it is paralyzed whenever it feeds on petals of zonal geranium (*Pelargonium* × *hortorum* Bailey), a preferred host plant. Quisqualic acid, the active compound responsible for intoxication of Japanese beetles, may somehow circumvent or not induce detoxification enzymes in Japanese beetles. We studied the connection between geranium and quisqualic acid intoxication, and the activities of cytochrome P450 (P450), glutathione S transferase (GST) and carboxylesterase (CoE) in the midgut of adult Japanese beetles. Beetles fed varying quantities of toxic petals of geranium flowers or agar plugs containing quisqualic acid were assayed for midgut enzyme activities. Activities of P450, GST and CoE were induced in Japanese beetles that consumed both geranium petals and quisqualic acid. Peak enzyme activity occurred after 24H but peak paralysis occurred after just 12H. Our study suggests that induction of detoxification enzymes by toxic geranium and quisqualic acid does not completely protect Japanese beetle from being paralyzed by geranium.

3.1 Introduction

Japanese beetle *Popillia japonica,* is a polyphagous scarab that feed on over 300 plant species with agricultural or horticultural importance, however some host plants have significant fitness costs for these beetles. For example, when adult Japanese beetles feed on flower petals of zonal geranium (*Pelargonium* × *hortorum* L. G. Bailey), paralysis results (Ballou, 1929, Held and Potter, 2003). Beetles prefer to feed on geranium flowers even in the presence of non-toxic highly preferred host, linden. Over time, repeated bouts of paralysis can reduce the fecundity and survival of Japanese beetles. Interestingly, some beetles recover from geranium paralysis under lab conditions and repeatedly choose geranium despite the post-ingestion malaise (Potter and Held 1999). Ranger et al. (2011) identified quisqualic acid as the compound in geranium petals that induces paralysis in Japanese beetles. Quisqualic acid is an agonist of glutamate receptors in muscles that causes excitotoxicity (Miyamoto et al., 1985). This neuromuscular action produces the extended legs and general paralyzed state of beetles originally described by Ballou (1929) and later confirmed by Held and Potter (2003).

Recovery from paralysis can occur whether beetles feed upon petals of geranium flowers (Potter and Held, 1999, Held and Potter, 2003) or quisqualic acid incorporated into artificial diet (Rangers et al., 2011). Beetles regardless of their experience with geranium-induced paralysis still become paralyzed following consumption. However, beetles experienced with geranium feeding can consume greater amounts of geranium petals than naïve beetles and have a lower incidence of paralysis relative to naïve beetles. The activity of detoxification enzymes in Japanese beetles has been suggested as a possible mechanism for recovery from this paralysis (Potter and Held, 1999).

Japanese beetles possess post-ingestive capabilities in form of detoxification enzymes; cytochromeP450 (P450), glutathione S transferase (GST) and carboxylesterase (CoE), for circumventing host plant allelochemicals (Ahmad, 1983) when feeding on plants. Detoxification enzymes are widely accepted to afford insect herbivores, the liberty to consume plants that would otherwise be toxic (Karban and Agrawal, 2002; Feyereisen, 2005; Schoonhoven et al., 2005 Li et al., 2007; Xie et al., 2010). Multiple detoxification enzymes systems (P450, GST and CoE) are induced in Japanese beetle's midgut in response to feeding on host plants and in response to feeding on a diversity of host plants (Ahmad, 1983). In Chapter 2, it was reported

that feeding on mixed diets from diverse plant families, generally induced higher titers of P450, CoE and GST in the midgut of Japanese beetle. Despite these defenses (P450, GST and CoE) against allelochemicals, the beetle is still severely paralyzed by consuming 1-2 geranium petals with a relatively small amount (~4.9 ng) of L-quisqualic acid (Ranger et al. 2011). Toxic plant allelochemicals when consumed by herbivores may interfere with the activities of detoxification enzyme systems like cytochrome P450 and glutathione S transferase (Neal and Beranbaum 1989,; Lee 1991, Snyder and Glendinning, 1996). The ability of *Manduca sexta* to recover from initial toxicity by nicotine is linked to increased activities of P450 activty (Snyder and Glendinning, 1996).

Therefore, it is necessary to study the possible connections between consumption of paralysis-inducing geranium flowers and the activities of detoxification enzymes in adult Japanese beetle. It is our hypothesis paralysis and recovery of Japanese beetles following consumption of geranium flowers or quisqualic acid interferes with the activity of at least one of the major detoxification enzymes. In this study, we examined the activities of three super-families of P450, GST, and CoE in the midgut of Japanese beetles in response to feeding on geranium flowers, and quisqualic acid incorporated into artificial diet.

3.2 Materials and Methods

3.2.1 Reagents and Supplies

Except where mentioned, all reagents and supplies were purchased from VWR Scientific and were of high grade and quality.

3.2.2 Source of insects

Adult beetles were collected from Town Creek Park in Auburn AL using traps (Pherocon® JB trap, Trécé, Adair, OK) baited with a food lure (2-phenyl-ethyl propionate, eugenol, and gereniol; 3:7:3 ratio). There were no plantings of geraniums at this location. The beetles were examined behaviorally in the lab to be active and void of any morphological defect before been used for feeding assays. Mixed sex beetles starved for 4-6 hours after field collection was used the feeding assays.

3.2.3 Source of plant material

Geranium (*Pelargonium x hortorum* Patriot Bright Red) plants were grown in black plastic pots filled with soilless media (Earthgro potting Soil, Maryvile, OH) in the greenhouse facilities (25-27 °C) at Auburn University from March to July, 2014. Plants were watered daily, fertilized twice with lo-start fertilizer 15-9-12 (eveRRiS: Dublin, OH), and no pesticides were applied during this time. Petals of *Rosa x radazz 'Knockout'* (Rosaceae) were freshly collected from different plantings at Rose Garden, Comer Hall, and Auburn University. Rose petals are a preferred plant (Held and Potter 2003) and were used as a control. Petals used in the feeding experiments were freshly harvested < 6 h before each experiment. To ensure representative samples, each petal was plucked from separate rose and geranium flowers from different plant stands.

3.2.4 Activities of P450, GST and CoE associated with consumption of geranium petals

This experiment compared the differences in enzyme activities that accompany consumption of either geranium petals or rose petals by Japanese beetles. Beetles were provided freshly harvested petals and allowed to feed for 6h, 12h or 24h. A group of beetles were held without food in similar conditions for the same time periods as another level of experimental control. Beetles were individually held in 37 ml sterile waxed-bottom transparent plastic cups at 25-27 °C with a 14:10 L: D photoperiod. Moistened filter paper was placed at the bottom of each cup to prevent desiccation and provide humidity. Each beetle received two petals of either plants pinned to the center of the cup (Held and Potter, 2003). The mass of petal tissue (geranium or rose) in mg consumed by each beetle was determined by weighing the petals before and after feeding using a weighing balance and a digital imaging software estimate the area of c petal consumed (Image J, http://imageJ.net), this was converted to equivalent fresh weight using a mass/area ratio. Prior observation showed no significant desiccation or weight loss of petals within 24 hrs, when held with moistened filter paper. The number of beetles paralyzed by geranium at each time period was scored. A beetle was considered paralyzed when it could not move or extend it legs and was unable right itself when placed on its back (Held and Potter, 2003; Ranger et al., 2011). Each treatment group (i.e. host plant × time) had 15-20 beetles in a randomized complete block experimental design with four replicates.

3.2.5 Activities of P450, GST and CoE associated with consumption of quisqualic acid

The second experiment had a similar design to the one described above, but evaluated enzyme activities associated with feeding on agar plugs (Ranger et al. 2011) containing toxic but non-deterrent doses of quisqualic acid and Pipernoyl butoxide (PBO). PBO is a known inhibitor of cytochrome P450 and also act as insecticide synergist (Snyder and Glendinning, 1996; Feyerisen, 1999). In a preliminary test, we determined that 10 µg of quisqualic acid per agar plug was sufficient to induce paralysis yet not deter feeding. This concentration of quisqualic acid was used in the experiment. Similarly, 1 µl of 28mM PBO was also determined as the maximum rate of PBO that would not affect feeding (unpublished data). These concentrations of quisqualic acid and PBO were used in the experiment.

Both PBO and quisqualic acid were administered to beetles via artificial diet i.e. agar plug (Ranger et al., 2011; Ladd 1986). Each agar plug (diameter of Xmm) contained 10µl of 0.1M of sucrose, a phagostimulant for Japanese beetle (Ladd 1986). The average weight of the agar plugs used in the experiment was 317 ± 10.26 mg, all agar plugs used in this experiment was made form preparation thereby ensuring consistency in the concentration of agar in each plug. Controls in this experiment were agar plugs containing sugar but with no other additives. Quisqualic acid (QA) and PBO was injected into individual agar plugs using a pipette, to ensure the amount per plug.

