Effects of Variable Host Plant Toxins on *Aphis nerii* Performance

Jennifer N. Cottle, Stephanie Birnbaum, and Patrick Abbot

KEYWORDS. *Aphis nerii,* genetic interaction, secondary metabolites, symbioses

BRIEF. This study investigated the performance effects of plant secondary metabolites on insect performance, and the molecular mechanisms insects use to overcome these toxins.

ABSTRACT. The effects of plant secondary metabolites on herbivorous insects have been widely studied in the field of ecology. However, the effect of variation in natural plant toxin concentrations on the performance of specialized insects is not clearly defined. Do the specialized insects thrive on toxic plants because they can circumvent plant defenses, or do they suffer costs at high toxicity levels? To test the hypothesis that Aphis nerii suffers performance costs at higher cardenolide concentrations, fitness tests were done with two lineages of A. nerii on three species of milkweed with varying toxicity levels, Asclepias incarnata (low toxicity), Asclepias curassavica (medium toxicity), and Gomphocarpus physocarpus (high toxicity). Time to reproductive maturity, lifetime fecundity, and survival of aphids growing on different host plant species was measured. Results indicate that lineage and toxicity of host plant had a significant effect on the time it took for the aphids to mature to adulthood, while toxicity level only had a significant effect on fecundity and survival. By creating a better understanding of the interactions between herbivorous insects and plant defensive toxins, the interactions between agriculturally detrimental insects and commercial insecticidal toxins can be more accurately understood and predicted.

INTRODUCTION.

The coevolution of plant-herbivore interactions is a major theme within the fields of evolution and ecology, as plants often evolve defensive adaptations as a result of herbivory; in turn, herbivores evolve adaptations to overcome plant defensive mechanisms [1]. The specifics of the genetic relationship underlying these interactions between plants and herbivores are lacking, however. In particular, there is a piecemeal understanding of how insects evolve to tolerate host plant toxins, and functional studies on the evolution of herbivore biochemical defense mechanisms within a single insect lineage are lacking [6].

This study addresses the ways in which insect herbivores interact with and tolerate toxins secreted by milkweed plants. Milkweed plants (Apocynaceae) are well studied for their production of toxic secondary metabolites known as cardenolides. While milkweed plants are toxic to most animals, a small number of diverse insects have evolved to live on these plants. The milkweed-oleander aphid, *Aphis nerii* (Aphididae), interacts with and tolerates the toxins secreted by its milkweed host plant. *A. nerii's* methods of tolerating cardenolides are relatively unknown [2]. It is known that the way in which other insects tolerate cardenolides, most notably the monarch butterfly via amino acid substitution, is absent in *A. nerii* [9]. This suggests that *A. nerii* likely expresses an entirely novel mechanism for tolerance.

This research was interested in describing the effect of varying host plant toxicity on *A. nerii* performance. To better understand the effect of host plant toxicity level on *A. nerii,* we performed three experiments on *A. nerii* at increasing levels of cardenolides. We found that aphid performance decreased as host plant toxicity increased. To more fully understand the genetic basis of *A. nerii* tolerance, we examined transcriptomic gene expression of aphids reared on different host plant species. We found a number of genes that were differentially expressed and hold potential to be involved in *A.* nerii cardenolide toleration. Discovering the method of toleration that insects use against naturally encountered toxins will be important in predicting how they may evolve toleration against agricultural pesticides.

MATERIALS AND METHODS.

Performance Experiments.

Two *Aphis nerii* populations (i.e., MIA14 and GH14) distinguished by varying locations of origin, were used in the initial performance experiment. These aphid populations were reared on three host plant milkweed species of varying toxicity [3]: *Asclepias incarnata* (low toxicity), *A. curassavica* (medium toxicity), and *Gomphocarpus physocarpus* (high toxicity). Prior to the start of the experiment, aphids of each lineage were reared on their respective host plant species for six generations to acclimate them. A total of 114 host plants were used, with four to seven adult aphids on each plant.

The performance cost of living and feeding on host plants across the three toxicity variations were measured using three response variables: time to reproductive maturity, survival rate, and lifetime fecundity. We statistically analyzed these variables to assess whether differences in lineage or host plant toxicity could account for variability in these outcomes.

