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Host suitability and diet mixing influence activities of detoxification enzymes in adult Japanese beetles

Adekunle Adesanya^a, Nannan Liu^a and David W. Held^{a,*}

^aDepartment of Entomology and Plant Pathology, Auburn University, 301 Funchess Hall,
Auburn AL 36830

* Corresponding author

ABSTRACT

Induction of cytochrome P450, glutathione S transferase (GST), and carboxylesterase (CoE) activity was measured in guts of the scarab *Popillia japonica* Newman, after consumption of single or mixed plant diets of previously ranked preferred (rose, Virginia creeper, crape myrtle and sassafras) or non-preferred hosts (boxelder, riverbirch and red oak). The goal of this study was to quantify activities of P450, GST and CoE enzymes in the midgut of adult *P. japonica* using multiple substrates in response to host plant suitability (preferred host vs non-preferred hosts), and single and mixed diets. Non-preferred hosts were only sparingly fed upon, and as a group induced higher activities of P450, GST and CoE than did preferred hosts. However, enzyme activities for some individual plant species were similar across categories of host suitability. Similarly, beetles tended to have greater enzyme activities after feeding on a mixture of plants compared to a single plant type, but mixing *per se* does not seem as important as the species represented in the mix. Induction of detoxification enzymes on non-preferred hosts, or when switching between hosts, may explain, in part, the perceived feeding preferences of this polyphagous insect. The potential consequences of induced enzyme activities on the ecology of adult Japanese beetles are discussed.

Keywords: Cytochrome P450, Glutathione S transferase, Carboxylesterase, Host suitability, Diet mixing

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 mechanisms; 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 (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, are involved in phase I (P450, CoE) and phase II (GST) stages of xenobiotic metabolism in insects (Yu, 2008). Phase I metabolism generally reduces the toxicity and changes the functionality of lipophilic substrates and Phase II functions to increase the solubility of toxic lipophilic substrates and hasten excretion. These enzymes also perform other physiological activities and housekeeping functions in insects, such as acting on endogenous substrates like hormones and lipids (Feyereisen, 2005; Yu, 2008; Ranson and Hemingway, 2005).

Elevated activities of detoxification enzymes have been linked with host plant utilization and diet heterogeneity (Brattsten, 1992; Ahmad, 1983; Snyder and Glendenning, 1996; Yu, 1989; Yu et al., 2003). For example, consumption of carrot, a non-preferred host plant, by the generalist caterpillar *Spodoptera eridania* induced higher P450 enzyme compared lima bean, a more favored host (Brattsten, 1992). *Bemisia tabaci* (B-biotype), a generalist whitefly, has significant variation in CoE, GST, and P450 activities among five host plants that varied in suitability (Xie et al., 2011). Caterpillars and certain hemipterans (e.g, aphids and whiteflies) are among the more common taxa used in empirical studies relating detoxification enzymes to diet,

with fewer examples among adult insect life stages of coleopterans. Lepidopteran larvae and plant-feeding hemipterans (Krieger et al., 1971; Mullin, 1986; Rose, 1985) show increased activities of P450 and GST (Yu; 1982, 1983, 1984, 1996; Lee, 1991) upon feeding on non-preferred or less suitable host plants. When assayed using model substrates (Feyersien, 2005), polyphagy among insect herbivores results in higher enzyme activity compared to monophagy (Krieger et al., 1971, Mullin, 1986; Ahmad, 1983; Yu, 1996). Higher enzyme activities may be induced by the diversity of plant toxins in phylogenetically dissimilar plants in mixed diets (Feyereisen, 2005). Selective or inducible enzyme systems, however, could provide generalist herbivores an ecological opportunity to utilize secondary plant compounds before expending metabolic costs for detoxification. Generalist herbivores are considered to benefit from polyphagy by having access to more plant nutrients (nutrient balance) to maximize fitness, escape predation from natural enemies, or diversification of chemical defenses acquired from multiple plant sources (toxin dilution) (Bernays, 1988; Bernays et al., 1994; Bernays and Minkenberg, 1997, Mason and Singer 2015).