Feeding assays were conducted with individual beetles held individually held in petri dish (90 mm diameter) at 25-27 °C with 14:10 L: D photoperiod. Agar (with or without QA and/or PBO) were individually weighed before and after feeding and the difference (in mg) determined the amount consumed. Unused agar plugs were left as a control test to account for weight loss, however this was insignificant. The incidence of paralysis, amount of food ingested and the activities of detoxification enzymes in beetles' gut were determined at 12 h and 24 h. Non-feeding beetles were removed from the data set before analysis. Each treatment group had 15-20 beetles and the experiment was replicated three times.

3.2.5 Dissection for removal of midguts

After feeding, beetles were pinned on an ice-chilled waxed-bottom petri dish covered with Parafilm to prevent contamination and dissected under a binocular microscope. Beetles'

guts were excised using sterilized dissecting scissors and forceps. The guts from beetles fed the same diet were immediately transferred into 1.5 ml Eppendorf tubes placed on dry ice. After dissection, the gut tissues were stored in -80 °C freezer.

3.2.6 Extraction of microsomes and cytosols

Beetle guts were homogenized in 10 ml of ice-cold homogenization buffer (0.1 M phosphate buffer pH 7.5, 10% glycerol, 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF and 1 mM PTU). The homogenate was filtered through a double-layered cheesecloth into a 15 ml centrifuge tube. The filtrate was centrifuged at 10,000 g for 30 min at 4 °C (Beckman Allegra 25R equipped with a type TA-14-50 rotor). The pellet (nuclear debris, and other materials) was discarded and the supernatant was transferred into another centrifuge tube and spun in an ultra-centrifuge (Sorvall, Discovery 90SE,) at 100,000 g for 1 h at 4 °C. The resulting supernatant (cytosolic fraction) was removed and used as the source of enzymes for the GST and CoE activity assays. Pellets (microsomes), the source of P450 enzyme, were dissolved in a re-suspension buffer (0.1 M phosphate buffer pH 7.5, 20% glycerol, 1mM EDTA, 0.1 mM DTT and 1 mM PMSF). All the steps described were performed on ice. The microsomes and cytosols (source of GST and CoE) were temporarily stored at -80 °C.

3.2.7 Protein content determination

The method of Bradford (1976) with Bio-Rad Dye reagent, was used to determine the amount of protein present in the microsomes and cytosols of each treatment group using bovine serum albumin as the standard. A minimum of triple determinations was done for each sample using a UV-VIS spectrophotometer (Du 640, Beckman Coulter).

3.2.8 P450 activity assays

P450 activity was assayed on the ability of microsomes from beetles' gut to covert 7-ethoxycoumarin to 7-hydroxycoumarin by O-deethylation (ECOD). The method used was modified from earlier studies by Lee and Scott (1992) and Guo et al., (2014). A sample (30–50 µl) of the microsomal fraction and 40 µl of ECOD buffer (50mM Tris-base, 150mM KCl and 1mM EDTA, pH 7.8) containing 4nM of 7-ethoxycoumarin (7-EC) was added to each well of a 96-well plate (Black flat-bottom BD Falcon). The reaction was initiated by adding 0.1 µM NADPH (freshly prepared) to each well. The reaction mixture was incubated for 30 min at 30

°C. The fluorescence of NADPH was quenched by adding 0.3 µM oxidized glutathione and a 0.5 unit of glutathione reductase (Sigma-Aldrich) to each well. The reaction was stopped after 10 min at 25 °C by adding 120 µl of stop solution (50% acetonitrile and 50% 0.05 M, pH10 Trizma-base buffer). The amount of 7-hydroxycourmarin produced was measured using a microplate reader (Cyataion 3: Biotek) at a wavelength of 390 nm and 465 nm for excitation and emission respectively. The control was the reaction between NADPH and 7EC without microsomal fraction. A product standard curve was also generated by using seven different dilutions of 100 µM of 7-hydroxycoumarin under the aforementioned conditions. ECOD P450 activity was expressed as pmoles of product formed per min per mg protein.

3.2.9 GST activity assay

GST activity was assayed by the ability of cytosolic fractions to catalyze the conjugation of glutathione (GSH) and chroloro-2, 4-dintrobenzene (CDNB), a method modified from Habig et al., (1976). The reaction was carried out in a transparent 96-well plate. A phosphate buffer (0.1M pH 6.5) containing EDTA (1mM), PMSF (1mM) and DTT (1mM) was used. For each reaction, freshly prepared GSH (dissolved in buffer) was mixed with 5 µl of the enzyme source in a final volume of 200 µl and a final GSH concentration of 2.5 mM. Reactions were incubated for 3 min at 25°C, after which 10 µl of 30 mM CDNB (dissolved in alcohol and freshly prepared) was added. The control reaction is the non-enzymatic reaction between GSH and CDNB under the same conditions. The mixture was shaken slightly and the change in absorbance was measured for 5 min using a plate reader (Cyataion 3: Biotek) set at 340 nm. GST activity was expressed as micromoles CDNB conjugated per min per mg protein using the extinction co-efficient of $5.3mM^{-1}cm^{-1}$ for S-(2,4-dintrophenyl) glutathione.

3.2.10 CoE activity assays

This was based on the hydrolysis of α- naphthylacetate (α-NA) to α-naphthol by modified methods of Van Asperen (1962), and Zhang et al (2007). The reaction was carried out in a transparent flat bottom 96-well plate. The working concentration of the substrate (α-naphthylacetate) was 0.3 mM (containing 0.3 mM eserine), diluted from 0.03 M stock solution. Each assay reaction contained: 60 µL phosphate buffer (0.04M, pH7.0), 50 µL of the supernatant (enzyme source) and 80 µL of 0.3mM of substrate solutions. The reaction (slightly shaken) was incubated at 37 ℃ for 30 min and stopped by adding 20 µL of stop solution (two parts of 1%

Fast Blue BB and five parts of 5% sodium dodecyl sulfate). Color change was allowed to develop for 15 min at room temperature, and the absorbance was measured at 600 nm for the hydrolysis of α-NA, using microplate reader (Cytation 3: Bio-Tek®). The products (α- and β naphthol) standard curve was generated by measuring optical absorbance at same wavelength for enzymatic reactions, at different concentrations. Each well was added 0, 1, 5, 10, 20 and 30 µL of 32mM of α-naphthol dissolved in acetone and also 20 µL of stop solution.

3.2.11 Data analyses

ANOVA was used to compare the average mass of petals or agar consumed by Japanese beetle and induced activity of P450, GST and CoE, across the tested time periods. The number of beetles paralyzed by consuming either geranium or quisqualic acid across different time periods were compared using logistic regression analysis. All analyses were done using JMP (SAS JMP version Pro 11, SAS Corp.).

3.3 Results

3.3.1 Activities of P450, GST and CoE associated with consumption of geranium petals

Consumption of geranium and rose petals induced activity of one or more enzyme groups but the magnitude of the induced responses varied over time and by plant species (Figure 3.1).

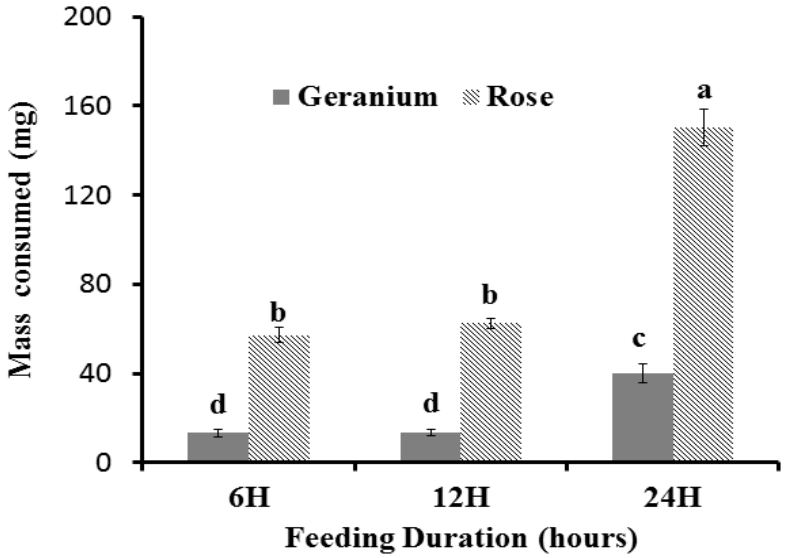

Figure 3.1: Average mass (mg) of rose and geranium petals consumed by Japanese beetles at different time periods. $F_{(5, 15)} = 132.7$, $P < 0.0001$. 15-20 beetles per treatment group.

