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Yasmine Guedira April 5, 2022

Analyzing the Developmental and Transcriptomic response of *Callosobruchus maculatus* to Alternative Bean Hosts

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Co-evolution between insects and their host plants has resulted in the development of a multitude of defense mechanisms in plants to protect themselves from herbivory and in insects to ameliorate the negative impacts of plant defenses. Transcriptomic analysis allows for the exploration of these relationships at the molecular level. This study aims to explore the transcriptional profile of adult *Callosobruchus maculatus* reared on alternative bean hosts. I couple this molecular investigation with the study of the development of these insects on the same alternative hosts. Significant differences in critical life history metrics of beetles reared on the alternative hosts indicate that plant defense strategies significantly delay larval development, and that the seed coat of the adzuki bean serves as an important barrier to herbivory. Transcriptionally, there was a relatively low number of differentially expressed genes across bean hosts. The overexpression of genes related to metabolism in adzuki reared beetles compared to black-eyed pea indicates that the unique amino acid composition of the beans could be influencing larval development, while the upregulation of transporters suggests that the bean beetle be transporting and sequestering toxic legume storage proteins.

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Acknowledgements

I would like to thank my mentor, Dr. Anna Zelaya, for her support and guidance throughout this project. I would also like to thank my advisor, Dr. Nicole Gerardo, and my committee members, Dr. Chris Beck and Dr. Timothy Read, for their comments that have shaped the direction of my project. In addition, I would like to thank Dr. Chris Beck for the datasets that were used in this study.

Finally, I would like to thank my family and friends for their encouragement throughout my research project.

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Introduction

Herbivorous insects represent one third of all described eukaryotic species (Hardy et al., 2020). The evolution of the dynamic relationship between insects and their plant hosts spans over 400 million years (Roy et al., 2016). Herbivorous insects often have life cycles that involve interaction with their hosts at all stages (Funk et al., 2002). Plant hosts can serve as a food resource, oviposition site, habitat, and mating site for herbivorous insects (Simon et al., 2015). Understanding the complexities of plant-insect interactions is critical to advancing the current understanding of herbivorous insect ecology.

The expansion of an insect's diet range could provide the benefit of having alternative hosts, but many factors limit the diet breadth of phytophagous insects. The plant chemical composition of current hosts, the recognition of the plant by females, and the fitness of the larvae that feed on the plant all impact an insect's ability to adapt to a new host (Näsvall et al., 2021). In particular, the use of chemical defenses by plants impacts all three of these factors and will be explored in this study.

Insect-Plant Host Coevolution

Coevolution between plants and insects has resulted in the evolution of unique defense mechanisms for protection (Agrawal & Zhang, 2021). Plants use various biochemical and morphological mechanisms to respond to herbivory that are dynamic and highly diversified (War et al., 2012). In turn, insects have developed counter mechanisms to detoxify or circumvent these novel defense strategies (Nishida, 2014). The molecular mechanisms that allow insects to overcome diverse ranges of toxic phytochemicals remain poorly understood.

Detoxification of plant toxins in the gut is one of the primary mechanisms insects have evolved to mediate the toxicity of secondary compounds present in their host diets (Berenbaum, 2002). The detoxification process typically involves chemical modification, degradation, and excretion using mechanisms that are suitable for the expansive range of phytochemicals that insects encounter (Roy et al., 2016). Findings suggest that transcriptome plasticity could aid in adaptation to novel host species (Hardy et al., 2020). The overexpression of detoxification genes is a mechanism certain insects use to overcome plant defensins (Heidel-Fischer & Vogel, 2015). Upregulation of genes can be detected through differential expression analysis and used to connect host plant use to gene networks that underlie the insects' response to phytochemicals. Gene expression analysis can also identify critical genes in the detoxification pathway and provide insight into chemical mechanisms that control transcriptome plasticity across various host plants.

Bean Beetle Ecology

Callosobruchus maculatus, commonly known as the bean beetle or cowpea weevil, is a generalist seed beetle that centers its life cycle around the seeds of Fabaceae. Females oviposit on the surface of the bean, and the larvae burrow into the seed, develop into pupa, and emerge as adults within 21-30 days (Figure 1), although development time varies with temperature and among diets. Adult beetles are aphagous and live around one to two weeks (Beck & Blumer, 2014).