Adult Japanese beetles have more than 300 suitable plant species in 79 plant families (Fleming, 1972; Potter and Held, 2002). Dietary range and host preferences of adult Japanese beetles are well documented from historical field observations (Fleming, 1972; Held, 2004) and feeding trials (e.g., Keathley and Potter, 2008; Ladd, 1987; 1989;), but relatively little is known about the underlying physiological responses such as induction of detoxification enzymes relative to host suitability or dietary mixing. Among plants within this host range, adult Japanese beetles prefer certain plants such as rose blooms and foliage of sassafras or Virginia creeper and consumption of these plants produce higher survival and greater fecundity (Held and Potter, 2004; Keathley and Potter, 2008; Ladd, 1987; 1989). Similarly, Japanese beetles that consume a

83 dietary mixture of suitable hosts have improved fecundity and adult longevity (Held and Potter,
84 2004). The mechanism, toxin dilution or nutrient balance, to explain these apparent benefits of
85 dietary mixing in Japanese beetles have not been well researched. Thus far, only Ahmad (1983)
86 has suggested a link between enzyme activity, diet choice and dietary mixing in adult Japanese
87 beetles. Midgut P450 activity in adult beetles is induced in response to feeding on three unrelated
88 plants (broccoli, sassafras, and phlox) as single and mixed diets. Enzyme activities are lowest in
89 unfed (starved) beetles, followed in magnitude by the single diets, which varied significantly
90 with respect to one another. Polyphagy under lab or field condition induced the greatest enzyme
91 activity. Those results support the hypothesis (Krieger et al., 1971; Mullin, 1986; Yu, 1989; Yu
92 et al., 2003) that polyphagous species, because they encounter more chemically diverse plant
93 toxins, tend to have inducible detoxification enzymes systems that have greater flexibility for
94 metabolism of host plant toxins compared to specialists. Since Ahmad (1983) evaluated only
95 P450 enzymes with a single substrate, it is not possible to determine the relative diversity of
96 detoxification systems used by this polyphagous scarab.

97 More recent studies (reviews, Feyereisen 2005; Ranson and Hemingway, 2005) suggest
98 that polyphagous insects have greater diversities of genes encoding for different isoenzymes of
99 detoxification enzymes with varying substrate specificity. Furthermore, insect herbivores
100 irrespective of the host breadth often deploy more than one detoxification enzyme system (Xie et
101 al., 2011; Yang et al., 2001). Activities of GST and CoE, which may also facilitate metabolism
102 of secondary plant chemicals and insecticides (Lee, 1991; Xie et al., 2011; Yu, 1996), have not
103 been previously studied in *P. japonica*. Biochemical characterization remains a crucial first step
104 in the study of detoxification enzymes, especially for insect species (i.e. Japanese beetles) whose
105 genome or transcriptome is unavailable (Feyereisen, 2005). This study sought to quantify

activities of P450, GST and CoE detoxification enzymes in the midgut of adult *P. japonica* using multiple substrates in response to host plant suitability (preferred host vs non-preferred hosts), and single and mixed diets. The work of Ahmad (1983) would predict greater enzyme activities for beetles consuming diets of non-preferred hosts and those with greater dietary heterogeneity.

2. Materials and methods

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 geraniol; 3:7:3 ratio). Multiple host plants from different orders and families varying in relative suitability to *P. japonica* were used (Fleming, 1972, Table 1). Plant selections for these experiments were based on previous studies (e.g., Held and Potter, 2004; Keathley and Potter, 2008; Ladd 1987; 1989;) that have verified host preference and beetle performance in lab or field choice and rearing tests. Despite their generalist habit, beetle longevity and fecundity are generally greater when fed rose blooms or foliage from Virginia creeper, and sassafras relative to other suitable hosts (Fleming, 1972; Held, 2004). 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.

