Transcriptional plasticity in a specialist, non-model insect
associated with living in a toxic environment.

By

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I would like to dedicate my Ph.D. dissertation to Dr. Nicole Gerardo (Emory University). I began my research career as a work-study student in her lab, was able to complete an honor's thesis with her, and worked for her for four years after graduating with my undergraduate degree. She allowed me the freedom to pursue my own research interests while working as her lab manager, and this experience wholly equipped me to be successful in graduate school. I can honestly say that I would not be a scientist were it not for her continued support and encouragement.

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CHAPTER I

INTRODUCTION

Plant-feeding insects account for a large proportion of the described species on earth and are incredibly diverse in phenotypes ranging from body morphology to niche adaptations. Most insect species use only a surprisingly narrow fraction of the plants available to them, and true generalists are the rare exceptions. There are a number of hypotheses for what explains variation in insect diet breadth and specialization, and in recent years, there has been growing interest in understanding the genetic mechanisms underlying host plant use. Milkweed-herbivore systems have been models for studies for decades due to the unique chemical ecology of cardenolide-producing milkweed plants and the adaptations of milkweed herbivores. Many milkweed herbivores possess target-site substitutions, *i.e.*, specific amino acid substitutions that reduce toxin binding, that confer resistance and/ or tolerance to milkweed cardenolide toxins. However, not all milkweed herbivores, such as the milkweed aphid, *Aphis nerii*, possess these specific, protective amino acid substitutions. Furthermore, a number of insect herbivores are agricultural pests, and it is well known that mechanisms of insecticide resistance mirror those employed by insects to overcome natural plant defenses. With these ideas in mind, I have applied transcriptomics to identify novel genes involved in insect-milkweed adaptations and to test the role of gene expression convergence in insect adaptations to plant toxins and insecticides.

Here, I use the non-model milkweed aphid species, *A. nerii*, to investigate alternative mechanisms for host plant adaptation and the relationship between insect adaptations towards plants and insecticides. Author contributions are listed at the beginning of each chapter. First, I describe how studies of gene expression, in particular global transcriptomic studies, can inform evolutionary ecology studies of plant-insect interactions (Chapter II). Then, I discuss background on the milkweed-herbivore system and why *A. nerii* is a unique model system in which to study insect adaptations towards toxic host plants (Chapter III; (Birnbaum & Abbot, 2018, *Entomologia Experimentalis et Applicata*)). Next, I outline methods for the use of aphids in laboratory and molecular studies (Chapter IV; Birnbaum, Rinker, & Abbot, 2018, *Journal of Visualized Experiments*)). In Chapter V, I discuss my findings on how, after long-term adaptation to host plants varying in toxicity, *A. nerii* experience reduced fitness and differentially express a narrow set of primarily detoxification related genes (Birnbaum, Rinker, Gerardo, & Abbot, 2017, *Molecular Ecology*). Next, in Chapter VI, I explore the role of transcriptional plasticity after one and five generations of exposure to more toxic host plants and two insecticides. I found that after five generations of exposure, genes differentially expressed in *A. nerii* in response to insecticides are largely encompassed by those genes differentially expressed in response to more toxic host plants. Last, I discuss what my experiments and data have contributed to our understanding of milkweed-herbivore adaptations and the role of transcriptional plasticity in the evolution of diet breadth, and remaining questions in the evolutionary ecology field that can be addressed using the milkweed aphid system (Chapter VII).

CHAPTER II

WHAT THE TRANSCRIPTOME TELLS US ABOUT THE EVOLUTION OF DIET BREADTH IN INSECTS

Author Contributions: Stephanie S.L. Birnbaum and Patrick Abbot equally contributed to the outline of this Chapter. S.S.L.B. performed the literature review and wrote the Chapter, and created all Boxes, Figures, and Tables. P. Abbot edited the Chapter.

Overview

Plant-feeding insects are among the most diverse organisms on the planet. Most species use only a surprisingly narrow fraction of the plants available to them, and true generalists are the rare exceptions. There are a number of hypotheses for what explains variation in insect diet breadth, and in recent years, there has been growing interest in understanding the relationship between variation in gene expression and host plant use. We provide an overview of the kinds of studies that are emerging on gene expression and insect herbivores, and identify areas that are in need of further inquiry. Addressing the roles that expression variation plays in insect diet breadth will have important implications for our understanding of the evolution of specialization and the complex map between genomes and ecological niches.

Insects, plants and genes: what's the question?