Feeding induced enzyme activity was most evident relative to the starved control beetles. Starved beetles had lower P450 and GST activities than beetles fed either rose or geranium petals at 6 and 12 h (Figure 3.2). This pattern was not consistent with CoE activity. CoE activity was

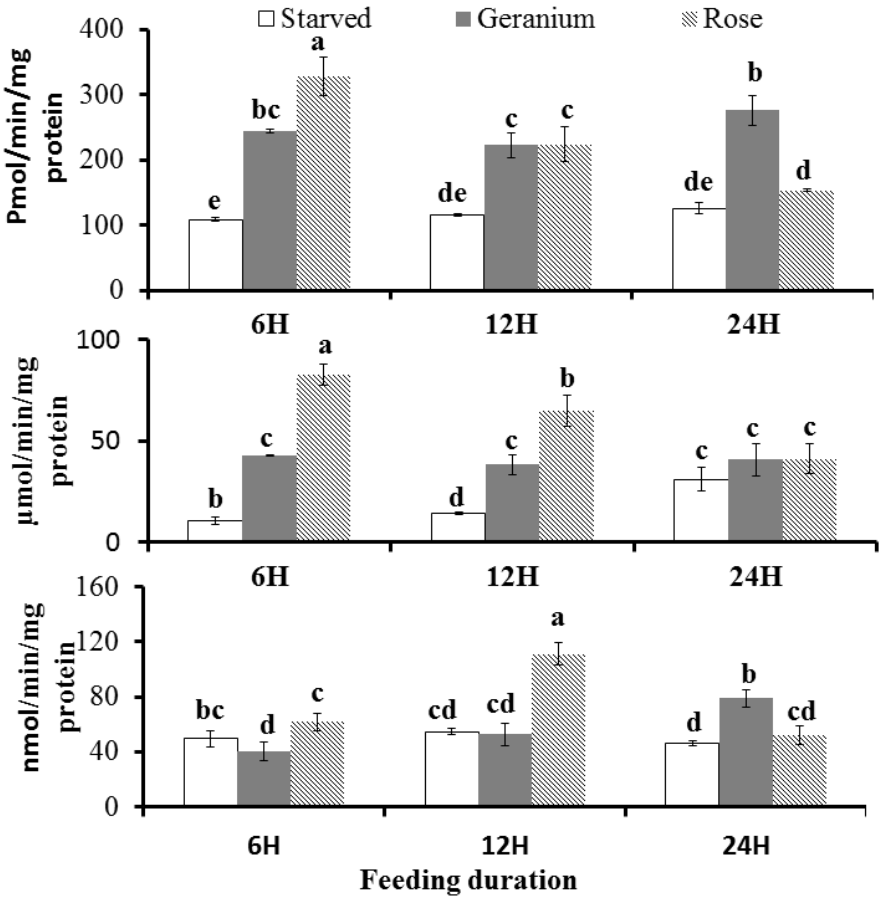

Figure 3.2: Activities of detoxification enzymes in the midgut of adult Japanese beetles that consumed petals of rose or geranium at different time periods. Top: P450 Activity: $F_{(9,60)} = 26.46$, $P < 0.001$. Middle: GST activity: $F_{(9,60)} = 17$, $P < 0.001$. Bottom: CoE activity: $F_{(9,60)} = 16.18$, $P < 0.001$. Each of the enzymatic was replicated 4 times with 15-20 beetles per treatment group.

relatively consistent in starved beetles over time, but beetles had significantly greater CoE activity at 12 h if consuming rose petals, and at 24 h for beetles consuming geranium petals. Regardless of time, beetles consumed more mass of rose petals than geranium because paralysis resulted from feeding on geranium petals. More than 50% of beetles were paralyzed at each time with significantly more paralyzed at 12h (87%) than at 6 or 24 h (χ^2=10.7 P<0.005; Figure 3.3).

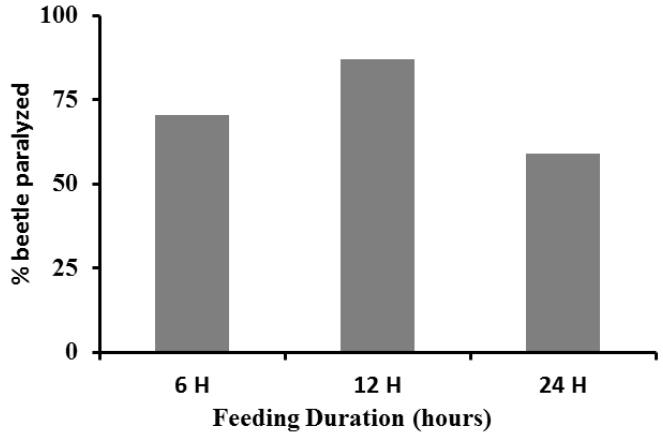

Figure 3.3: Time difference in the paralysis of Japanese beetle by geranium. (P<0.05, logistic regression). Paralysis of beetles by geranium was modeled as binary count and illustrated as percentage of total numbers of beetles tested.

There was insignificant difference in the number of beetles paralyzed at 6h (70%) and 24h (59%) (*P*=0.27) although consumption of geranium as well as rose petals increased significantly between 12 h and 24 h (Figure 3.2)**.** There was 3 fold increase in the amount of geranium consumed by beetles from 12h to 24h, compared to 2.5 fold increase in the average amount of rose consumed.

Activities of P450 and GST generally increased quickly at 6 then decrease from 6-24 h despite greater consumption at 24 h. Beetles that consumed geranium petals had relatively consistent activity for P450 and GST over time despite an increase in consumption between 12 and 24 h. Due to the rapid induction then waning, P450 and GST activity in beetles that consumed rose petals was significantly greater only at 6 h then equal to, or significantly lower than beetles that consumed geranium thereafter. CoE had significantly spikes in activity at 12h when beetles consume rose and at 24 h when beetles consume geranium petals.

3.3 Consumption of artificial diet in the presence and absence of quisqualic acid

Quisqualic acid did not cause a significant reduction in the amount of agar (artificial diet) consumed by Japanese beetle, both at 12 and 24 h, despite inducing paralysis. However, the amounts of agar diet at 24 h consumed was significantly higher compared to 12 h. Interestingly, the mixture of qusiqualic acid and PBO reduced the average mass of agar plug consumed by beetles at 12h by 25% relative to control diet but no effect at 24h. This suggests the possibility of P450 playing an important metabolic role at early stage of quisqualic acid intoxication (Figure 3.4). Consumption of quisqualic acid induced higher activity of P450, GST and CoE at 12 and

24 h compared to agar diet with/without PBO. Implying the induced response to quisqualic acid paralysis inform of induced enzyme activities (Figure 3.5). PBO significantly reduced P450 activity at 12 h with a resulting increase in GST and CoE activities compared to consumption of agar plugs only. The mixture of PBO and quisqualic acid, induced greater activities of GST and CoE especially at 24 h, suggesting that a multi-enzyme detoxification system in Japanese beetle against quisqualic acid intoxication.

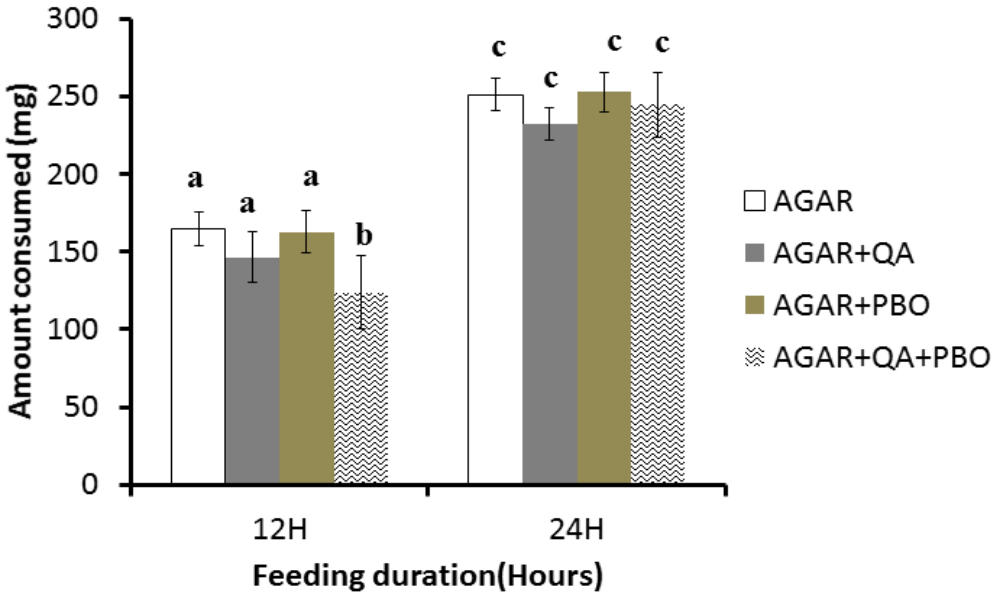

Figure 3.4. Average mass (mg) of agar plugs with/without quisqualic acid and/or PBO consumed by Japanese beetles at different time periods. $F_{(5, 15)} = 13.2$ $P < 0.0001$.