Statistical Methods.

Data from all three response variables were measured using the statistical package R v3.2.1(R-project.org), an open-source software for statistical data analysis. A generalized linear model (glm) with a quasi-binomial distribution was used to analyze the effects of both host plant species toxicity and aphid lineage on the time it took aphids to mature to adulthood. This procedure allowed both variables of lineage and host plant to be evaluated within the same test. A linear mixed effects model (lme) was used to analyze lifetime fecundity data, with variations between plants modeled as a random factor. A Cox proportional hazards model (coxph), which is a part of the survival package in the Rv3.2.1 program, was used to perform the survival analysis.

Differential Gene Expression.

Full transcriptome differential gene expression was performed to identify genes which may explain the underlying molecular mechanisms involved in *A. nerii* toleraance to toxic cardenolides in its diet. RNA was extracted utilizing the standard Trizol method for three lineages of *A. nerii* (MIA14, GH14, and UM14) reared on *A. incarnata* (low toxicity) and *G. physocarpus* (high toxicity) cDNA synthesis was performed with oligo dT primers, and using the Superscript III First Strand synthesis kit. Illumina whole transcriptome RNA sequencing was done at the Hussman Institute for Human Genomics (University of Miami Miller School of Medicine). The three lineage's sequences were assembled *de novo* separately using the Trinity software package. The EdgeR Bioconductor packet was used to identify significantly differentially expressed genes between the high toxicity and low toxicity diets. Protein annotation was performed on the transcriptomes with the Blast2Go tool for functional annotation. Organisms that were used to create the de novo transcriptome for *A. nerii* were *Acyrthosiphon pisum, Aedes aegypti, Anopheles gambiae, Apis mellifera, Bombyx mori, Drosophilia melanogaster, Danaus plexippus,* and *Tribolium castaneum.* All genomes came from the National Center for Biotechnology Information, with the exception of *Danaus plexippus* (http://monarchbase.umassmed.edu/resource.html).

RESULTS.

Performance Experiments.

Statistical testing of the three response variables (time to adulthood, lifetime fecundity, survival rate) were done for the purpose of determining the relation-

ship that exists between aphid performance and host plant toxicity level. Both aphid lineage and host plant species had a significant effect on the time it took aphids to mature to adulthood, with aphids of the GH14 lineage reared on *A. incarnata* maturing to adulthood the fastest (Figure 1). The effect of these two variable on aphid performance is significant.

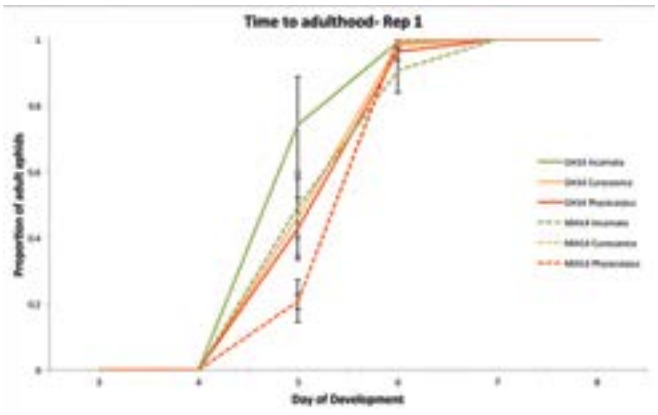

Figure 1. Proportion of aphids that matured to adulthood on each day for each of the six treatments (lineage/ host plant).

Both host plant and lineage had a significant effect on the time to adulthood (glm: host plant- F2,626 = 21.7, $p < 1x10^{-10}$; lineage - F1,628 = 27.8, $p < 1x10^{-7}$; interaction- F2,624 = 1.0, p = 0.36). The F value is a measure of statistically significant variance, with F > 1 indicating significance. Aphids reared on low toxicity host plants matured faster than those reared on medium and high toxicity, and GH14 aphids matured faster than MIA14.