Herbivorous insects account for a remarkable percentage of described species: about half of all described eukaryotes are insects that feed on a plant (Gilbert et al., 1979). Most are specialists, feeding on one or a group of related plant species (monophagy or various degrees of oligophagy) (Forister et al., 2015; Futuyma & Moreno, 1988). In any given habitat, there are a number of possible host plants for an insect species, and there seem obvious advantages to using them broadly (Janzen, 1970; Nyman, 2010). Why then colonize only a fraction of the available host plants? Why not a broad niche? The question of what limits niche breadth is one of the enduring problems in evolutionary biology (Futuyma & Agrawal, 2009; Sexton, Montiel, Shay, Stephens, & Slatyer, 2017; Wilkinson & Sherratt, 2016).

Original theories on the evolution of specialization in herbivorous insects centered on the importance of generalist predators in limiting diet breadth (Bernays & Graham, 1988), and studies in sequestering insects, perhaps one of the more dramatic scenarios of insect specialization on plants, have largely focused on the importance of this adaptation against natural enemies (Petschenka & Agrawal, 2016; Züst, Mou, & Agrawal, 2018). In recent years, there has been growing appreciation for the genetic and epigenetic mechanisms associated with host plant use, and the mechanisms involved in colonization of alternative hosts plants and host range expansion (Nylin et al., 2018). With advances in next-generation sequencing, studies of phenotypic plasticity have turned to focus on the molecular plasticity that underlies the shape of the reaction norms across alternative host plants (Morris & Rogers, 2013). Imagine an insect colonizes a novel host plant. Over the course of development, these colonists express genes in ways that differ from the typical, ancestral patterns on their normal host. What causes these differences and what are their consequences for changes in host range? Perhaps transcriptional plasticity facilitates colonization and confers a degree of non-genetic fit to a novel host (Nylin & Janz, 2007). Alternatively, perhaps the differences are maladaptive, random differences due to drift, or perhaps just noise (Ghalambor et al., 2015; Mallard, Jakšić, & Schlötterer, 2018; Price, Qvarnström, & Irwin, 2003).

Motivated by such questions, there has been a recent accumulation of studies, mostly focusing on patterns of gene expression, that collectively address how insect herbivores respond to the challenge of alternative host plants. Concepts and phrases borrowed from evo-devo, such as genetic accommodation, genetic assimilation, and Baldwin Effects, are increasingly commonplace in the literature on insect-plant interactions. However, as new studies are accumulating, biases are also emerging, in both questions and methods. The result is a diversity of experimental designs and objectives, producing a fragmented portrait of the relationship between gene expression and the insect herbivore niche. In short, the ecological annotation of genes for host use, range expansion, and diversification of insect herbivores is very much a work in progress.

Our aim here is to organize the rationales and existing data on the role of gene expression in diet breadth in insects, to define both the key questions and current approaches in the field, and to highlight experimental opportunities to address the intrinsic factors that govern the insect dietary niche. One of our primary insights is that, while many exciting studies are emerging, there remain to date remarkably few that directly characterize the response of specialist and generalist insects to their hosts plants within a single, coherent experimental framework. It is precisely these sorts of studies that will address long-standing ideas and current debates about the genetics and evolution of diet breadth in insect herbivores. Another theme that emerges is that studies of gene expression in insect herbivores address questions about the evolution of gene expression itself. But the fact is that less is known about the evolution of gene expression than may be always appreciated in the literature (Box 1), and debates are sure to emerge, much as they are elsewhere on the studies of gene expression and plasticity (Ghalambor et al., 2018; Mallard et al., 2018; van Gestel & Weissing, 2018).

Box 1. The evolution of gene expression- gene expression vs. gene regulation.

Changes in gene regulation that underlie adaptation to novel environments and lead to phenotypic diversity have been hypothesized to drive speciation and the diversification of organisms, but questions regarding the evolution of gene expression and its influence on speciation remain (Gilad, Oshlack, & Rifkin, 2006; Pavey, Collin, Nosil, & Rogers, 2010; Romero, Ruvinsky, & Gilad, 2012). The importance of various genetic and epigenetic mechanisms underlying gene regulation variation and the evolutionary forces that shape patterns of gene expression remain to fully characterized (Box 2; (P. W. Harrison, Wright, & Mank, 2012)). Adaptation to novel environments likely involves changes in gene expression, and the regulation of gene expression can occur at multiple points including chromatin condensation, histone modification, methylation, transcriptional initiation, alternative splicing, translational controls, or post-translational modification (Orphanides & Reinberg, 2002). Variation in gene expression can also result from changes in the genetic code, such as gene duplication or mutations in enzymes; one of the most important determinants of gene expression is transcriptional initiation (Wray, 2003).