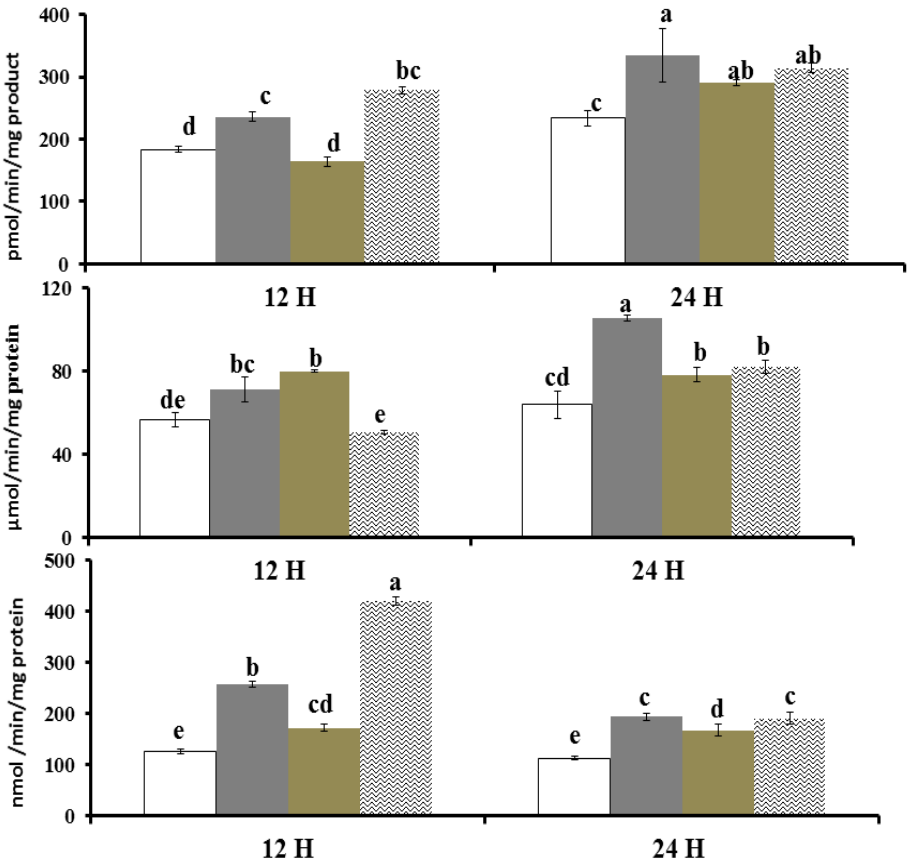

Figure 3.5: Activities of Detoxification enzymes in the midgut of adult Japanese beetles that consumed artificial diet with/without quisqualic acid (QA) or PBO, at different time periods. Top: P450 Activity: $F_{(9, 60)} = 13.4$, $P < 0.001$, Middle: GST activity: $F_{(7,38)} = 20.6$, $P < 0.001$.Bottom: CoE activity :. $F_{(9, 60)} = 160.7$, $P < 0.001$. Number of replicate is 3, there was 15-20 beetles per

Both Quisqualic acid and quisqualic acid plus PBO, induced peak paralysis of Japanese beetle at 12 h. However, more beetles were paralyzed by the combination of quisqualic acid and PBO at 24 h compared to quisqualic acid alone (Figure 3.6), suggesting the involvement of P450 in the metabolism of toxic quisqualic acid.

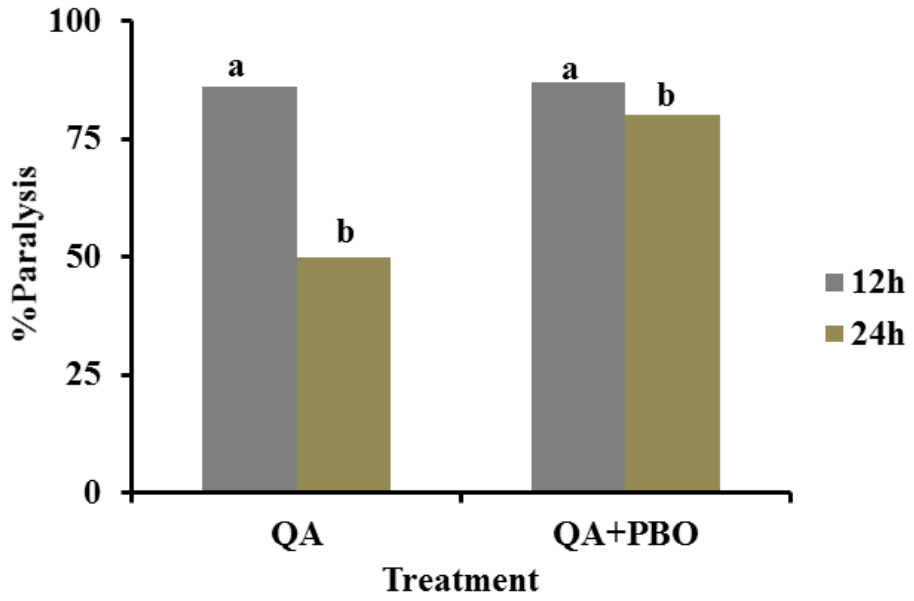

Figure 3.6: Time difference in the paralysis of Japanese beetle by quisqualic acid. ($P<0.05$, JMP Pro 11, logistic regression). Paralysis of beetles by quisqualic acid (QA) was modeled as binary count and illustrated as percentage of total numbers of beetles tested. 15-20 beetles per treatment group. Number of replicate is 3.

3.5 Discussion

Japanese beetle is a generalist herbivore that encounters diverse potentially toxic phytochemicals while foraging on host plants. Yet, consumption of geranium flowers delivers a dose of quisqualic acid sufficient to induce a temporal paralysis. Detoxification enzymes (P450, GST and CoE) are one adaptation used by Japanese beetles to accommodate this polyphagous habit (Ahmad, 1983). This study provides evidence that consumption of geranium flower petals, although intoxicating, also induces enzyme activities in the midgut of Japanese beetles. Potter and Held (1999) speculated an unknown allelochemicals (quisqualic acid was not yet identified from geranium) in flower petals may somehow circumvent the detoxification enzymes

previously reported in the digestive system of Japanese beetles. Nevertheless, the induction of these enzymes does not completely protect the beetles from geranium intoxication. It remains unknown if quisqualic acid is metabolized by Japanese beetle's detoxification enzymes. An incubation study would show the rate of metabolism or inhibition and the end products resulting from enzymatic degradation of quisqualic acid.

Quisqalic acid-induced paralysis in adult Japanese beetles is temporal in the lab and often fatal in the field (Held and Potter, 2003). A 10 µg dose of quisqualic acid in an agar plug with 0.1 M sucrose was sufficient to induce paralysis in 87% of the tested beetles (at 12 h), which was similar to what we observed for beetles feeding on geranium petals. It is likely that one or more enzyme groups act upon quisqualic acid in the Japanese beetle midgut. When tested with petals, Japanese beetles consume approximately 1.25 mg of quisqualic acid per g of petal tissue (Ranger et al., 2011) in addition to other phtyochemicals (e.g., flavonoids, geraniol) present in geranium flowers. Other phytochemicals may compete as substrates for detoxification enzymes once in the midgut of the beetle. Further investigation is required to identify other phytochemical(s) present in geranium that (could) act as synergist for the toxicity of quisqualic acid to Japanese beetles. The delay in recovery in spite of enzyme induction may occur as enzymes become liberated from primary substrates to conjugate quisqualic acid secondarily.

The experiment with agar plugs provides insight into enzymatic degradation of quisqualic acid independent of other phytochemicals. Both experiments allow for indirect comparison of *in planta* versus artificial doses of quisqualic acid relative to enzyme activity. It was clear that CoE activity, but not P450 or GST, was induced coincident with significant paralysis when beetles consumed agar plugs with quisqualic acid. CoE activity also increased over time when beetles consumed geranium petals and was greatest at 24 h when paralysis decreased. P450 and GST activity decrease over time when beetles consumed quisqualic acid *in planta* but increased over time when beetles consumed quisqualic acid in agar plugs. The increase in P450 and GST activity are coincident with a decrease in paralysis at 24 h in the agar plug experiment.

The recovery of geranium/quisqualic acid-paralyzed Japanese beetles varied with time as shown by the result of this study. The heterogeneity in recovery also reflects the physiological fitness of each beetle. P450, GST and CoE enzymes are likely involved in the recovery of the beetles from quisqualic acid intoxication. This is because the activities of these enzymes

generally peaked at 24 h when feeding or geranium or quisqualic acid and the incidence of paralysis was lowest at this same time. Previous studies (Lee, 1991; Neal and Berenbaum, 1989; Li et al., 2007) have also implicated these enzymes in nullifying or reducing the toxic effects of toxic alleochemicals on insect herbivores. However, in vitro studies are required to understanding the direct interaction of quisqualic acid and the gut enzymes of Japanese beetles. This will provide further insight on which of these enzymes play vital role in metabolism of quisqualic acid.