2.2 Reagents and supplies

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

2.3 Feeding assays

Only active, apparently healthy beetles free of any morphological defects were used in experiments. For each trial, mixed sex beetles (25 per replicate), were held individually in 100 × 15 mm sterile polystyrene petri dishes with host plant's 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 for 24 h) 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). For mixed plant diets, plant materials were

provided provisioned individually to ensure feeding experience with each host plant. Each experiment was replicated four times (100 beetles total) on different days.

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 had no significant water and weight loss as used in this study.

2.5 Dissection and enzyme extraction

2.5.1 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. Ahmad (1983) evaluated activity of P450 enzymes in midgut, fat body, and Malpighian tubules of adult Japanese beetles. Gut tissue has the greatest enzyme activity level among these three tissues and is generally has consistent mass in field collected insects relative to fat body. Our assays also focused on feeding which also made gut tissue a logic target tissue for enzyme activities. 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.

2.5.2 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.

2.5 Enzyme assays

2.5.1 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).

2.5.2 P450 activity assays

Activity of P450 was determined by reactions with two different substrates; *O*-deethylation of 7-ethoxycoumarin to 7-hydroxycoumarin (ECOD) and oxidative demethylation

of ethoxyresorufin to resorufin (EROD) (Guo et al., 2014; Lee and Scott, 1992). 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-hydroxycoumarin produced was measured using a microplate reader (Cytation 3: Biotek, Winooski, VT, USA) 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 pMol 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.

2.5.3 GST activity assays

GST activity was determined as the ability of cytosolic fractions to catalyze the conjugation of glutathione (GSH) and chloro-2, 4-dinitrobenzene (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 μ l of the enzyme source in a final volume of 200 μ l and a final GSH concentration of 2.5 mM. Reactions were incubated for 3 min at 25°C, after which 10 μ l 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⁻¹cm⁻¹ for S-(2,4-dinitrophenyl) glutathione.

2.5.4 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 μ L phosphate buffer (0.04M,

pH7.0), 50 μ L of the supernatant (enzyme source) and 80 μ L of 0.3 mM of substrate solutions. The reaction (slightly shaken) was incubated at 37 °C for 30 min and stopped by adding 20 μ L 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 μ L of 32mM of α - and β naphthol dissolved in acetone and also 20 μ L of stop solution. CoE α - and β -naphthylacetate.

2.6 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).

3. Results

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

Consumption of host plants 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 deprived of food (starved) had significantly lower activities of P450 EROD ($t = 4.61$, $P < 0.001$) and ECOD ($t = 4.1$, $P < 0.001$) compared with starved beetles that had fed (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 3).

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.

4. Discussion and implications

This study is the first to compare and report the activities of GST, P450, and CoE in midguts of adult *P. japonica* in response to feeding. It extends earlier work (Ahmad 1983) that first demonstrated feeding-induced P450 *p*-chloro-*N*-methylaniline activity in the beetle's midgut. By evaluating three enzyme groups using multiple substrates, we were able to support the hypothesis that generalist herbivores have flexibility in their detoxification mechanisms since they encounter a greater diversity of plant allelochemicals. In the absence of a robust genomic or transcriptome datasets for Japanese beetle, the experiments presented in this study are an

important first step for future work exploring the variation in isoenzymes of these enzyme systems within Japanese beetles in response to host plant feeding.

A working hypothesis at the onset of this study was that preferred hosts would induce less enzymatic responses than non-preferred hosts. The enzymatic costs associated with non-preferred hosts may explain the patterns of host usage from field and lab experiments. Based on group (preferred host and non-preferred host) means, consumption of preferred hosts typically induced lower activities of enzymes than non-preferred plants, however, that overall analysis doesn't represent the variation in enzyme activities for the selected plants. Beetles fed either foliage of sassafras or petals of roses, two preferred host plants, often had the lowest overall enzyme activity. Consumption of crape myrtle foliage, another preferred host, consistently resulted in greater enzyme responses relative to starved beetles and greater P450 and CoE activities relative to beetles fed other preferred hosts. However, enzyme activities for one or more of the non-preferred hosts also grouped with means of preferred hosts.