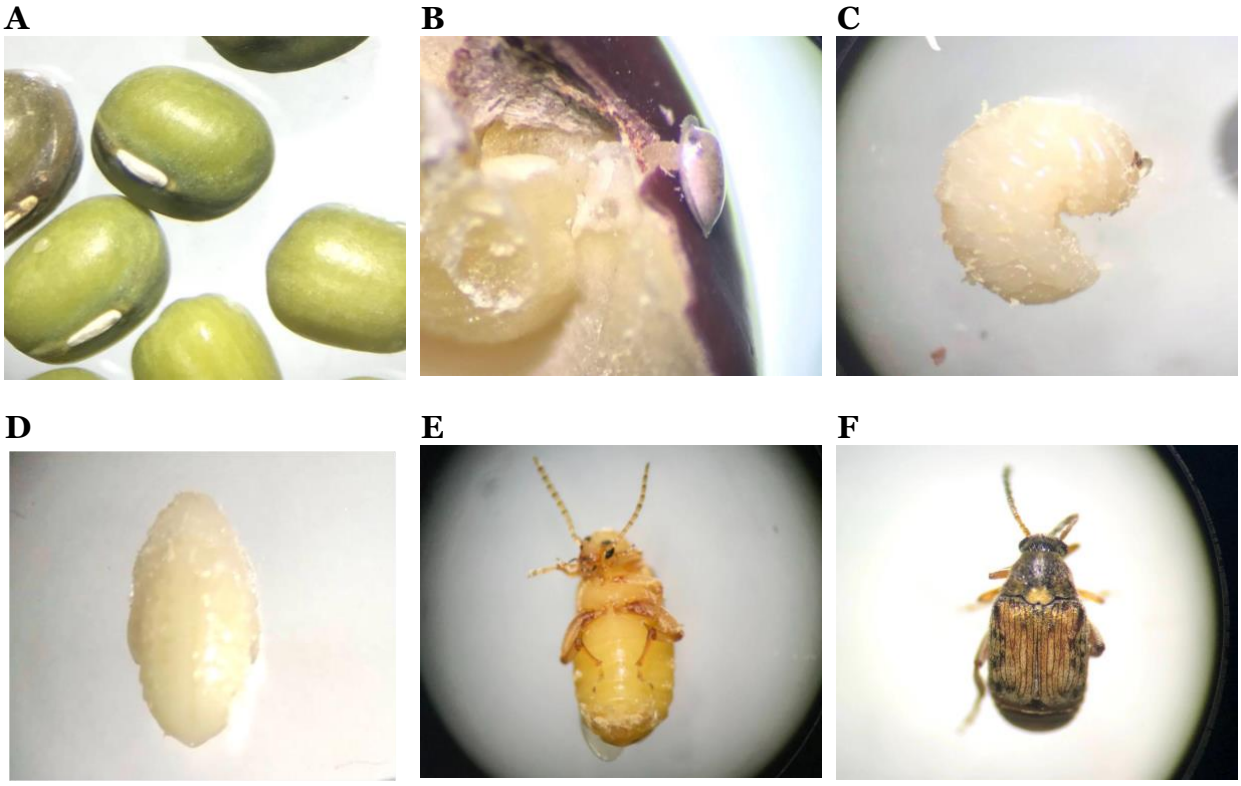

Figure 1. *Callosobruchus maculatus* life cycle. A) Egg laid on mung bean, B) larval emergence from egg into adzuki bean, C) fourth instar larva, D) early stage pupa, E) late stage pupa and F) adult male beetle.

The bean beetle causes significant damage to legume products in Africa and tropical regions of Central and South America (Alves et al., 2019). For example, in Niger alone, an estimated 2.4% of cowpea pods are lost in storage due to bean beetle infestations (Sanon et al., 2018). *C. maculatus* is the most common pest of stored *Vigna unguiculata*, commonly known as cowpea, seeds worldwide (Gad et al., 2021). Cowpeas are a major source of plant protein for both humans and livestock and are a critical source of income for farmers and distributors in many developing countries (Tochukwu et al., 2014).

The bean beetle is a highly effective pest due to its high fecundity, short developmental time and continuous generational turnover in stored bean products (Murdock et al., 2003). The larval stage of the beetle is the most destructive to the crop; the developing larva in the bean causes loss of weight and nutritional value in the bean product, making it unfit for consumption (Swella & Mushobozy, 2007).

Bean Beetle Transcriptome

Few studies have aimed to elucidate important functions of the bean beetle using transcriptomics (Cui et al., 2019; Pedra et al., 2003). Transcriptome sequencing on the bean beetle antennal gland uncovered 99 chemosensory genes, including genes from the three main families of insect chemoreceptor genes: odorant, gustatory and ionotropic receptors (Tanaka et al., 2022). This study also analyzed the differential expression between male and female adult beetles and found one gene differentially expressed in males that could contribute to sex pheromone reception and four genes differentially expressed in females that might be involved in host selection for oviposition (Tanaka et al., 2022). In addition, there is evidence that gene expression varies across developmental stages. In the larval stage, genes involved in digestion and the formulation of the larval cuticle were uniquely expressed. Glycoside and peptide hydrolases are the major digestive enzymes in the gut of *C. maculatus.* In the adult stage, olfaction proteins and gamete production genes were unique (Sayadi et al., 2016). Further analysis of these results and exploration into the functions of these genes could allow for the development of sustainable pest control strategies.

Experimental Design

My goal was to analyze plant host-dependent responses of the bean beetle on both the ecological and molecular levels. The analysis of several critical life history characteristics elucidates the impact of bean hosts across the bean beetle life cycle. Differential gene expression analysis of the adult developmental stage of *C. maculatus* explores the molecular responses to alternative hosts.

The majority of studies of the bean beetle have focused on analyzing the insect molecular response to black eyed peas, as this bean is the primary bean host of *C. maculatus,* and the damage of this bean causes tremendous economic loss worldwide (Amusa et al., 2018; Sales et al., 2001; Swella & Mushobozy, 2007). In this study, the use of unfavorable bean hosts facilitates exploration of alternative mechanisms that the bean beetle uses to overcome plant defenses. I focus on the consequences of feeding on black-eyed peas (*Vigna unguiculata*), mung beans (*Vigna radiata)*, and adzuki beans (*Vigna angularis*) as host plants. These legumes contain different classes and quantities of these toxic phytochemicals, and thus I can leverage this natural variation to study how various bean beetle enzymes may be involved in the detoxification process (Panzeri et al., 2022). This study makes a novel contribution to the current understanding of beetle metabolism as well as to the mechanisms that underlie the complex interactions between herbivorous insects and their hosts.

Methods

Life History Analyses

Laboratory stock cultures were reared on a single host type for more than five years prior to the start of the experiment. Females were reared on their ancestral host type in isolated Petri dishes and allowed to oviposit for 48 hours. The eggs were incubated at 30℃ for the duration of their life cycle.

Under aseptic conditions, larvae were extracted from the beans at each of the four larval instar stages, photographed, weighed, and measured. These measurements were used to track the timing of larval development across different host types. Larvae were homogenized using a sterile pestle and frozen at -80°C in DNA/RNA Shield (Zymo Research©). Male virgin adults were isolated within 24 hours of emergence, homogenized using a sterile pestle, placed in DNA/RNA Shield (Zymo Research©), and frozen at -80°C.

To measure time to emergence, emergence rate, and lifespan of adult beetles, individual beans with one egg were placed in 12-well tissue culture plates and monitored every 24 hours. Time to emergence was calculated as the number of days between oviposition and emergence of the adult beetle from the bean. Adult lifespan was calculated as the number of days between emergence of the adult beetle from the bean and the death of the beetle. After all the beetles had emerged from the bean and died, percent emergence was calculated as the percent of beans that had one egg that resulted in the full emergence of a beetle over the total number of eggs laid.

R programming language version 4.1.2 was used for all statistical analyses. A one-way analysis of variance (ANOVA) test with a confidence interval of 95% was used to compare the

time to emergence of vigin adult male beetles across bean types. Tukey's posthoc tests were used to compare differences across all pairwise combinations.

RNA Extraction

RNA was extracted using the SV Total RNA Isolation System Kit (Promega). Four replicates of both larval and adult stage beetles across each diet were individually sequenced for a total of 24 experimental samples. To assemble the larval reference transcriptome, two larvae from each instar across the three beans types were extracted individually and combined to one sample. For the adult transcriptome reference, four virgin male beetles from each bean type were extracted individually and combined into one sample for sequencing. Larval samples were processed according to the manufacturer's protocol. Adult samples were processed according to the manufacturer's protocol with an added incubation in Proteinase K at 72°C for 10 minutes during the cell lysis stage. Total RNA was quantified and quality checked using an Agilent 4200 TapeStation System or an Agilent 2100 Bioanalyzer System at Emory University's Integrated Genomics Core (EIGC). *De novo* transcriptome sequencing and differential gene expression analysis of these samples will be conducted in a future study.