The mechanisms by which gene expression plasticity can evolve (*i.e.*, expression of a gene/ groups of genes that were initially plastically expressed become constitutively up- or down- regulated) remain to be characterized in detail. Cis- and trans-regulatory elements act proximally and distally, respectively, to control gene expression. Divergence of these regulatory elements has been shown to influence the differential expression of genes involved in differing adaptive phenotypes between wine and laboratory strains of *Saccharomyces cerevisiae* (Fraser, Moses, & Schadt, 2010) and closely related *Drosophila* species (McManus et al., 2010). To experimentally characterize changes in transcriptional regulation, integrated measurements of gene expression, binding of transcription factors to promoters, variation in promoter sequence, and information of protein interactions are required (Cavalieri, 2009).

The evolutionary processes acting on transcription and gene expression are complex. In model organisms and primates, the expression levels of most genes appear to be evolving under stabilizing selection (Gilad et al., 2006), however, there is evidence that some genes are evolving under directional (positive) selection (Romero et al., 2012). In *Drosophila* species, adaptive changes in gene expression are more frequent in functional classes with clear ties to adaptive functions, such as sensory perception and sexual behavior (Nourmohammad et al., 2017).

Microarrays provided the first genome-wide gene expression assays (Harrington, Rosenow, & Retief, 2000), and now, RNAseq allows genome-independent quantitative characterizations of whole mRNA differences, *i.e.*, differential gene expression, in a wide variety of non-model organisms (Finotello & Di Camillo, 2015). Through these studies, patterns of constitutive and inducible expression can be characterized between biotypes, closely related species, or over time to identify plastic and evolved gene responses important in the evolution of diet breadth. Genes differentially expressed in the same biotype/ species in response to a novel host plant represent a plastic, induced response, whereas expression differences between biotypes/ species are constitutive, induced differences. By examining gene expression changes over time during adaptation to novel plants, differentially expressed genes can be grouped into responses determined by selection history as compared to responses driven by host plant feeding.

Specialists, generalists, and genomes

Traditionally, ideas about host specialization and diet breadth have centered on host-use trade-offs and plant defenses – the idea that perhaps the adaptations that insects express to locate and exploit host plants diminish the ability to find and exploit alternatives (Lynch & Gabriel, 1987). However, evidence for host-use trade-offs is not universal (Bennett & Lenski, 2007; Forister, Dyer, Singer, Stireman, & Lill, 2012; Whitlock, 1996), and if nothing else, the existence of extremely polyphagous generalists suggests that trade-offs are not inevitable (Normark & Johnson, 2010). A consequence of years of effort to discern general rules governing plant-insect interactions is a pluralism of approaches, accompanied by a more refined set of questions about the relative importance of different contributing factors at work over the course of adaptation to host plants (Nylin et al., 2018; Sexton et al., 2017). For example, negative correlations across host plants may be less important in governing host range than the accumulation of alleles that are beneficial on specific hosts and conditionally neutral otherwise (Gompert et al., 2015). Geographic co-occurrence and the legacy of traits that fortuitously confer a degree of fit between insects and alternative hosts may explain apparent conservation in host use better than phylogeny (Agosta & Klemens, 2009; Calatayud et al., 2016). There are vigorous debates about the dynamics and consequences of historical and ecological effects on diet breadth (Hamm & Fordyce, 2016; Hardy, Peterson, Ross, & Rosenheim, 2017; Nylin & Janz, 2007), and indeed, in precisely what way the distribution of diet breadth requires special explanation at all (Forister & Jenkins, 2017).

Patterns of gene expression are refined by host plant use, but also may constrain host range

Gene regulatory perspectives on diet breadth are centered on the notion that patterns of gene expression provide windows into host plant adaptation and the evolution of niche breadth in insects. Variation in gene expression is generally divided along different axes – those that represent a form of plasticity that affect the fit of an organism to its environment, and those that represent a form of noise in gene expression that may have little to do with adaptation. Both have been reviewed extensively (Box 2; (Hedrick, 2006; Morris & Rogers, 2013; Renn & Schumer, 2013)). The generalist niche is presumably more heterogeneous than the specialist one, and all else being equal, more broad niches should accompany and be facilitated by genomic flexibility (Sexton et al., 2017). This is the basis of the expectation that a jack of all trades should have a toolbox with instruments for many occasions. Important clues to the dietary niche should be recorded in insect genomes.