Keathley and Potter (2008) used a similar treatment structure of four preferred (e.g., Virginia creeper and sassafras) and four non-preferred host plants for adult Japanese beetles. That study evaluated plant phytochemical and nutritional profiles as well as adult Japanese beetle longevity and fecundity, yet found no consistent patterns in nutrition or phytochemicals within groups classified as preferred or non-preferred hosts. In essence, the selected preferred plants had similar or even greater amounts of tannins or saponins as plants considered as non-preferred. Also, preferred plants in that study had greater nitrogen content and similar water content to non-preferred hosts. Beetle performance, measured as fecundity and longevity, was significantly greater for preferred hosts. Beetles fed rose blooms or sassafras foliage have greater fecundity and survival relative to other host plants (Held and Potter, 2004; Keathley and Potter, 2008;

Ladd, 1987). Future studies attempting to discern patterns in overall enzyme activity in relation to patterns in host suitability for adult Japanese beetles should consider studies with individual plant species (e.g. rose) instead of the multi-plant species approach of this study.

Japanese beetles are polyphagous yet they have distinct preferences for certain plants such as rose blooms and sassafras, within their host range (Held and Potter, 2004; Ladd, 1987) and may consume those plants despite the presence of other hosts nearby (Fleming 1972). Our working hypothesis was that enzyme activities would be increase with greater diet heterogeneity. Ahmad (1983) showed increasing P450 activity ranging from the lowest activity for starved beetles, intermediate activity when single species were consumed, and the highest P450 activity among beetles allowed to consume multiple plants. Activity of GST, P450, and CoE in Japanese beetle midguts increases 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, crape myrtle foliage consumed alone often produced similar or lower enzyme activities to mixed diets containing crape myrtle. This is consistent with performance assays with Japanese beetles involving single and mixed species diets. Rose blooms when fed to beetles individually or in diet mixtures with other plants can produce greater longevity and fecundity however mixtures do not often improve performance relative to rose alone (Held and Potter, 2004). This is consistent with the meta-analysis by Lefcheck et al. (2013), which concluded that mixed diets only improve herbivore performance relative to an average diet but not relative to the highest quality single food source. In this study, consumption of rose petals in two or three plant mixtures generally produces greater enzyme activity than rose petals alone. The possible link between beetle performance and enzyme activity was not tested in this study but future experiments that consider enzymes, detoxification of specific phytochemicals in rose, and beetle performance in the same experiment

would determine the relative contribution of these enzymes in driving host preference and performance in this generalist scarab.

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REFERENCES CITED

- Analytical Software (2004) Statistix for Windows v. 8. Analytical Software, Florida
- Ahmad, S., 1983. Mixed-function oxidase activity in a generalist herbivore in relation to its biology, food plants, and feeding history. *Ecology* 64, 235–243.
- Ahmad, S., Pardini, R.S., 1988. Evidence for the presence of glutathione peroxidase activity toward an organic hydroperoxidase in larvae of the cabbage looper moth, *Trichoplusia ni*. *Insect Biochemistry and Molecular Biology* 18(8), 861-866.
- Bernays, E.A., Bright, K.L., Gonzalez N., Angel J., 1994. Dietary mixing in a generalist herbivore: tests of two hypotheses. *Ecology*, 75 (7), 1997-2006.
- Bernays, E.A., 1988. On the evolution of host specificity in phytophagous arthropods. *Ecology*, 69(4), 886-892.
- Bernays, E. A., Minkenberg, O. P. J. M., 1997. Insect herbivores: different reasons for being a generalist. *Ecology*, 78(4), 1157-1169.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248–254.