Differential Gene Expression Analysis

RNA-seq reads sequenced by Illumina in 2013 from a previous study were obtained from Dr. Chris Beck at Emory University. The 2013 dataset was used to update and optimize a functional analysis pipeline for the samples that were extracted in 2022. As the RNA-Seq results have not been completed at the time of this writing, the results of the 2013 analysis will be presented here. These results will also help to serve as a comparison between two different generational stocks of adult bean beetles fed the same diets. Each of the three experimental

groups, black eyed pea (BEP), adzuki bean (ADZ), and mung bean (MUNG) had three replicates and were analyzed in a pairwise fashion.

The quality of the raw RNA-seq reads was analyzed using FASTQC to analyze quality scores, base N, and to test for the presence of adaptors. Fastp was used to trim out adaptors. The previously sequenced and annotated reference transcriptome file for *C. maculatus* (Sayadi et al., 2016) was indexed using kallisto and mapped to experimental samples (Bray et al., 2016). DEseq2 was used with default parameters; DESeq2 fits negative binomial generalized models for each gene and uses the Wald test for hypothesis testing (Love et al., 2014). The Benjamini-Hochberg adjusted p-value method was used to account for multiple comparisons. Genes with a $\log_2$ fold change > |1| and $p_{adj} < 0.05$ were considered to be significantly differentially expressed between treatments and were used for downstream functional enrichment analysis. Principle component analysis was used to visualize the DEseq2 output for exploratory data analysis. Experimental groups were analyzed in a pairwise fashion, which resulted in three comparisons, ADZ-BEP, ADZ-MUNG, and MUNG-BEP, where the first bean type in each pairing represents the treated group while the second bean type represents the baseline group. Upregulated genes in each condition represent genes that are up-regulated in the first bean type as compared to the second bean type. Gene Ontology (GO) annotations and predicted protein descriptions included in the annotation of the reference transcriptome file were used for functional classification of differentially expressed genes. Gene Ontology terms were assigned to each gene that was upregulated in the ADZ-BEP condition and were visualized by Revigo. Revigo uses multidimensional scaling to visualize GO terms and find connections between related groups of genes (Supek et al., 2011). The interaction plot shows how upregulated GO terms are interconnected with each other.

Results

Life History

Larval development was impacted by bean type (Figure 2). Larvae weighing more than 0.1 mg were discovered as soon as seven days after oviposition on black eyed peas. However, the first larva weighing more than 0.1 mg was at 13 and 12 days after oviposition for larvae reared on mung and adzuki beans, respectively. Throughout larval development, black-eyed pea beetles generally had higher weights than larvae on the other two bean types. Pupation began around day 19 for black-eyed peas, day 22 for mung, and day 24 for aduzki. For all three bean types, there was considerable variability in the weights of larvae at the same number of days after oviposition. At 21 days post oviposition, for example, 11 larvae from mung bean were harvested with recorded weights ranging from 0.3 mg to 7.4 mg.

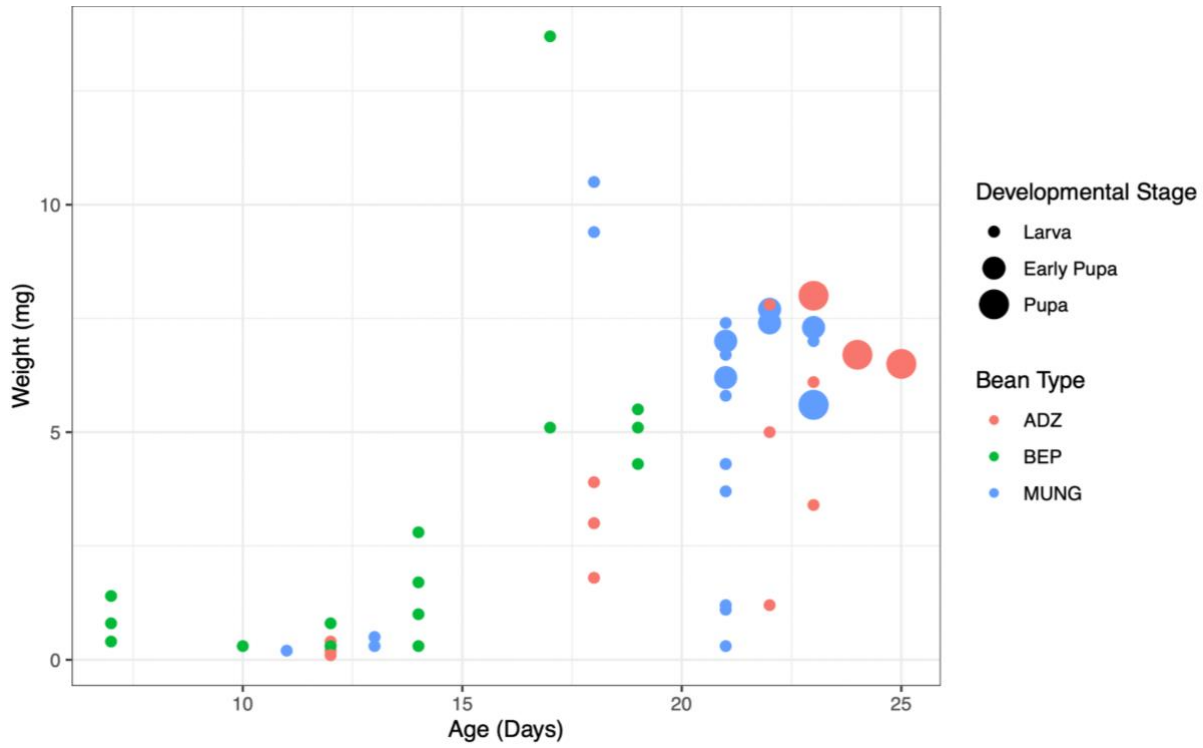

Figure 2. Age (days) and weight (mg) of developing larvae. Color corresponds to the bean type and the size of the circle indicates the stage of larval development.

There was a substantial difference in the emergence rates among the three host types. Black-eyed pea and mung bean had emergence rates of 86.7% and 79.2% respectively, while adzuki had an emergence rate of 23.3% (Table 1). It was visually observed in the lab that the majority of the adzuki eggs that had not fully developed to adult beetles were the result of early-stage larvae that had not crossed through the seed coat into the bean.

Table 1. Emergence rate of adult beetles across bean types.