Beetles consuming a dose of quisqualic acid in agar show an increase in gut P450 and GST activity between 12 and 24 h but not beetles feeding on geranium petals. P450 activity is relatively constant and GST activity decreases during a feeding bout of similar duration on geranium petals. Activity of CoE increases over time when beetles consume petals but decrease over time when they consume agar plus quisqualic acid. Neal and Beranbaum, (1989) reported that gut enzymes from *Papilio polyxenes* were less sensitive to phytosynergists like myristicin and safrole, present in umbellifers that increases the toxicity of furanocouamrins. Francis et al., (2005), also observed differences in the titers of GST in aphis that was fed brassica plants and aphis that fed on brassicaceae allelochemicals incorporated into artificial diet. Further investigations will be required to identify other phytochemicals present in geranium petals and their interaction with insect detoxification enzymes.

Allelochemicals in geranium petals may not be good substrates for GST enzymes. Thus, the delayed response of enzymes to geranium consumption likely resulted in the toxicity of geranium to Japanese beetle, as the case of nicotine on the tobacco budworm (Snyder et al., 1994) and indole 3-carbinol on fall armyworm (Glendinning and Slansky, 1995). Larvae of *Spodoptera frugiperda*, had a delayed behavior when challenged with artificial diet containing toxic but non-deterrent dose of indole 3-carbinol, which is a plant allelochemical that induced P450 activity required for continued feeding (Glendinning and Slansky, 1995). It is also possible that the rate of digestion of these diets may vary in the midgut, which likely affect enzyme activities.

Ranger at al., (2010) quantified the amount of quisqualic acid present in one petal of geranium to be 4.62 ± 0.11 ng. However, a higher dose of synthetic quisqualic acid 10 µg was required to induce paralysis in Japanese beetles in this study. There was no complete (100%)

paralysis at any tested time in this study as against the study of Ranger at al., (2010). This difference might be due partly to variations in the populations of beetles used in the studies and also in the formulation and stability of the synthetic form of QA used for the assays.

Beetles were more paralyzed at 12 h of feeding on geranium and quisqualic acid but with higher enzyme activities of P450, GST and CoE at 24 h. This shows that beetles responded physiologically to the high level of intoxication at 12 h with elevated activities of enzymes, which resulted in higher consumption and the reduced paralysis at 24 h of feeding. Snyder and Glendinning (1996) reported that tobacco hornworm, failed to significantly increase its consumption of nicotine diet until 36 h, which is also the time for peak activities of P450 enzymes that reduced the toxic effect of nicotine. The reduced paralysis of beetles at 24 h suggests that isoforms of P450, GST and CoE induced at this time are more efficient at metabolizing geranium and QA relative to enzymes induced earlier (6 and 12 h). However, further investigation is needed to verify this speculation.

Chapter 4

Influences of Life stage and Time of Feeding on the activities of Detoxification enzymes in Japanese beetle

Abstract

In this study, we characterized the expression profiles of three super-families of detoxification enzymes: cytochrome P450 (P450), Glutathione S transferase (GST) and carboxylesterase (CoE) in the immature life stages of Japanese beetles using model substrates and also examined the influence of feeding duration and starvation on the activities of P450, GST and CoE in the midgut of adult Japanese beetle using a preferred host plant Virgina creeper. The activities of these enzymes are developmentally expressed in the different life stages of Japanese beetles, with enzymes from each life stage having varying affinity to the substrate and reaction speed. Furthermore, a feeding bout of 24 h was enough to induce optimal activities of P450, GST and CoE in the midgut of adult beetles. The consequences of the observed pattern of enzyme activities in the physiology and ecology of the Japanese beetle are discussed.

4.1 Introduction

Cytochrome P450, glutathione S transferase and carboxylesterase are the most important superfamilies of enzymes that are widely studied for their metabolic roles in insects (Li et al., 2007). Apart from metabolizing various classes of xenobiotics, detoxification enzymes are also known for performing other physiological functions by metabolizing endogenous substrates like hormones, lipids, pheromones and also aid in combating oxidative stress (Li et al., 2007; Feyereisen, 2005; Ranson and Hemmingway, 2005). Throughout growth and development, the activities of detoxification enzymes markedly vary in different tissues among the various life stages of a particular species. Scott and Lee, (1993) reported varying levels of P450 monooxygenase activities in different tissues of houseflies in response to phenobarbital treatment. The variation in titers of these enzymes across insect tissues provides an insight into their functions (Ranson and Hemmingway, 2005). For example, the detection of P450 enzymes in housefly antennae by Ahmad (1987) suggested the enzymes role in pheromone metabolism. Interestingly, tissue specific expression of detoxification enzymes have important consequence on a species phenotype, for example deltamethrin resistance in *Tribolium castenum* is due over expression of a brain specific P450 protein (Zhu et al., 2010). There are also studies that have reported differences in the expression profiles of P450, GST and CoE across the life stages of insects (Ahmad, 1983; Ting and Liu, 2011). Pottier et al., (2012) also reported differential expression of P450 genes in the antennae of *Spodoptera littoralis* in response to plant odors and also variation in larval and adult expression of certain P450 genes. These studies showed difference in the expression of P450 between larval and adult; suggesting the various physiological roles played by detoxification enzymes. The activities of the detoxification enzymes in different body tissues of the insect is suggestive of the role(s) played by these enzymes and also this can reveal the localization of genes encoding these enzymes.

The ecologies of the adult and larval Japanese beetles are distinct. The adults are polyphagous, feeding on diverse host plants while the larval are restricted to feeding on roots of turfgrass and mainly herbaceous annuals and perennials (Potter and Held, 2002). However, it is still largely unknown how the expression profiles of these enzymes changes across the life stages and tissues of this insect and also the temporal induction of these enzymes by feeding especially

in the adult Japanese beetles. Ahmad (1983) reported a 2 fold difference in gut P450 activity between actively feeding early third instar Japanese beetle larvae compared to overwintered late instar larvae. The difference in P450 activity was attributed to induction by feeding, since the late 3rd instar had reduced. Using the same P450 substrate i.e. *p*-chloromethyl aniline (PCNMA), Ahmad (1983) observed peak activities of P450 in midgut tissue, especially those that fed on multiple host plants, while quiescent life stages like pre-pupa, pupa and newly emerged adults had low P450 activity. Later studies, reviewed by Feyereisen (2005) and Ranson and Hemingway (2005) suggest that insect herbivores rely on multiple superfamilies of detoxification enzymes which exit in isoforms with varying substrate specificity. Hence, it is important to study the expression of other detoxification enzymes (GST and CoE) and also P450 (using substrate other than PCNMA) in life stages of Japanese beetle. This will provide better insight on the biochemical defense in this insect species and the role they play in the physiology and ecology of Japanese beetle.

Different aspects of the feeding behavior of adult Japanese beetles have been the focus of many studies (review, Potter and Held, 2002). Male and female adult Japanese beetles usually pulse during feeding for different reasons. Males will mate-guard females for long periods without food (Kruse and Switzer, 2006) and the females will stay without food during oviposition in the soil, before returning to host plant for feeding (Fleming, 1972). However, it is unknown the effect of feeding duration and starvation on the activities detoxification enzymes in adult Japanese beetle.

This chapter present the life stage expression of P450, GST and CoE activities in the egg, first, second and third instar larva of Japanese beetle. We also, we studied the effect of feeding duration on the induction of these enzymes in the adult midgut.

4.2 Materials and Methods

4.2.1 Source of insects

Male and female Japanese beetles were collected from sites within Town Creek Park in Auburn, Alabama using traps (Pherocon® JB trap, Trécé, Adair, OK) baited with a food lure (2-phenyl-ethyl propionate, eugenol, and gereniol; 3:7:3 ratio). Beetles from the traps were reared in

plastic tubs containing sterilized sifted silt loam soils at 25-27 °C with a 14:10 L: D photoperiod. Each plastic tub contained mixed adult beetles that were allowed to naturally mate, and the females oviposited into the soil. The soil in the rearing chamber was moistened every 12h to provide humidity and prevented beetles from desiccation, this also allowed for conducive soil ambient for eggs laid by beetles. After 72 h of mating and oviposition, eggs were collected from the soil by scooping through the soil with a moist camel's hair brush, eggs collected were placed in 1.5ml centrifuge tub and stored immediately in -80 °C freezer. Eggs left in the soil tub were left unperturbed for 2-3 weeks to hatch into first instar larvae, some of the first instar grubs were collected into 1.5 ml centrifuge tube and also stored in -80 °C freezer. To collect second instars, rye grass was planted in small transparent plastic cups (manufacturer and city) was infested with first instars and they were allowed to grow into second instars by feeding on the roots of rye grass. Pre-wintered third instars (early third instar grubs i.e. **ETI**) were collected from infested *Poa pratensis* turfgrass in WI from sites that has not been treated with pesticides for 12 months. Overwintered third instar grubs (late third instar i.e. **LTI**) were collected from the roots of infested rye grass grown in an outdoor screen house in Auburn AL, which had no pesticide application.