- 401 Brattsten, L.B., 1992. Metabolic defenses against plant allelochemicals, in: Rosenthal, G.A.,
 402 Berenbaum, M.R. (Eds.), *Herbivores: Their interactions with secondary plant metabolites* 2nd
 403 edition, Academic Press Inc., San Diego, pp. 199-270.
- 404 Dowd. P.F., Smith, C.M., Sparks, T.C., 1983. Detoxification of plant toxins by insects. *Insect*
 405 *Biochemistry and Molecular Biology*, 13(5), 453-568.
- 406 Feyereisen, R., 2005. Insect cytochrome P450, in: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.),
 407 *Comprehensive Molecular Insect Science Volume 4*, Academic Press Inc., San Diego, pp. 1-77.
- 408 Fleming, W.E., 1972. Biology of the Japanese beetle. USDA Tech. Bull. No. 1545. 65pp.
- 409 Fraenkel, G.S., 1959. The raison d'être of secondary plant substances. *Science*, 129(3361), 1466-
 410 1470.
- 411 Guo, L., Xie, W., Wang, S., Wu, Q., Li, R., Yang, N., Yang, X., Pan, H., Youjun, Zhang, Y.,
 412 2014. Detoxification enzymes of *Bemisia tabaci* B and Q: biochemical characteristics and gene
 413 expression profiles. *Pest Management Science*, 70(10), 1588-1594.
- 414 Habig, W.H., Michael, J.P., Jakoby, W.B., (1974). Glutathione S-transferase: The first enzymatic
 415 step in mecapturic acid formation. *Journal of Biological Chemistry*, 249, 7130-7139.
- 416 Held, D.W., 2004. Relative susceptibility of woody landscape plants to Japanese beetle
 417 (Coleoptera: Scarabaeidae). *Arboriculture and Urban Forestry*, 30, 328-335.
- 418 Held, D.W., Potter, D.A., 1999. Absence of food aversion learning by a polyphagous scarab,
 419 *Popillia japonica*, following intoxication by geranium, *Pelargonium × hortorum*. *Entomologia*
 420 *Experimentalis et Applicata*, 91, 83-88.
- 421 Held, D.W., Potter, D.A., 2004. Floral affinity and benefits of dietary mixing with flowers for a
 422 polyphagous scarab, *Popillia japonica* Newman. *Oecologia*, 140, 312-320.
- 423 Ali, J.G., Agrawal, A.A., 2012. Specialist versus generalist herbivores and plant defense. *Trends*
 424 *in Plant Science*, 17(5), 293-302.
- 425 JMP analytical software from SAS version 11.
- 426 Karban, K., Agrawal, A.A., 2002. Herbivore offense. *Annual Review of Ecology and*
 427 *Systematics*, 33, 641-664.
- 428 Keathley, C.P., Potter, D.A., 2008. Quantitative Resistance Traits and Suitability of Woody Plant
 429 Species for a Polyphagous Scarab, *Popillia japonica* Newman. *Environmental Entomology*,
 430 37(6), 1548-1557.
- 431 Krieger R.I., Fenny P.P., Wilkinson, C.F., 1971. Detoxification enzymes in the guts of
 432 caterpillar; an evolutionary answer to plant defenses? *Science*, 172, 579-581.
- 433 Ladd, T.L., 1987. Japanese beetle (Coleoptera: Scarabaeidae): Influence of favored food plants
 434 on feeding response. *Journal of Economic Entomology*, 10, 1014-1017.