	Adzuki Bean	Black Eyed Pea	Mung Bean
Total beans isolated	129	45	48
Total emergences	30	39	38
Total beans with no emergences	99	6	10
Emergence percent	23.3%	86.7%	79.2%

Time to emergence differed significantly across the three host bean types for male and female adult virgin beetles (Figure 3); ANOVA $p < 6.85e\text{-}13$). The adzuki condition had the latest average time to emergence and the widest interquartile range. (Figure 3).

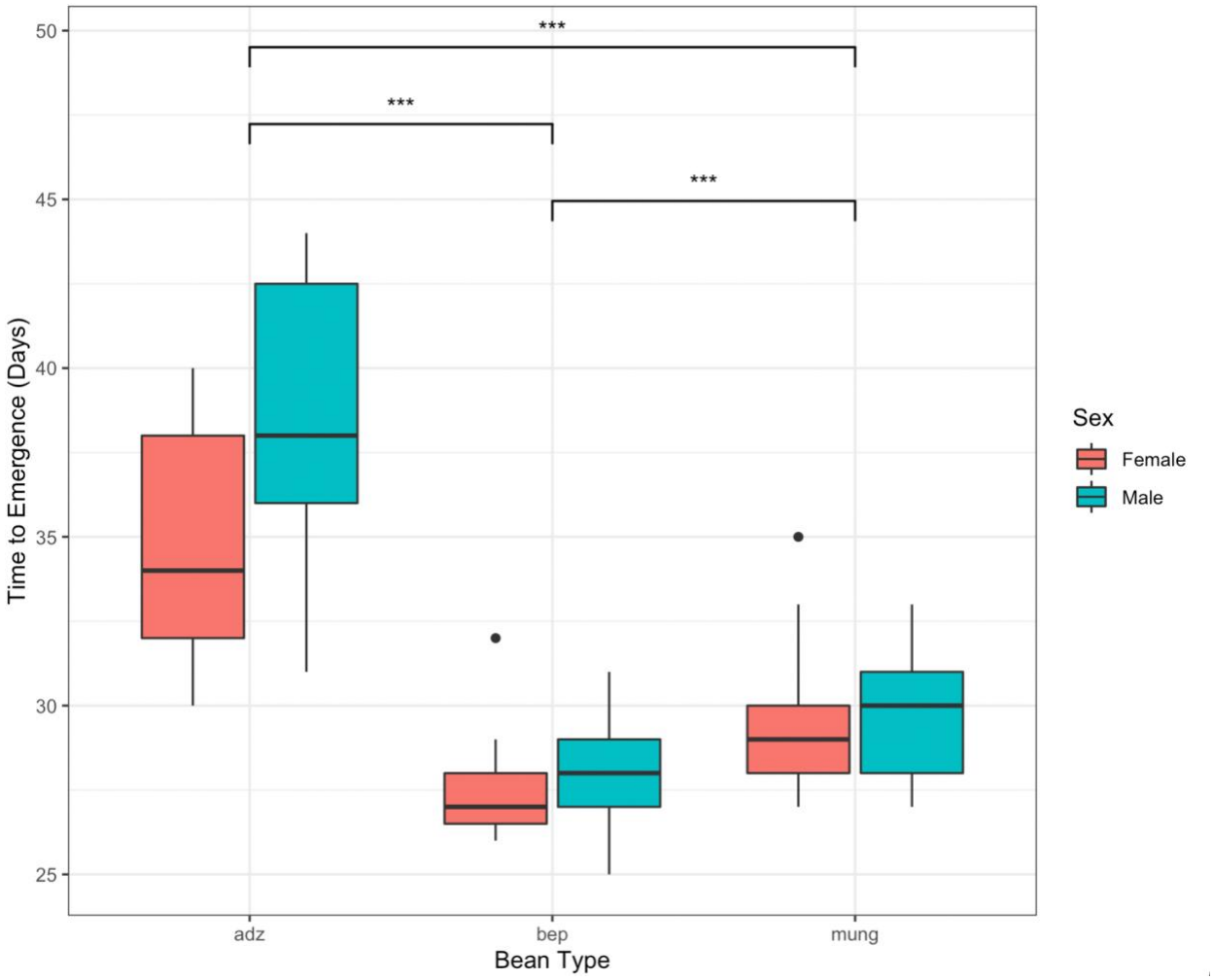

Figure 3. Time to emergence of virgin adult beetles after oviposition across bean types. (adz: n=16, bep: n=38, mung: n=38)

Tukey's multiple comparison tests indicate that there is a significant difference in the time to emergence of adult male beetles in the BEP-ADZ and MUNG-ADZ comparisons (Table 2).

Table 2. Tukey pairwise comparison test for the time to emergence across bean types of virgin male beetles.

			95% confidence interval	
Group Comparison	**Mean Difference**	**Adj *p*-value**	**Lower Bound**	**Upper Bound**
BEP-ADZ	-10.745342	0.000000	-13.20533562	-8.285348
MUNG-ADZ	-8.924370	0.000000	-11.48365385	-6.365086
MUNG-BEP	1.820972	0.0502721	-0.00178178	3.643726

The bean type had little impact on adult beetle lifespan (Figure 4). One way ANOVA analysis comparing the lifespan of adult beetles on different bean hosts indicated that there was no statistically significant difference in the group means at 95% confidence level ($p = 0.92$).