Box 2. Phenotypic plasticity in the context of transcriptional plasticity and the evolution of diet breadth.

Phenotypic plasticity is environmentally-induced phenotypic variation. That is, if an organism's differential response to a novel environment is plotted as a reaction norm (Fig. 1), plasticity can be visualized as a change in the slope of the reaction norm. When organisms experience novel environments, optimal trait values likely differ from those in ancestral environments. Plasticity can result in changes in trait values towards (adaptive) or away from (non-adaptive) the new optima. Whether plasticity is adaptive or not has important

implications for the likelihood of persistence in novel environments, and subsequent evolutionary changes (Ghalambor, McKay, Carroll, & Reznick, 2007).

With respect to transcriptomes, plasticity can be measured through analyses of differential expression in response to novel environments. Transcriptional plasticity is hypothesized to underlie variation in insect diet breadth and the evolution of specialization versus generalization. Specialists and generalists may demonstrate similar patterns of plasticity (Fig. 1, plasticity, no divergence), or they may differ in their plastic responses in a number of described processes. The idea that evolution can act upon plastic responses and proceed in the direction of the induced plastic response is known as the Baldwin effect (J. M. Baldwin, 1896a; 1896b; Crispo, 2007). Specialists and generalists may differ in their plastic response, which is illustrated by a difference in the y-intercept of their reaction norms, and represents a significant species effect but not species x environment effect (Fig. 1, Baldwin effect 1), or a difference in the slope of their reaction norms, *i.e.* a significant species x environment effect (Fig. 1, Baldwin effect 2), in common compared to novel environments. Alternatively, in the case of genetic assimilation, theoretically, transcriptional plasticity that initially allows an organism to survive a novel environment becomes canalized or constitutively expressed (Fig. 1, genetic assimilation- dashed line; (Crispo, 2007; Waddington, 1942; 1953)). Direct comparisons of transcriptional plasticity in specialist and generalist insect herbivores or in diverged populations are necessary to discern the role and shape of transcriptional plasticity in the evolution of diet breadth.
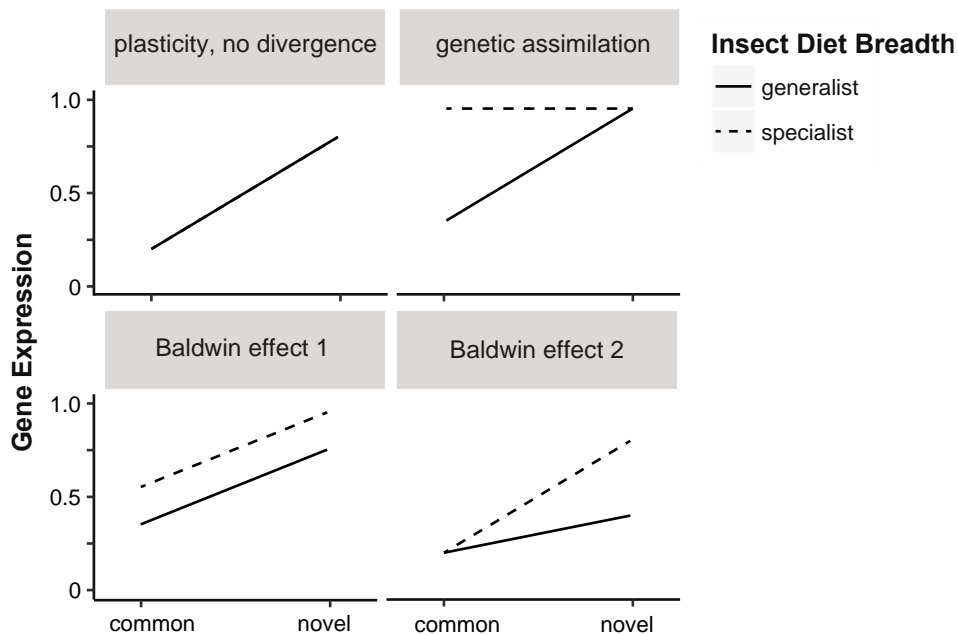
Figure 1. Gene expression reactions norms for generalist and specialist insects with **(A)** plasticity, no divergence, **(B)** genetic assimilation, **(C)** and **(D)** Baldwin effect. Note that these graphs depict patterns involving upregulation in specialists, although patterns involving downregulation are also expected (Crispo, 2007; K. Hoang et al., 2015).