- 435 Ladd, T.L., 1989. Japanese beetle (Coleoptera: Scarabaeidae): feeding by adults on minor host
436 and nonhost plants. *Journal of Economic Entomology*, 82; 1616-19.
- 437 Lee, K., 1991. Glutathione *S*-transferase activities in phytophagous insects: induction and
438 inhibition by plant phototoxins and phenols. *Insect Biochemistry and Molecular Biology*, 21,
439 353-361.
- 440 Lee, S.S.T., Scott, J.G., 1989. An improved method for preparation, stabilizing and storage of
441 housefly (Diptera: Muscidae) microsomes. *Journal of Economic Entomology*, 82, 1559-1563.
- 442 Lefcheck, J.S., Whalen, M.A., Davenport, T.M., Stone, J.P., Duffy, J.E., 2013. Physiological
443 effects of diet mixing on consumer fitness: a meta-analysis. *Ecology*, 94, 565-572.
- 444 Mullin, C.A., 1986. Adaptive divergence of chewing and sucking arthropods to plant
445 allelochemicals, in: Brattsten, L.B., Ahmad, S. (Eds.), *Molecular Aspects of Insect-Plant*
446 *Interactions*. Plenum, New York, pp. 175-209.
- 447 Potter, D.A., Held, D.W., 2002. Biology and management of the Japanese beetle. *Annual Review*
448 *of Entomology*, 47, 175-205.
- 449 Ranson, H., Hemingway, J., 2005. Glutathione transferases. in: Gilbert, L.I., Iatrou, K., Gill, S.S.
450 (Eds.), *Comprehensive Molecular Insect Science Volume 5*, Academic Press Inc., San Diego, pp.
451 383-402.
- 452 Ranger, C.M., Winter, R.E., Singh, A.P., Reding, M.E., Frantz, J.M., Locke, J.C., Krause, C.R.,
453 2010. Rare excitatory amino acid from flowers of zonal geranium responsible for paralyzing the
454 Japanese beetle. *Proceedings of the National Academy of Sciences USA*, 108(4), 1217-1221.
- 455 Schoonhoven, L.M., van Loon, J.A., Dicke, M., 2005. *Insect-Plant biology*, second ed. Oxford
456 Press, Oxford.
- 457 Singh S.P., Coronella, J.A., Benes, H., Cochrane, B.J., Zimniak, P., 2001. Catalytic function of
458 *Drosophila melanogaster* glutathione *S* transferase DmGSTS1-1(GST2) in conjugation of lipid
459 peroxidation end products. *European Journal of Biochemistry*, 268, 2912-2923.
- 460 Snyder, M.J., Glendenning, J.I., 1996. Causal connection between detoxification enzyme activity
461 and consumption of a toxic plant compound. *Journal of Comparative Physiology A*, 179, 255-
462 261.
- 463 Van Asperen K., 1962. A study of housefly esterase by means of a sensitive colorimetric
464 method. *Journal of Insect Physiology*, 8, 401-406.
- 465 Xie, W., Wang, S., Wu, Q., Feng, Y., Pan, H., Jiao, X., Zhou, L., Yang, X., Fu, W., Teng, H. Xu,
466 B., 2011. Induction effects of host plants on insecticide susceptibility and detoxification enzymes
467 of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Pest Management Science*, 67(1), pp.87-93.
- 468 Yang, X., David, C.M., Zhu, K.Y., Buschman, L.L., 2001. Host plant-induced changes in
469 detoxification enzymes and susceptibility to pesticides in the two spotted spider mite (Acari:
470 Tetranychidae). *Journal of Economic Entomology*, 94(2), 381-387.