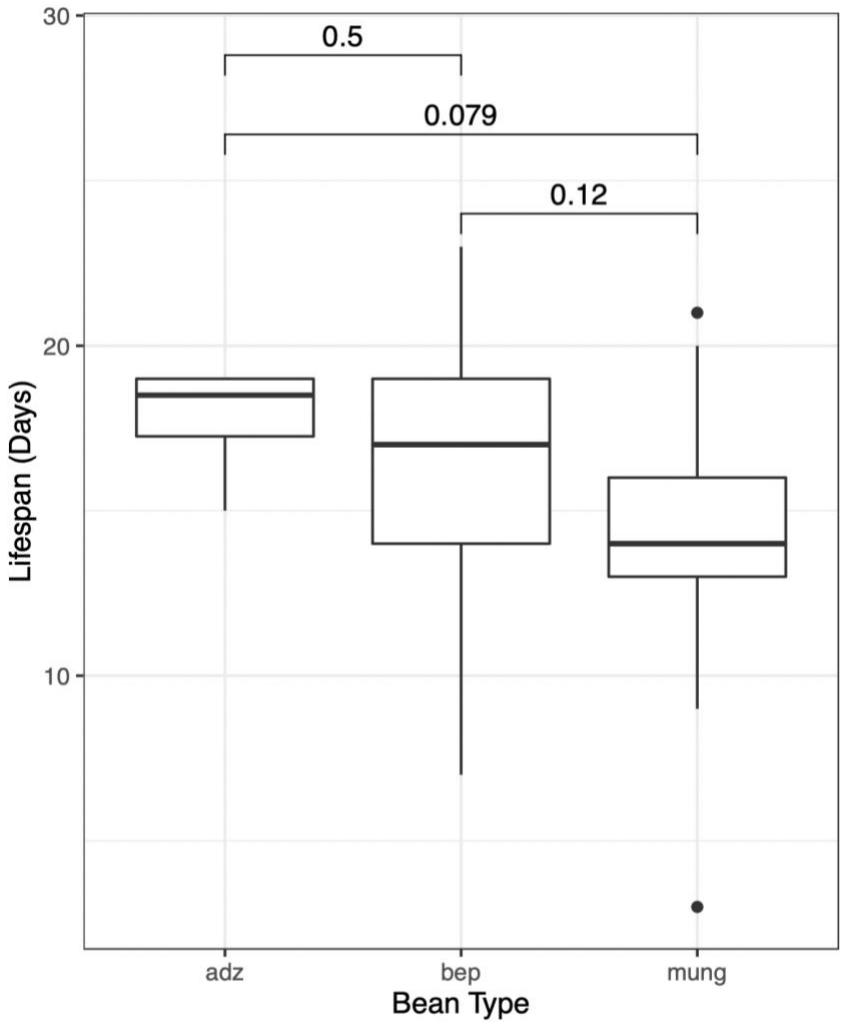

Figure 4. Lifespan in days after emergence of adult beetles across bean types (adz: n=14, bep: n=22, mung:n=18)

Differential Gene Expression Analysis

Across the three conditions, 22 total differentially expressed genes were found in the BEP-ADZ condition, 19 were found in the ADZ-MUNG, and 18 were found in MUNG-BEP conditions (Table 3). Principal component analysis of the gene expression data does not show distinct clusters based on bean type (Figure 5).

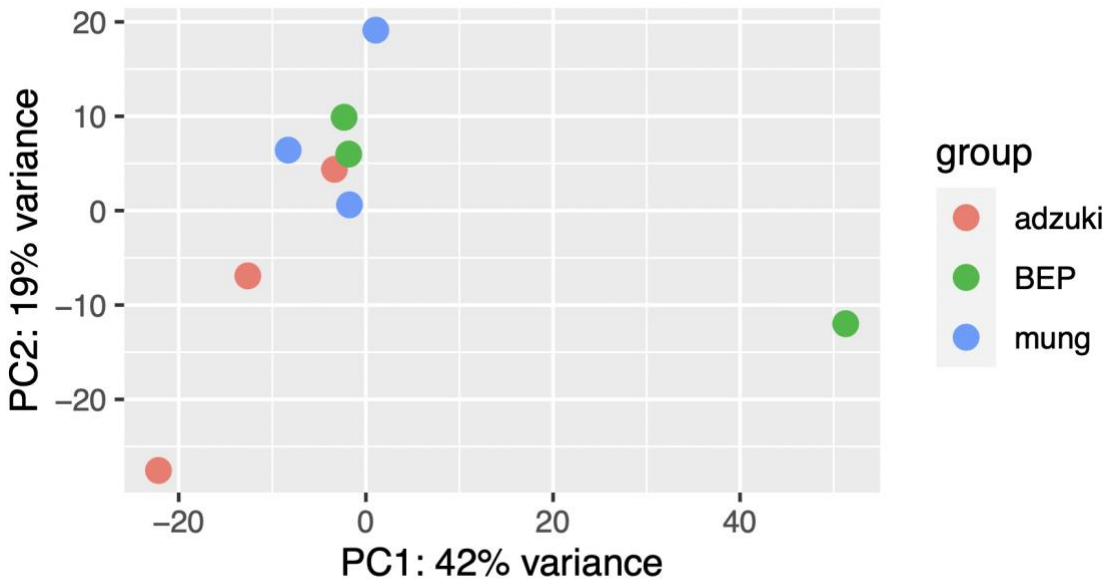

Figure 5. Principal Component Analysis representing the overall structure of the gene expression data and similarity between experimental groups.

For the majority of the downstream analysis, the ADZ-BEP and MUNG-BEP conditions were examined due to the significant delay larval development that was observed in mung and aduzki reared beetles relative to black-eyed pea reared beetles.

Table 3. Number of Differentially Expressed genes in each bean group condition

Group Comparison	Number of Upregulated Genes	Number of Downregulated Genes	Total Number of DEG
ADZ-BEP	14	8	22
ADZ-MUNG	11	8	19
MUNG-BEP	7	11	18

Overall, 14 genes were upregulated in ADZ-BEP, and seven genes were upregulated in MUNG-BEP based on the constraints set for differentially expressed genes (Table 4). Three genes in each experimental group had a $\log_2$ fold change greater than 20, which was significantly higher than the rest of the differentially expressed genes. No genes were differentially expressed across all three pairwise comparisons. Three upregulated genes were shared in ADZ-MUNG and ADZ-BEP comparison and four upregulated genes were shared in the ADZ-BEP and MUNG- BEP comparisons (Figure 6).

Table 4. Differentially Expressed Genes that were (A) upregulated in the adzuki bean fed beetles compared to black eyed pea group and (B) upregulated in mung bean group compared to the black eyed pea group. Genes with a $\log_2$ fold change greater than 20 are shaded in gray.

A

Transcript ID	Protein Function Description	Log_2 Fold Change	p_{adj}
TR17087-c0_g2_i10	a disintegrin and metalloproteinase with thrombospondin motifs 9 isoform x2	21.68962	0.000496
TR57344-c1_g3_i3	protein unc-80 homolog	21.18422	0.000768
TR32782-c0_g1_i1	ornithine carbamoyltransferase, putative	20.06644	0.002276
TR65694-c0_g3_i20	ankyrin repeat-containing partial	9.060667	2.59E-05
TR51648-c0_g1_i14	205 kda microtubule-associated	8.999927	0.0005
TR8526-c0_g12_i6	hypothetical protein YQE_09842, partial	8.83084	0.048849
TR23135-c4_g12_i1	protein roadkill isoform x4	8.503961	4.43E-05
TR38157-c0_g1_i10	PREDICTED: uncharacterized protein K02A2.6-like	8.495009	0.032375
TR72369-c2_g5_i5	histone-lysine n-methyltransferase partial	8.202914	4.43E-05
TR4234-c0_g2_i1	probable glutamate--trna mitochondrial	7.374991	0.005186
TR64680-c0_g4_i1	megator	7.328309	0.014322
TR3577-c5_g1_i3	creb-regulated transcription coactivator 1-like isoform x4	6.646688	0.040432
TR6314-c0_g1_i2	ecdysone-induced protein 74ef isoform a isoform x1	6.204638	0.042325
TR24644-c0_g1_i1	PREDICTED: uncharacterized protein KIAA1109	2.266182	0.038221