The first experiment examined the activities of detoxification enzymes across the pre-adult life stages of Japanese beetles using its egg, first instar, second instar and third instars (ETI and LTI) grubs as the source enzymes. These immature life stages were sourced as described above and the enzymes (P450, GST and CoE) fractions extracted from each life stage was characterized using multiple model substrates. The enzyme kinetic characteristics includes; activity (amount in moles of products produced per min per mg protein of enzyme source under defined condition), Michaelis constant (K_m) which reflect the affinity of an enzyme to a particular substrate under defined condition and maximum velocity (V_{max}) which indicates how fast an enzyme can catalyze a reaction. Each replicate had a pool of 50 eggs, 20 first instars and 5 second instars, and 4 ETI and 4 LTI. There were two-three replicates of each life stage depending on availability.

The second experiment tested the influence of feeding duration on the activities of detoxification enzymes (P450, GST and CoE) in the midgut of adult Japanese beetle. Virginia creeper *Parthenocissus quinquefolia* (Vitaceae), a documented highly preferred host plant

Japanese beetle was used as the model diet (Held, 2004). Actively feeding mixed sex adult beetles collected as describe above were individually held in a 100mm × 15mm sterile polystyrene petri dish laid with a moistened filter paper. A filter paper (Fisher brand) was also placed in each petri dish and was moistened with distilled water via a pipette to prevent desiccation of the foliage of host plant fed and also provide humidity for the beetles. This experiment was carried out in the laboratory at a temperature of 25-27 °C and 14:10 L: D photoperiod. The beetles were divided into different treatment groups based on time of feeding and starvation periods after feeding i.e.12, 24 and 48 h. The starved groups were initially fed for period of time equal to their subsequent starvation period, for example beetles starved for 24h was initially allowed to feed for 24 h. Freshly harvested foliage of Virginia creeper free of any pesticide application and disease attack was provided to each adult beetle *ad libitum*. After feeding, beetles belonging to the same treatment group was pooled together and dissected for their midguts with the aid of sterile forceps under a stereo light microscope as described in Chapter 2 and 3. Each treatment group had 18-25 beetles and this experiment was replicated three times. Non-feeding beetles were excluded from this experiment during the feeding assay.

4.2.2 Quantifying consumption of Virginia creeper: foliage were weighed and scanned individually prior to use and the mass per unit leaf area was determined. After feeding, the remnant tissue was re-scanned, missing area was computed with digital imaging software (Image J, http://imagej.net), and the amount consumed (mg) by each beetle was determined.

4.2.3 Extraction of microsomes and cytosols

Whole body of immature life stages or adults beetles guts were homogenized in 10 ml of ice-cold homogenization buffer (0.1 M phosphate buffer, pH 7.5; 10% glycerol, 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF and 1 mM PTU). The homogenate was filtered through a double-layered cheesecloth into a 15 ml centrifuge tube. The filtrate was centrifuged at 10,000 g for 30 min at 4 °C (Beckman Allegra 25R, equipped with a type TA-14-50 rotor). The pellet (nuclear debris, mitochondria and other materials) was discarded and the supernatant was transferred into a 30ml PC Oak Ridge centrifuge tube (Thermo Scientific, Asheville, NC) and spun in an ultra-centrifuge (Sorvall, Discovery 90SE, equipped with a TI 865 rotor) at 100,000 g for 1 h at 4°C. The resulting supernatant (cytosolic fraction) was removed and used as the source of enzymes for the GST and CoE activity assays. Pellets (microsomes), the source of P450 enzyme, were

dissolved in a re-suspension buffer (0.1 M phosphate buffer, pH 7.5; 20% glycerol, 1mM EDTA, 0.1 mM DTT and 1 mM PMSF). All steps were performed on ice. The microsomes and cytosols (source of GST and CoE) were temporarily stored at -80°C.

4.2.4 Enzyme assays

4.2.4.1 Protein content determination

Protein concentrations present in the microsomal and cytosolic fractions of each experimental group were determined by the Bradford (1976) method using Bio-Rad dye reagent and bovine serum albumin as the standard. A minimum of triple determinations was done for each sample using a UV-VIS spectrophotometer (Du 640, Beckman Coulter, Brea, CA).

4.2.4.2 P450 activity assay: activity was based on the ability of microsomal fraction from each treatment groups to catalyze the *O*-deethylation of 7-ethoxycoumarin to 7-hydroxycoumarin (ECOD). The reaction mixture contained 30-50 µl of microsomal fractions and 40mM of 7-ethoxycoumarin and 0.1 µM NADPH (freshly prepared). The reaction was carried as described in Chapter 2 and 3 in a 96-well plate (Black flat-bottom BD Falcon; Thermo Scientific, Asheville, NC). P450 activity was expressed as *p*moles of product formed per min per mg protein. Km and Vmax for P450 ECOD were measured from double reciprocal plot of velocity (activity) vs substrate concentration (Lineweaver-Burk plots) using 5 concentrations of 7 EC dissolved in ECOD buffer.

4.2.4.3 GST activity was determined as the ability of cytosolic fractions to catalyze the conjugation of glutathione (GSH) and chroloro-2, 4-dintrobenzene (CDNB), a method modified from Habig et al., (1976). For each reaction, freshly prepared GSH (dissolved in buffer) was mixed with 5 µl of the enzyme source in a final volume of 200 µl and a final GSH concentration of 2.5 mM. Reactions were incubated for 3 min at 25°C, after which 10 µl of 30 mM CDNB (dissolved in alcohol and freshly prepared) was added. Change in absorbance was measured for 5 min using a plate reader (Cyataion 3: Biotek) set at 340nm. GST activity was expressed as micromoles (µM) CDNB conjugated per min per mg protein using the extinction co-efficient of 5.3 $mM^{-1}cm^{-1}$ for S-(2,4-dintrophenyl) glutathione. The kinetic constants K_m and V_{max} determined from double reciprocal plots of velocity and substrate, GSH concentration was kept constant and CDNB was diluted to 4-6 concentrations.

4.2.4.4 Carboxylesterase activity assays: activity was based on the hydrolysis of α- and β-naphthylacetate (α- and β-NA) to α- and β-naphthol by modified methods of Van Asperen (1962), and Zhang et al., (2007). The reaction was carried out in a transparent flat bottom 96-well plate. The working concentration of the substrates (α- and β-naphthylacetate) was 0.3 mM (containing 0.3 mM eserine), diluted from their respective 0.03 M stocks. Each assay reaction contained: 60 µL phosphate buffer (0.04M, pH7.0), 50 µL of the supernatant (enzyme source) and 80 µL of 0.3 mM of substrate solutions. The reaction (slightly shaken) was incubated at 37 ℃ for 30 min and stopped by adding 20 µL of stop solution (two parts of 1% Fast Blue BB and five parts of 5% sodium dodecyl sulfate). Color was allowed to develop for 15 min at room temperature, and the absorbance was measured at 600 nm for the hydrolysis of α-NA, and at 550 nm for β-NA using microplate reader (Cytation 3: Bio-Tek). K_m and V_{max} was measured for each substrate (α- and β-NA) using 4-6 concentrations by serially diluting the stock solutions in buffer.

4.2.5 Data Analyses

In the first experiment, the mean activities of P450, GST and CoE in each immature life stage of Japanese beetles were compared using ANOVA, followed by LSD test for means separation. In the second experiment, ANOVA was used to compare the average mass of Virginia creeper foliage consumed, and also activities of P450, GST and CoE in adult Japanese beetle that were fed or staved at varying durations. The LSD post hoc test was used to test for significance among the average mass of Virginia creeper foliage and activities of P450, GST and CoE, at $\alpha = 0.05$(SAS JMP version Pro 11, SAS Corp.).

4.3 Results

4.3.1 Activities of detoxification enzymes in pre-adult life stages of Japanese beetle

All immature life stages of Japanese beetle expressed detoxification enzymes activities (P450, GST and CoE) except the eggs (no P450 and CoE activities) and first instars (no CoE activities) (Tables 1, 2 and 3). P450 activity was highest in the third instars (ETI and LTI) with no

difference between the activity in the first and second instar. The K_m for binding 7 EC by microsomes was 1.98 µM and 1.95 µM for first and second instars respectively and while their Vmax values was 56.8 pmol/min per mg protein and 43.77 pmol/min per mg protein respectively. In other words, P450 enzymes from both first and second instars of Japanese beetle have similar affinity and metabolize 7 EC at the same rate. However, the pre-winter third instar grubs (ETI) had a 5 fold lower affinity compared to the LTI, but LTI metabolize 7 EC 2.5 fold faster. Second instar grub had the highest GST activity, affinity for CDNB and also the fast rate of conjugating GSH and CDNB. It appears that GST activity increase from the egg stage and climaxed at 2nd instar before leveling back to the initial level in the late third instar (Table 2). ETI had the second highest binding affinity for CDNB followed by first instar and egg while the overwintered third instar grub showed the least binding affinity to the substrate despite having the same level of GST activity with egg and first instar. Second instar grubs had the highest α-NA and β-NA activities and binding affinity to the both substrates. ETI and LTI had similar activities of α-NA but LTI had more β-NA activity (Table 3).