- 471 Yu, S.J., Nguyen S.N., Abo-Elghar, G.E., 2003. Biochemical characteristics of insecticide
472 resistance in the fall armyworm, *Spodoptera fugiperda* (J.E. Smith). Pesticide Biochemistry and
473 Physiology, 77, 1-11.
- 474 Yu, S.J., 1996. Insect glutathione S-transferases. Zoological Studies, 35(1), 9-19.
- 475 Yu, S.J., 1989. Purification and characterization of glutathione transferases from five
476 phytophagous Lepidoptera. Pesticide Biochemistry and Physiology, 35:97-105.
- 477 Yu, S.J., 1984. Interactions of allelochemicals with detoxification enzymes of insecticide-
478 susceptible and resistant fall armyworms. Pesticide Biochemistry and Physiology, 22, 60-68.
- 479 Yu, S.J., 1983. Induction of detoxifying enzymes by allelochemicals and host plants in the fall
480 armyworm. Pesticide Biochemistry and Physiology, 19, 330-336.
- 481 Yu, S.J., 1982. Host plant induction of glutathione S-transferase in the fall armyworm. Pesticide
482 Biochemistry and Physiology, 18 (1), 101-106.
- 483 Yu, S.J., 2008. Principles of pesticides metabolism. In: the toxicology and biochemistry of
484 insecticide. Eds Yu S.J. CRC Press.
- 485 Zhang, L., Gao, X., and Liang P., 2007. Beta-cypermethrin resistance associated with high
486 caboxylesterase activities in a strain of house fly, *Musca domestica* (Diptera: Muscidae).
487 Pesticide Biochemistry and Physiology, 80: 65-72.
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Table 1. Japanese beetle's host plants of varying preference evaluated for induction of detoxification enzymes

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

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Table 2. Mixed-plant and single plant diet regimes for Japanese beetle

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)

Table 3. Average amount (mg) of preferred and non-preferred host plants consumed by Japanese beetle

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

‡Data shows means $\pm$ 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). ^cStudent's t-test (1 df) for orthogonal comparison between groups. *** P<0.0001,

Table 4. Orthogonal contrasts for P450 , GST and CoE activities for Japanese beetles fed with preferred (PH) and non-preferred (NPH) hosts

^a Groups	P450 activity		GST activity	CoE activity	
	ECOD pmol/min/mg protein	EROD pmol/min/mg protein	CDNB μmol/min/mg protein	α-NA nmol/min/mg protein	β-NA nmol/min/mg protein
Starved (S)	102.2±18	43.8±7	47.13±2.5	35.19±4.3	63.56±3.2
Fed (F) 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***

^aGroups 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

^bstudent's *t*-test for orthogonal comparison between groups. *** P<0.0001, **P<0.001, *P<0.05

^{ns}-no significant difference between compared groups.

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Table 5. Average amount (mg) of host plants consumed by Japanese beetle under different feeding regime

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

‡Data shows means $\pm$ 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).

^cStudent's t-test (1 df) for orthogonal comparison between groups. ***

P<0.0001, ^{ns}-no significant difference between compared groups

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Table 6. Orthogonal contrasts for P450, GST, and CoE activities for Japanese beetles fed with single and mixed diets

^a Groups	P450 activity ECOD pmol/min/mg protein	EROD pmol/min/mg protein	GST activity CDNB μmol/min/mg protein	CoE activity α-NA nmol/min/mg protein	β-NA nmol/min/mg protein)
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***

^aGroups mean ± standard error for P450, GST or CoE activities^bstudent'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.

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Figure 1. Midgut P450 activity in response to single plant diets. 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. Midgut GST activity in response to single plant diets. 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 3. Midgut CoE activity in response to single plant diets. 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.

Figure 4. Midgut P450 activity in response to single and mixed diets. 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 5. Midgut GST activity in response to single and mixed diets. 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 6. Midgut CoE activity in response to single and mixed diets 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

617 polyphagous diets. Letters above bars represent a means separation by Tukey's HSD ($P<0.05$).
618 Letters on x axis denote host plants feeding regimes (Table 2) as follows: S-starved beetles; C-
619 crape myrtle, V-Virginia creeper, R-rose. Number of replication=4, 25 beetles per diet
620 treatment.

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Figure 1.

Figure 2.

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Figure 4.

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Figure 6.

Host suitability and diet mixing influence activities of detoxification enzymes in adult Japanese beetles

Adesanya A. W., Liu N., and Held, D.W.