B

Transcript ID	Protein Function Description	Log_2 Fold Change	p_{adj}
TR57344-c1_g3_i3	protein unc-80 homolog	23.83193733	5.36022E-05
TR32782-c0_g1_i1	ornithine carbamoyltransferase, putative	22.83264741	0.000171075
TR17087-c0_g2_i10	a disintegrin and metalloproteinase with thrombospondin motifs 9 isoform x2	21.91470099	0.000393799
TR6130-c2_g1_i14	PREDICTED: uncharacterized protein LOC100141760 isoform X1	8.936371851	0.000584096
TR1104-c1_g1_i8	potassium voltage-gated channel protein shaker isoform x1	8.18993863	0.002259497
TR4234-c0_g2_i1	probable glutamate--trna mitochondrial	7.069002534	0.018471384
TR4936-c0_g2_i3	lian-aa1 retrotransposon protein	6.727844886	0.02229741

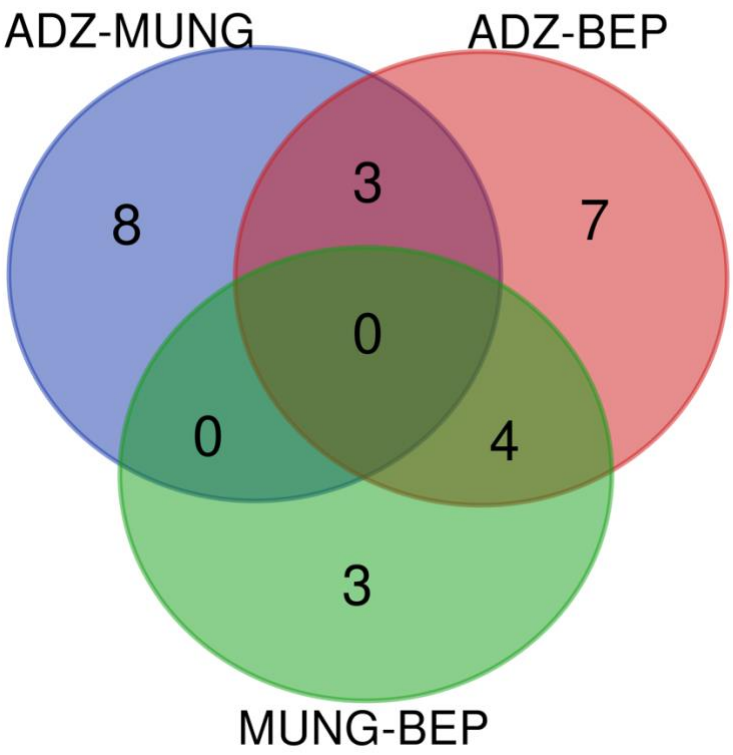

Figure 6. Venn Diagram showing the number of differentially expressed genes that are shared between each pairwise comparison and unique to each comparison.

Functional Enrichment Analysis

Of the upregulated GO terms, a few formed distinct clusters that indicate that the process as a whole is upregulated (Figure 7). Many genes related to metabolism and transport were upregulated and interconnected.

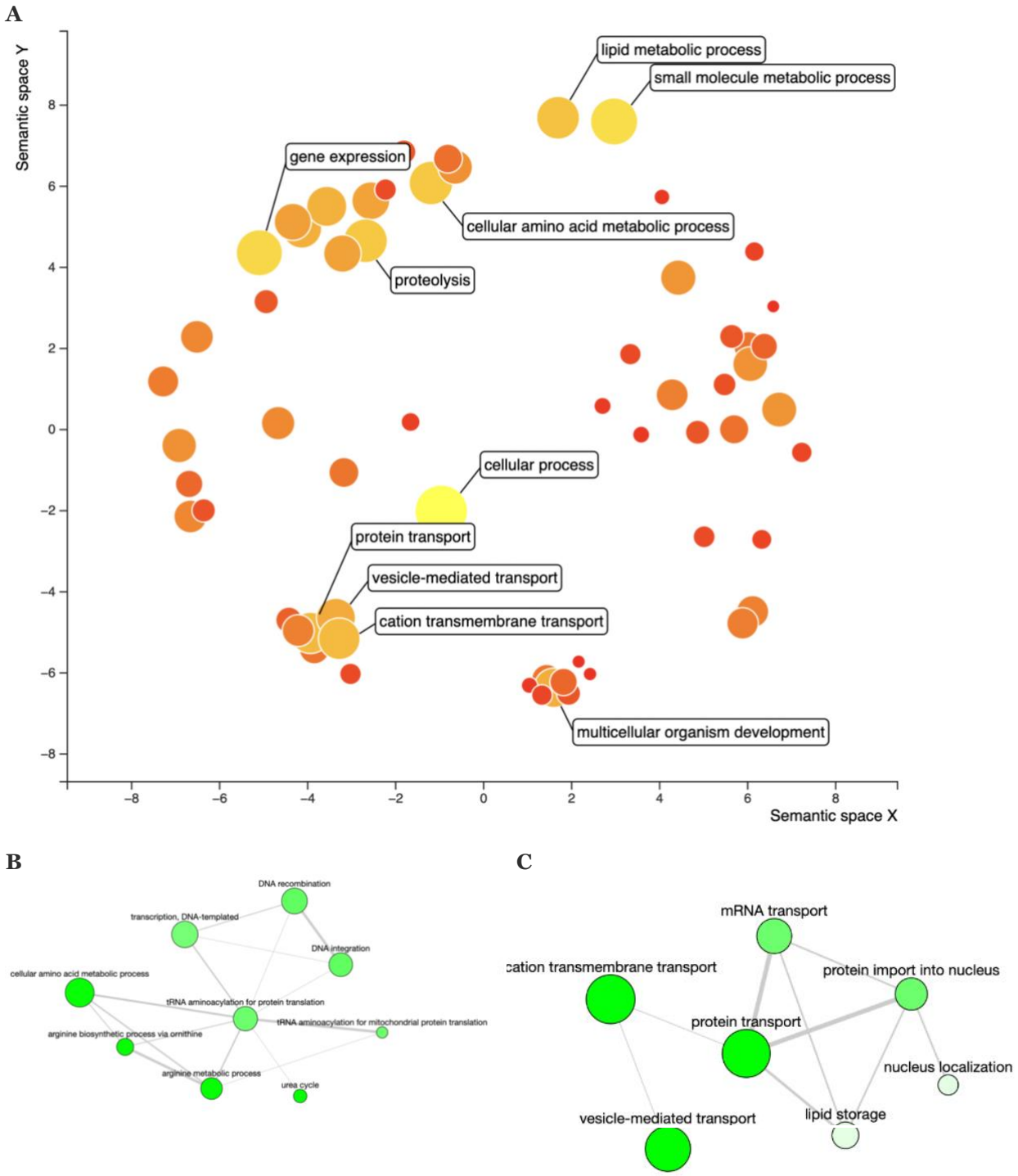

Figure 7. Distribution of Gene Ontology (GO) terms in ADZ-BEP condition. Bigger circles correspond to a larger log fold change. A) Overall plot of GO terms with significant terms labeled. B) Interaction plot of metabolic and transcription-related GO terms and (C) interaction plot of transport-related GO terms.