At the beginning of the experiment, aphids reared on the low treatment had more offspring daily than aphids reared on medium and high treatments, and high treatment aphids had the fewest offspring (Figure 2a). This trend was reversed at day 15, with aphids on high toxicity host plants producing the most offspring daily for the rest of the aphids' lifespan. Despite this, aphids reared on the low toxicity treatment (*A. incarnata*) had the most offspring total over the span of the experiment, and aphids reared on the high toxicity treatment (*G. physocarpus*) had the least total offspring (Figure 2b).

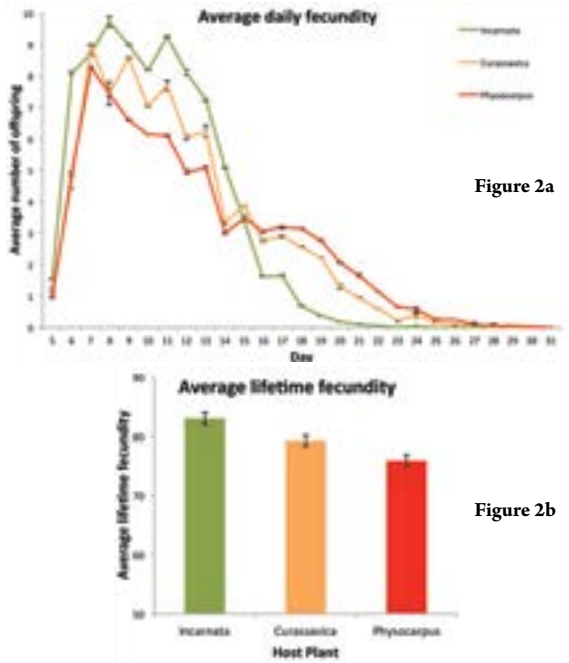

Figure 2a

Figure 2b

Figure 2. The average offspring per aphid per day for each host plant species.

2a. (Combined lineage fecundity) Lineage was not a significant factor in linear mixed effect models (lme: lineage- p = 0.69), but host plant was significant (lme: host plant- p = 0.01) **2b.** Aphids reared on low toxicity host plants had more offspring than medium and high toxicity plants, and aphids reared on high toxicity host plants had the fewest offspring.

Lifetime survival of *A. nerii* was significantly affected by host plant toxicity level, but lineage did not have a significant effect (Figure 3). *A. nerii* reared on *G. physocarpus* had the highest survival rate, *A. curassavica* had an intermediate survival rate, and *A. incarnata* had the lowest survival rate. However, until day 20 of the aphids' life, the low toxicity treatment *A. incarnata* had the highest rate of survival (Figure 3). Host plant toxicity had a significant effect on aphid survival ($< 1x10^{-13}$) while lineage did not (p = 0.88).

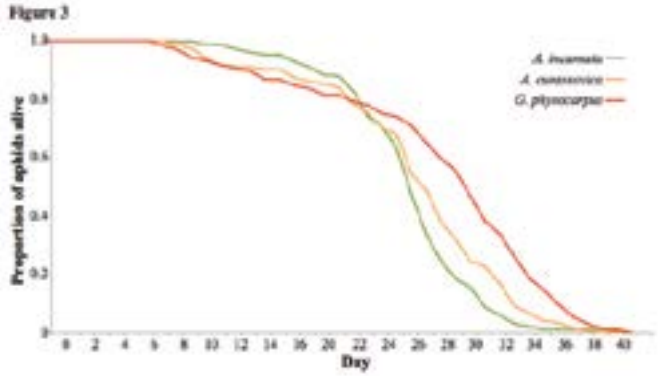

Figure 3. The proportion of surviving aphids from each host plant treatment over time.

Analyzed using a survival analysis (coxph model), lineage was not a significant factor in aphid survival (lineage - p = 0.88), but host plant was significant (host plant- $p < 1x10^{-13}$). Aphids reared on high toxicity host plants have higher rates of survival

Overall, it was concluded that *A. nerii* reared on *A. incarnata* (low treatment) had significantly elevated performance over aphids reared on *A. curassavica* (medium treatment) and *G. physocarpus* (high treatment).

Differential Gene Expression.