Table 4.1. P450 activity and kinetic parameters in different life stages of Japanese beetle

Life stage	ECOD Activity pmol/min/mg protein	K_m µM	V_{max} pmol/min/mg protein
Egg	ND	-	-
1st instar	41.13 ± 1.8^b	1.98	56.8
2nd instar	37 ± 1.54^b	1.95	43.77
ETI	116.6 ± 17^a	14.75	249.24
LTI	113.9 ± 19^a	2.99	96.62
$F_{3, 9}$	16.37***		

Table 4.2. GST activity and kinetic parameters in different life stages of Japanese beetle

Life stage	CDNB activity (µmol/min/mg protein)	K_m mM	V_{max} µmol/min/mg protein
Egg	26.87 ± 3.7[c]	6.75	34.72
1st instar	13.67 ± 0.9[c]	8.67	40.54
2nd instar	176.4 ± 6.9[a]	2.67	232.6
ETI	85.26 ± 20[b]	4.49	148
LTI	30.83 ± 6.7[c]	19.35	165.6
$F_{4, 12}$	41.31***		

Table 4.3. CoE activities and Kinetic parameters in different life stages of Japanese beetles

Life stage	α-NA activity µmol/min/mg protein	K_m µM	V_{max} µmol/min/mg protein	β-NA activity µmol/min/mg protein	K_m µM	V_{max} µmol/min/mg protein
Egg	ND	-	-	ND	-	-
1st instar	ND	-	-	ND	-	-
2nd instar	619 ± 34[a]	235.3	3333	235.6 ± 0.1[a]	285.9	729.3
ETI	118.5 ± 5[b]	206.8	875	87.67 ± 5[c]	605.3	698.6
LTI	123 ± 15[b]	198.1	286	147.5 ± 11[b]	361.3	428
$F_{2,6}$	183.78***			82.85***		

*** Significant difference (P<0.001) among mean enzyme activities of immature life stages of Japanese (LSD post hoc comparison)

4.3.2 Effect of feeding duration on the activities of detoxification enzymes in the midgut of adult Japanese beetle

Beetles consumed significantly higher amount of Virginia creeper foliage at 48 h compared to 12 and 24 h (Figure 1) but the amount of foliage consumed at 12 and 24 h was generally the same. Beetles that were starved after feeding, had similar consumption pattern compared to their non-starved counterpart at 12, 24 and 48 h. P450 ECOD activity increased 2 fold from 12 h of feeding to a peak at 24 h of feeding and still remained high after 24 h of starvation (Figure 2), however it waned at 48 h of feeding to level similar to 12 h. P450 EROD activity had similar pattern to P450 ECOD activity, except that there was a significant decrease of 1.5 fold from 24 h of feeding when the beetles was further starved for 24 h, (Figure 2). GST activity toward CDNB did not significantly change across the feeding durations (Figure 3) however starvation of beetles for 24 h or beyond induced higher GST activity. Beetles had peak activities of α and β -NA CoE after 24 h of feeding, which dropped at 48 h to a level similar to 12 h. Starvation significantly decreased the titers of α and β -NA CoE activities by 1.5 and 2.5 fold respectively at 24 h but not at 12 and 48 h (Figure 4).

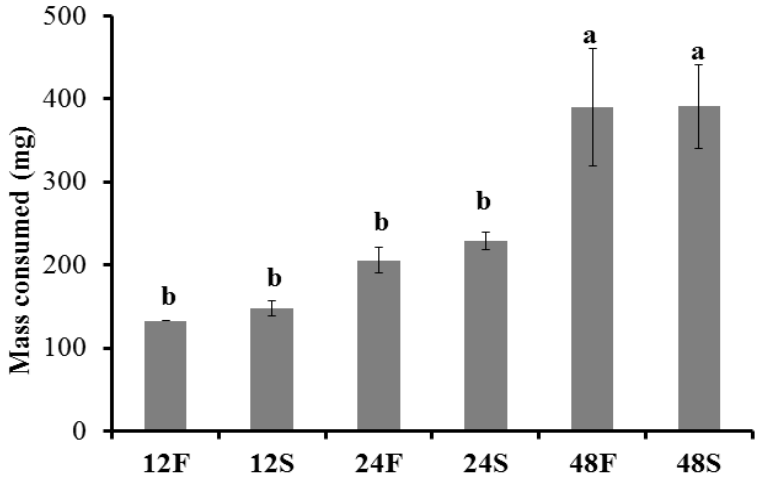

Figure 4.1: Average mass (mg) of Virginia creeper foliage consumed by Japanese beetles at different time periods. $F_{(5, 10)} = 9.745$, $P < 0.001$. Number of replicate is 3. Letters above each bar represent means separation by LSD ($P<0.05$). Letters/numbers on x axis denote duration of feeding and starvation after feeding, e.g. **48F**-beetles that fed 12hours, **48S**-beetles that were starved for 48 hours after feeding for 48 hours.

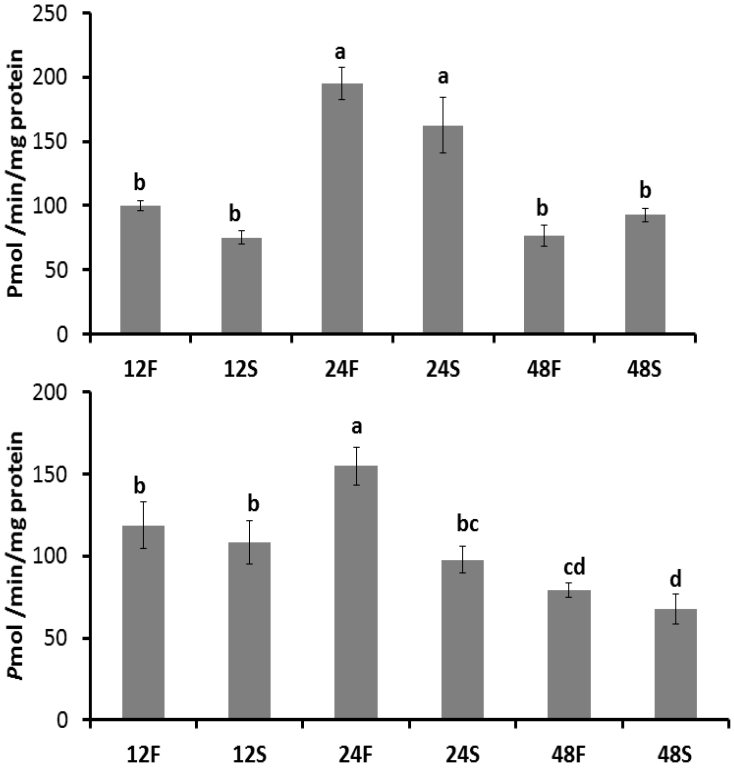

Figure 4.2. **P450 ECOD** (upper) ($F_{(5,28)}$=17.72) and **EROD** (lower) ANOVA($F_{(5,28)}$=9.79) Activity of adult Japanese beetles that fed on foliage of Virginia creeper for different time periods. Letters above bars represent a means separation by LSD (P<0.05). Letters on x axis denote duration of feeding and starvation after feeding, e.g. **12F**-beetles that fed 12hours, **12S**-beetles that were starved for 12hours after feeding for 12 hours.