One particular enzyme of interest, ornithine transcarbamoylase, is upregulated by a $\log_2$ fold change of 20.06 in ADZ-BEP and 22.83 in MUNG-BEP (Table 4). This enzyme catalyzes the reaction between ornithine and carbamoyl phosphate to form citrulline and phosphate and generally functions in amino acid metabolic processes (Figure 8).

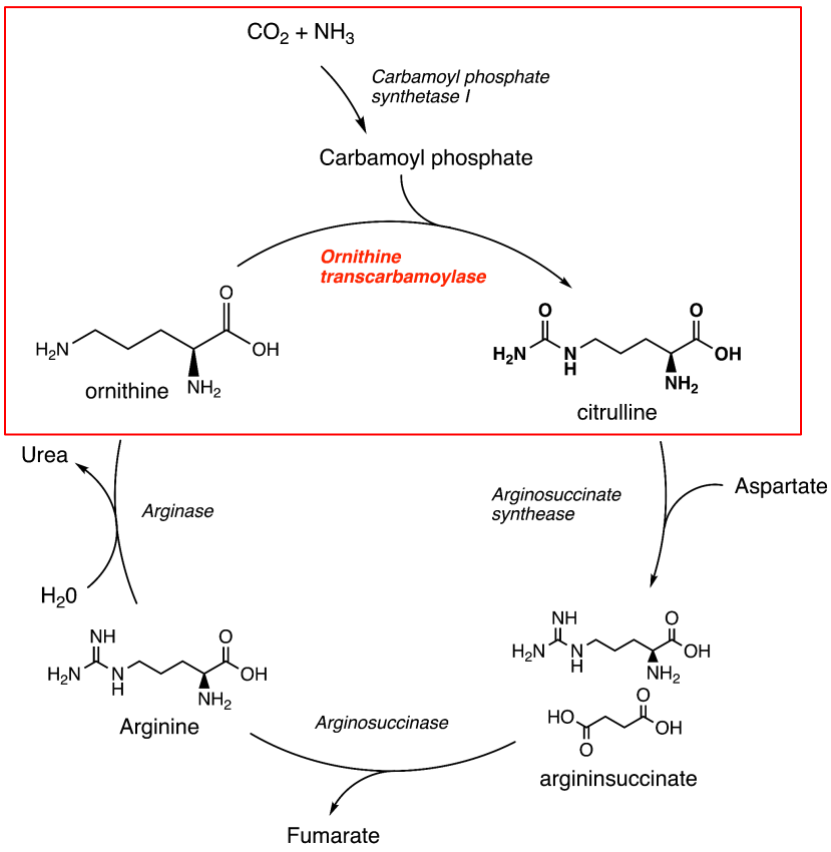

Figure 8. Urea cycle showing the conversion of ammonia to urea. Reactions in the box are a predicted mechanism that is upregulated in beetles reared on adzuki and mung beans. Enzymes are indicated in italics. Ornithine transcarbamoylase, indicated in red, is upregulated by a $\log_2$ fold change of over 20 in ADZ-BEP and MUNG-BEP comparisons.

Discussion

Life History

This study aimed to explore the ecological and molecular effects of host plant utilization by *C. maculatus.* There was a delay in larval development of *C. maculatus* on specific bean hosts. Certain plant toxins have been shown to negatively impact larval development. A developmental delay of larval growth was observed in the winter moth larvae *Operophtera brumata* after the tannin content of their diet was increased (Feeny, 1968). Similarly, various α-amylase inhibitors in *Phaseolus vulgaris* resulted in mortality at the first instar or a developmental delay in the larvae of the pea weevil *Bruchus pisorum* (Morton et al., 2000). Tannins and α-amylase inhibitors are present in the *Vigna* genus that contains the three bean hosts utilized in this study (Panzeri et al., 2022; dos Santos et al., 2010). The variation of functions, quantities and locations of these plant defenses in the bean across the three bean hosts are potential explanations for the delay in larval development of beetles reared on mung and adzuki beans.

The distribution of different plant defense compounds in the legume seed may underlie the differences exhibited in larval growth measured in this study. The delay of larval development when fed on mung and adzuki was most pronounced in the beginning of development. The majority of adzuki-reared beetles that did not emerge were the result of larvae that failed to penetrate the seat coat. Gutierrez Uribe et. al (2011) observed that in cowpea, seed coats contain at least 5 to 10 times more free and bound phenolics compared to the whole seeds. Tannins are present on the seed coat, and α-amylase inhibitors are located in the cotyledon of the black-eyed pea seed (Lattanzio et al., 2005). Aduzki beans have a tannin content of 21.7mg

catechin/g while mung beans have a tannin content of 5.4-13.9 mg catechin/g (Yousif et al., 2007). Thus, tannins and phenolic compounds of the seed coat of adzuki beans could contribute to the significant delay in development that occurs at the start of larval development when fed on these beans, and α-amylase inhibitors within the cotyledons of the bean could have contributed to the delayed time to emergence in mung and adzuki reared beetles.

The low emergence rate in adzuki reared beetles indicates that adzuki is a poor host for *C. maculatus*. Previous studies have shown that *C. maculatus* can rapidly adapt to specific legumes (Messina & Jones, 2009). For example, a population of bean beetles transferred from their ancestral host of mung beans to lentils had an initial survival rate of less than two percent. After 30 generations of development on lentils, the survival rate had increased to more than 90% (Messina & Jones, 2009). -This study indicates that *C. maculatus* is able to overcome the chemical defenses of certain legume types over time. In contrast, the beetles reared on adzuki in this study have been reared on adzuki for many generations, but only 23% of eggs laid on adzuki beans developed fully into adult beetles. This suggests despite the fact that successive generations of bean beetle were reared on adzuki, there were elements of the chemical and physical composition of adzuki beans did not allow for alternative host utilization to be as successful as in other legumes.