Differential gene expression analysis was performed using transcriptome sequences from three lineages of *A. nerii* (GH14, MIA14, and UM14) reared on low toxicity (*A. incarnata)* and high toxicity *(G. physocarpus*) host plant species. Eight genes were selected for future qPCR verification (Table 1), based on their degree of differential expression and the likelihood of their function relating to cardenolides toleration. The genes that held the most potential of these eight were a hydrolase activity gene, a UDP-glucuronosyltransferase (detoxification) gene, and a glutathione dehydrogenase gene. Genes involved in hydrolase activity (AGAP007243-PA) may be important because some organisms have been known to evolve mechanisms to detoxify cardenolides by way of hydrolysis [5]. Previous studies have revealed that the minimum structure for a cardenolide is a steroid nucleus composed of four fused carbon rings, and a hydroxyl group that drives the toxicity [2]. With hydrolysis being able to disrupt this steroid nucleus, this hydrolase gene is a strong candidate for further analysis. Genes similar to the UDP-glucuronosyltransferase gene identified in the transcriptome here encode proteins known to be active in the detoxification of multiple different compounds [8]. Additionally, glutathione dehydrogenase genes encode proteins known to be active in the detoxification of formaldehyde [1], which can be a byproduct of the breakdown of cardenolides [4]. It is also interesting to note the neuropeptide receptor gene that was differentially expressed was derived from the monarch butterfly (*Danaus plexippus),* the only insect within the de novo assembly to also feed on milkweed and tolerate cardenolides. This gene may be indicative of convergent evolution in neuronal tissue structures between *A. nerii* and *D. plexippus* as a result of the shared cardenolide exposure in their host plants.

Table 1. A table describing the genes selected for qPCR verification and further analysis

Transcript ID	Protein Name	Source Organism	LogFC	P-value
mia1|TR18012|c1_g3	AGAP007243-PA	African malaria mosquito	13.735	3.07E-21
mia1|TR4128|c1_g7	Triosephosphate isomerase	Mountain pine beetle	11.1571	1.16E-13
gh1|TR18072|c0_g2	UDP-glucuronosyltransferase	Pea aphid	3.593	0.0005
mia1|TR62071|c0_g1	S-(hydroxymethyl) glutathione dehydrogenase	Pea aphid	2.516	0.0005
mia1|TR4128|c1_g6	Triosephosphate isomerase	Mountain pine beetle	1.894	0.011
gh1|TR23200|c0_g2	Neuropeptide receptor A16	Monarch butterfly	1.721	0.047
mia1|TR29502|c0_g2	Signal transducer and activator of transcription	Pea aphid	-6.605	0.004
gh1|TR49590|c0_g1	FK506-binding protein	Fruit fly	-2.272	0.005

The Transcript ID number was generated based on the de novo transcriptome; the protein name denotes function based on homologous sequences. The source organism is the organism genome from which the gene annotation was derived. LogFC is a measure for the degree of differential expression (positive indicating up-regulation and negative indicating down-regulation). P-value is the measure of significance, with values of $p < 0.05$ being significant.

DISCUSSION.

Two of the three response variables measured in the performance experiments supported the hypothesis that *A. nerii* performance declines with increased levels of toxicity in host plants. The time it took to mature to adulthood was shortest in *A. incarnata,* intermediate in *A. curassavica,* and longest in *G. physocarpus* (Figure 1). There was a significant difference between lineages in how cardenolide levels affected maturation rate. These results suggest that development occurs more rapidly in aphids that do not need to invest energy into tolerating higher toxicity host plants. The ability to invest energy into growth and development is a characteristic of elevated performance.

The lifetime fecundity, or amount of offspring the aphids had during their lifetime, also supported the hypothesis that *Aphis nerii* will experience a performance cost when raised on higher toxicity host plants. Aphids reared on *A. incarnata* had the most total offspring over their lifetime, while *A. curassavica* had an intermediate amount, and *G. physocarpus* had the least (Figure 2). These results suggest that aphids can invest more energy into reproducing as a result of the smaller energy investment required to live on host plants of low toxicity. Perhaps, out of all three response variables, lifetime fecundity is the most important one to consider when assessing the performance of a given population. This is because the amount of offspring a population is able to produce will have the most direct impact on the health and survivorship of the overall population.