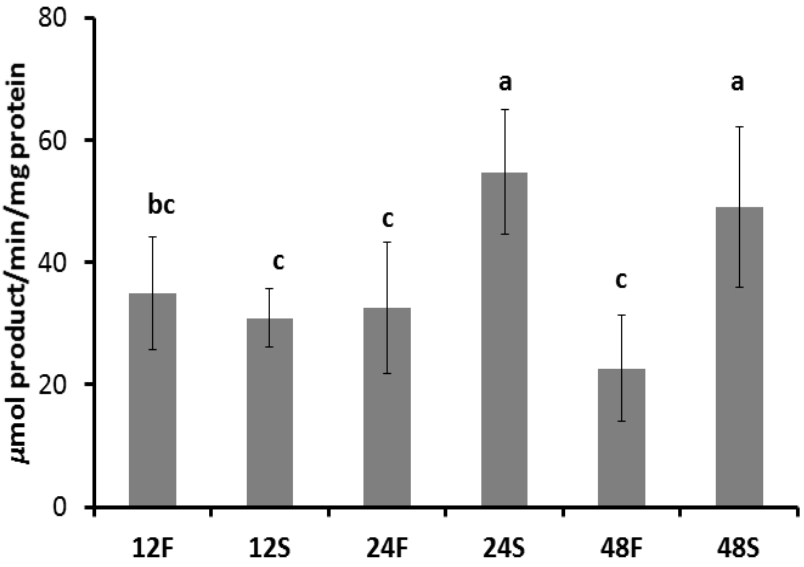

Figure 4.3. GST activity ($F_{(5,28)}$=4.88) activity of adult Japanese beetles that fed on foliage of Virginia creeper for different time periods. Letters above each bars represent means separation by LSD (P<0.05). Letters/numbers on x axis denote duration of feeding and starvation after feeding, e.g. **24F**-beetles that fed 24hours, **24S**-beetles that were fed for 24hours and then starved for 24hours

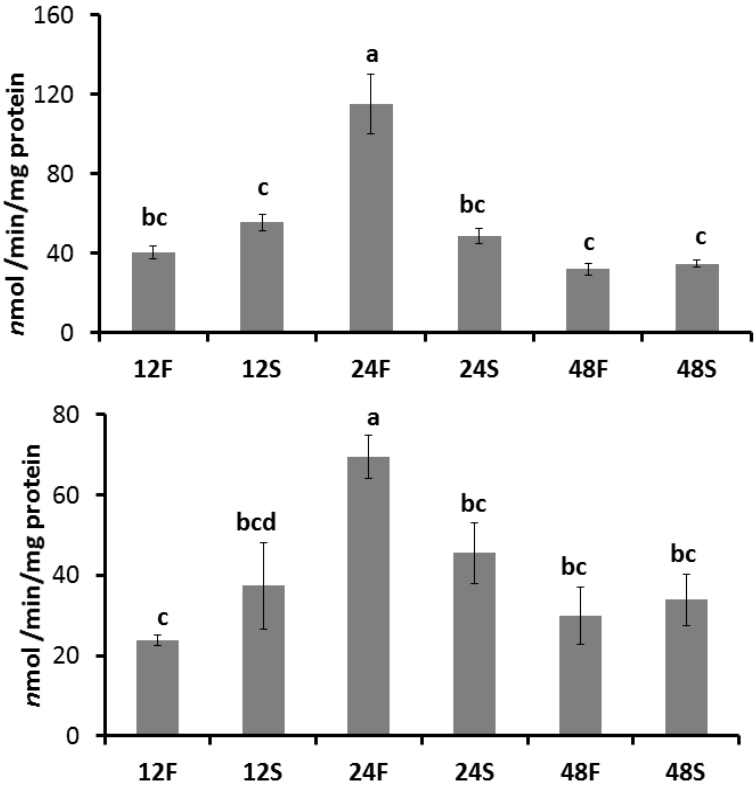

Figure 4.4. CoE β- NA (upper) ($F_{(5,28)}$=20.9) and α- NA (lower) ANOVA($F_{(5,28)}$=17.95) activity of adult Japanese beetles that fed on foliage of Virginia creeper for different time periods. Letters above each bars represent means separation by LSD (P<0.05). Letters/numbers on x axis denote duration of feeding and starvation after feeding, e.g. **48F**-beetles that fed 12hours, **48S**-beetles that were starved for 48 hours after feeding for 48 hours.

4.4 Discussion

The outcomes of this study clearly demonstrate the temporal distribution of the activities of detoxification enzymes in the immature life stages of Japanese beetle and also in the midgut of adult Japanese beetle. The activities of these metabolic enzymes are developmentally regulated in Japanese beetles from life stage to another, just as in other insect species in previous studies (Scott et al., 1996; Gunner et al., 2013). These results suggest that Japanese beetle relies on the suit of detoxifying proteins (P450, GST and CoE) across its life stage to perform diverse physiological functions in the course of its life cycle.

Japanese beetle, sequentially expresses activities of metabolic enzymes as it develop from egg to adult, GST was only the active enzyme detected in the egg using a universal model substrate (CDNB); Sanil et al., (2014) also detected GST activity the eggs of *Anopheles stephensi*. This possibly make a case for non-xenobiotic function of GST enzymes, though the exact physiological function of GST in a developing embryo cannot be stated within the context of this study. It is also possible that active GST enzyme in egg stage act defensively against any toxic chemicals that comes in contact with egg in the soil. Development of immature insects involves the actions of molting and juvenile hormones, both (even their precursors and metabolites) are candidate substrates for detoxification enzymes (Ranson and Hemingway, 2005; Feyereisen, 2005). The observed variation in the expression patterns of these enzymes (P450, GST and CoE) across the developing life stages in Japanese beetles is indicative that these enzymes are important physiological players in the growth and development of this insect. However, further studies are required to further understand the exact function played by each of these enzymes.

Ahmad (1983) reported a sharp decline in P450 activity in the gut of ETI compared to non-feeding LT1, using *p*-chloro-N-methylanaline (PCNMA) as the test substrate. This study, using whole body mass of ETI and LTI found no difference in P450 activity with 7 EC as test substrate, however, the LTI had higher binding affinity for the substrate while the ETI that is actively feeding, had a higher rate of metabolizing the substrate. Neal and Berenbaum (1989) had earlier reported that rapid metabolism of substrates is a crucial mean via which insect can tolerate toxic endogenous and exogenous chemicals that they encounter in the course of their life cycle. The difference between the observations in our study and that of Ahmad (1983) likely result from the different body tissue and chemical substrates used. Furthermore, P450 enzymes (and also GST and CoE) exit in multiple isoforms with varying degrees of substrate specificity and are expressed differently in various tissues (Feyereisen, 2005).

Adult Japanese beetle is a generalist herbivore that will frequently feed on foliage, fruits and flowers of over 300 plant species with diverse defensive chemical profiles. The outcome of the second experiment provides evidence for the involvement of multiple detoxification enzymes in the utilization of host plant. Beetles were only able to consume more host plant material after 24 h, which is also the period for peak activities of the three assayed enzymes (P450, GST and

CoE). Ahmad (1983) also reported that a 24 h feeding bout is enough to induce optimal activity of P450 PCNMA activity. Apart from studying the time for peak activities of two additional enzymes (GST and CoE), we also observed the influence of starvation on the titers of these enzymes. GST is the only enzyme groups that is induced in the absence of food especially beyond 12 h, the significance of this induction has earlier been discussed in Chapter 2 (metabolism of toxic ROS produced during oxidative stress). The optimal induction of detoxification enzymes in the midgut of Japanese beetle after 24 h affords it to consume more mass of host plant materials. Inductive effect of these enzymes by feeding is evident by the decline in their titers in the absence of food and increase in the presence in food.

Chapter 5: Conclusion

This current project established that Japanese beetle irrespective of its life stage, do rely on multiple enzyme systems for successful herbivory and xenobiotic metabolism. Prior to this study, there was no empirical evidence that for the involvement of multiple detoxification enzymes in the physiology of Japanese beetle. In this project, we used multiple model chemical substrates to study the induction and involvement of three-super families of detoxification enzymes (P450, GST and CoE) in adult and immature life stages of Japanese beetles.

Broadly speaking, we observed that multiple detoxification enzymes system is involved in Japanese beetles herbivory whether feeding on multiple host plants or host plant of varying preference. Earlier reports and observations have reported low performance of Japanese beetles on its non-preferred host plants. Thus, it is likely that high induction of enzymes by non-preferred host plants is metabolically expensive. Increase in the activities of these enzymes on mixed diets plausibly represent a price paid for benefitting from a nutritive mixed diets, as opposed to being restricted to a single diet. We also investigated the interaction between geranium/quisqualic acid paralysis of Japanese beetle and detoxification enzymes. Surprisingly, feeding on toxic geranium induces enzyme activities, but it appears quisqualic acid is not fully metabolized by enzymes. It remains to be known the mechanism involved in the recovery of beetles from geranium/quisqualic acid paralysis, especially in the lab.

The enzymes groups studied in this project are known to exist in isoforms with varying degrees of activity toward substrates and also functions. The current biochemical technique used in this study, could not determine the diversity of isoforms of each of the detoxification enzymes and the individual isozyme(s) that is (are) directly involved in the metabolism certain host plant allelochemicals, especially toxic quisqualic acid. However, the advent of cost effective next generation sequencing technologies is now available to study at the molecular levels, the genes involved in the metabolism of xenobiotics, especially when working with non-model species like Japanese beetle with no sequenced genome. This can be easily done using transcriptomics methods like RNA-Seq using such powerful techniques could help identify other groups of proteins like ABC transporters, UDP-glucosyltransferase that could also be involved in xenobiotic metabolism by Japanese beetle. Furthermore, the performance of Japanese beetles on

different test preferred and non-preferred host plants still needs to empirically evaluated, to test the ‘metabolic load’ hypothesis of enzyme induction.

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