While the monitoring of larval development over time, the emergence rate, and time to emergence show that larval development was impacted by host bean type, adult lifespan was not. This may not be surprising given that the adults are not feeding on the beans. However, this would not preclude long last effect of suboptimal diets on later life history characteristics. It would be interesting for future researchers to explore whether impacts are seen when adults are reared in more stressful environments (e.g., higher temperature or in presence of pathogens).

Differential Gene Expression Analysis

Across the three different bean hosts, there was a relatively low number of differentially expressed genes. One of the most significantly differentially expressed groups of genes in beetles reared on adzuki beans are those involved in metabolism, which includes small molecule, lipid, and amino acid metabolic processes. Genes involved in localization and transport, such as vesicle-mediated and protein transport, were also significantly upregulated in the beetles reared on adzuki beans as compared to those reared on black- eye peas.

In the ADZ-BEP and MUNG-BEP conditions, only three genes had a $\log_2$ fold change of more than 20; all three were shared between the two conditions. These genes were: 1) ornithine carbamoyltransferase, 2) a disintegrin and metalloproteinase with thrombospondin motifs 9 isoform x2, and 3) protein unc-80 homolog. However, these three enzymes were not differentially expressed between the hosts that showed delayed larval development, mung beans and adzuki beans. Because of this relationship, this could indicate that there are some transcriptional changes associated with feeding on these alternative hosts.

I. Metabolism

Ornithine carbamoyltransferase (OTC) was upregulated by a $\log_2$ fold change of 20 and 23 in adzuki and mung bean fed beetles, relative to black eye pea fed beetles, respectively. This enzyme catalyzes the reaction between ornithine and carbamoyl phosphate to form citrulline and phosphate (Legrain et al., 1977). In mammals, this is one of the key enzymes in the urea cycle and serves an important role in the metabolism of nitrogen containing compounds. There is evidence indicating that insects contain some or all of the amino acid intermediates and enzymes of the urea cycle and that they excrete uric acid as a nitrogenous waste product, but there is large

variation in the presence of these molecules across species (Bursell, 1967). The specific pathway of nitrogen metabolism of seed beetles has not been characterized. Although primary plant metabolites are not often considered to be significant factors in host plant specificity, amino acid composition of a plant host has a direct impact on insect physiology. Even a deficiency in one essential amino acid in the diet of herbivorous insects can cause unbalanced nitrogen metabolism (Roy et al., 2013). In addition to the essential protein amino acids, non-protein amino acids could be contributing to the overexpressed Gene Ontology annotation of "cellular amino acid metabolic process." Non-protein amino acids play a critical role in development, nitrogen compound storage, and the defense response of plants against herbivores. L-ornithine is an important non-protein intermediate in primary plant metabolism (Huang et al., 2011). The presence of this amino acid could be a contributing factor to the overexpression of OTC. The unique amino acid composition of mung and adzuki beans could be stimulating the overexpression of OTC and impacting the nitrogen metabolism processes of *C. maculatus*.

II. Transport

A disintegrin and metalloproteinase with thrombospondin motifs 9 isoform x2 was another protein that was significantly differentially expressed in both beetles reared on adzuki and mung beans. This protein cleaves large proteoglycans and promotes protein transport from the endoplasmic reticulum to the Golgi apparatus (Yoshina et al., 2012; Somerville et al., 2003). This protein is involved in proteolysis and vesicle-mediated transport, which are two processes that were highly upregulated in the adzuki and mung bean beetle conditions (Figure 7). Transporters allow herbivores to control toxin accumulation. Plant toxins can be sequestered through transport mechanisms by insect herbivores to use as self-defense compounds against

predators (Erb & Robert, 2016). Specifically, vicilins are storage proteins found in legume products that associate with chitinous structures in the insect's midgut and cause delays in larval development (Sales et al., 2001). While feeding, *C. maculatus* larvae transport vicilin found in black-eyed peas to their internal environment (Uchôa et al., 2006). Vicilin was found in the fat body of the adult bruchids despite the fact that feeding ends in the larval developmental stage. The sequestration of toxic legume storage proteins could be a function of the upregulated expression of transport proteins that are involved in the bean beetle's response to mung and adzuki beans.

The low number of differentially expressed genes could indicate that there is a lack of large-scale transcriptomic changes happening during the adult developmental stage of the bean beetle. The primary upregulated genes include metabolism and transport genes. Two significantly upregulated proteins, ornithine carbamoyltransferase and a disintegrin and metalloproteinase could be involved in amino acid metabolism and toxin sequestration processes that are elements of the bean beetle response to alternative bean hosts.

Future Directions

This study explored differential gene expression in adult bean beetles. Throughout the life cycle of the beetle, there are many significant changes in morphology that are brought about by changes in gene expression (Sayadi et al., 2016). Therefore, we aim to expand this study to include more replicates and the larval stage of the bean beetle. Understanding transcriptional plasticity across both the larval and adult developmental stage would give a more complete picture of the molecular mechanisms involved with host plant use.

The life history metrics collected in this study give further justification for the importance of the larval stage in the analysis of alternative plant hosts. Time to adult emergence, percent emergence, and larval development were significantly different across bean hosts, which suggests that differences across hosts significantly impact larval development. Differences in the composition, quantities, and location of plant defense compounds of each bean indicate that a detailed analysis of the gene expression is required to understand the specific detoxification mechanisms and pathways that are involved in the degradation of these compounds. In addition, we plan to explore the microbial symbionts present in the bean beetle life cycle to understand the potential impact of bacterial gene expression on the metabolism of secondary compounds. Previous studies have indicated that many herbivorous insects have bacterial symbionts that contribute to detoxification as a whole as well as to the specific plant defenses in the *Vigna* genus (Hosokawa et al., 2006; Dowd & Shen, 1990). The inclusion of the larval stage and the bacterial transcriptome many result in a higher number of differentially expressed genes that will allow for a more complete analysis of the Gene Ontology annotations and the addition of pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database.

Additionally, we are assembling and annotating a *de novo* reference transcriptome to have a complete and updated transcriptional reference. The current reference transcriptome was conducted on the South India SI4 reference population in 2016 and the beetles had an ancestral host of mung beans. Having a complete reference transcriptome across all three host types using the same reference population as the experimental samples will allow for the identification of possible new transcripts that are expressed exclusively in beetles reared on black-eyed peas or adzuki beans.

The expansion of this study to include a *de novo* transcriptome as well and the transcriptomes of the larval stage and bacterial symbionts, will extend the evidence presented in the paper and provide novel contributions to the current understanding of *C. maculatus* host utilization.

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