The survival of aphids over time on their host plants did not sustain the hypothesis that aphids will have higher lifetime survival rates on host plants of lower toxicity. *A. nerii reared on A. incarnata* had the highest rate of survival up until day 22. After this, the trend reverses itself, and aphids reared on *G. physocarpus* have the highest survival rate and the longest average life span (Figure 3). This, however, does not negate the overall hypothesis that aphid performance will be increased on host plants of lower toxicity. A possible reason why this unexpected trend was seen may have be related to the pattern of fecundity that was influenced by host plant secondary chemistry. Aphids reared on *A. incarnata* had a larger number of offspring total (Figure 2b), and were able to produce the bulk of this total during a more condensed period, near when they had reached maturity (Figure 2a). Furthermore, populations reared on all host plants began to die approximately 2 days after reproduction ceased. Because aphids reared on *A. incarnata* were able to reproduce much more quickly and with greater output, they stopped reproduction more quickly, and therefore died more quickly. Moreover, it is highly unlikely that *A. nerii* under natural conditions would survive the length of the experiment presented here. *A. nerii* are highly predated and parasitized in the field, thus reproduction early in their life would be most important [7].

From the performance experiment, it can be suggested that the molecular mechanisms *A. nerii* use to overcome toxic cardenolides in its diet are costly (Figures 1,2,3). Further analysis of the differential gene expression sequencing in future work will yield further insight into the nature of *A. nerii's* adaptive mechanisms against cardenolides.

One previous study concluded that there was not a significant performance effect of variation in toxicity on aphids raised on two species of milkweed [10]. However, this previous study concluded that its ability to detect performance variation was limited, and it did not use milkweed species with as broad a toxicity variation as this one did. A novel characteristic of this current work is that it investigated the exact nature of the effects of performance and tested a more extreme spectrum of toxicity levels in the milkweed species.

The eight genes that were selected to be possibly relevant to *A. nerii* toleration of toxic cardenolides will be confirmed with qPCR analyses. Once these differentially expressed genes are confirmed, the protein annotation of the transcriptome data will be used to generate hypotheses in future studies regarding candidates involved in tolerating cardenolides. These genes then will be further investigated regarding their functions in cardenolides detoxification, toleration, and sequestration that *A. nerii* are exposed to in their diet. The data gained from this analysis can be used to help to provide insights into the co-evolution between *A. nerii* and milkweeds. Generally, this work will provide a better description of specialist feeding insects that share a close evolutionary relationship with plants they obligately feed on.

Ultimately, knowing the answer to how insects evolve mechanisms to detoxify naturally occurring plant toxins can assist in agricultural efforts. Insects have been known to develop molecular mechanisms to overcome commercially used insecticides by using the molecular mechanisms they evolved to overcome natural plant toxins [8]. By understanding these detoxification systems , we will be able to better predict how and when insects will utilize those mechanisms to tolerate commercially used insecticides. These "super-pests" therefore can be more efficiently managed, and damage done to crops will be mitigated.

ACKNOWLEDGMENT. We would like to thank Dr. Eeds for her guidance and the School for Science and Math at Vanderbilt.

REFERENCES.

1. Hakima Achkor et al., *Plan. Physiol.* 132 (2003)
2. Anurag A. Agrawal et al, *New Phytol.* 194 (2012)
3. Rasmann S., Agrawal, *Ecol. Lett.,* 14 (2011)
4. D. H. G. Crout et al., *Journal Chem.* Society. 0 (1963)
5. Johann J. Framm et al., *Plant Cell Physiol.* 41 (2000)
6. Douglas J. Futuyma and Anurag A. Agrawal, *PNAS.* 106 (2009)
7. Yeargan and Colvin, *Journal of Kan. Entom. Society,* 86 (2013)
8. Yoshitomo Kikuchi et al., *PNAS.* 109 (2012)
9. C. D. King et al., *Current Drug Metab.* 1 (2000)
10. Ying Zhen, *Science.* 337 (2012)

Jennifer Cottle is a senior at John Overton High School in Nashville, Tennessee; she attended the School for Science and Math at Vanderbilt.