Relative to specialists, the genomes of most polyphagous species share common features, including often dramatic expansion of genes involved in chemoreception and detoxification of plant compounds (Grbić et al., 2011; Xu et al., 2016), and differential regulation of genes involved in protein synthesis, digestion, and detoxification (Celorio-Mancera et al., 2013). In omnivorous Diptera and Hymenoptera, as well as some Coleoptera and Orthoptera, genes encoding detoxification enzymes are expanded relative to those that feed on more narrow or innocuous diets (nectar and pollen) (Rane et al., 2016). In certain cytochrome P450 subfamilies, both gene number and functional diversity correspond to dietary breadth and complexity (Calla et al., 2017).

When faced with challenging, novel host plants, it is generally assumed that generalist insects should display more environmentally plastic patterns of gene expression, facilitating broad niche occupancy, compared to specialists, which are hypothesized to have evolved a more targeted gene repertoire (Box 2). Whole genome transcriptomics can provide important insights into host plant specialization and the evolution of diet breadth, but the appropriate comparisons must be made amongst both plants and insects. Population-level comparisons might reveal how environmentally-responsive gene expression confers a form of plasticity in response to the non-optimal or stressful conditions that novel plants must represent (Berenbaum, 2002; Levis & Pfennig, 2016; Nylin & Janz, 2007). Studies comparing how host plant variation (e.g. ancestral vs. diverged host plants or plants varying in toxicity) affect gene expression in phylogenetically-related specialist and generalist species are required to understand how transcription plasticity influences the evolution of dietary specialization. However, given problems associated with gene expression comparisons between species, valuable information can also be gained by comparing differences in gene expression between specialists and generalists within species, e.g. biotypes or diverged populations. Studies comparing the transcriptomic responses of adapted and non-adapted insect populations to variation in host plants, or those comparing diverged populations from the field, will help elucidate how variation in gene expression contributes to or prevents host plant switching.

Experiments examining the herbivore transcriptomic response to alternate or novel host plants, ancestral vs. derived host plants, or plants differing in secondary chemicals or toxicity all have the potential to elucidate critical genes and processes involved in host plant adaptation. Compared to generalists, specialist herbivores are generally thought to utilize detoxification enzymes with targeted substrate specificity and/ or increased efficiency, reflecting refinement and adaptation towards narrow host plant ranges. Furthermore, differences in gene expression are expected during short-term vs. long-term adaptation towards new host plants. In both cases, these hypotheses remain largely conjectural, and must be comprehensively tested across a range of insect herbivores, feeding guilds, and host plants. On sub-optimal hosts, specialists may experience greater reductions in fitness compared to generalists, but they may ultimately achieve the highest fitness if adaptation on the sub-optimal host occurs. One might expect the greatest gene expression changes early in adaptation and plasticity to decrease as adaptation progresses, however, this hypothesis has not been tested across a variety of systems (Dermauw et al., 2013; Wybouw et al., 2015). Changes in the direction and scale of differential gene expression during adaptation need to be characterized in a range of generalists and specialists to understand how expression plasticity facilitates host shifts (Fig. 1). The phylogenetic relatedness of the plants that are being compared should also be considered, and, when appropriate, studies should include comparisons between both closely and distantly related host plant species.

Other factors contributing to gene expression variation in insects

Developmental plasticity and variation in developmental gene expression play roles in the evolution of novel traits and speciation (Moczek et al., 2011; Rifkin, Kim, & White, 2003). Given changes in host plant feeding behavior through development in holometabolous insects, variation in diet-related gene expression between larval and adult stages should be expected. In contrast, hemimetabolous insects that feed on their host plants for the entirety of their lifetime may consistently express diet-related genes through different life stages. Moreover, gene expression variation differences are expected between different feeding guilds, i.e. sucking (phloem feeders) vs. chewing insects. In general, sucking insects induce plant salicylic acid (SA) defenses as opposed to jasmonic acid (JA) defenses and, overall, have a less drastic effect on induced plant responses. Comprehensive, comparative studies using insects across the specialization spectrum in both phloem-feeding and leaf-chewing insects are needed to understand gene expression differences associated with these differences.

Using transcriptomics to analyze diet breadth in insects

The ideal transcriptomic studies should compare differential expression across time in a range of generalist to specialist insect species or biotypes feeding on host plants that vary in defenses or evolutionary

history. Moreover, appropriate life history and fitness parameters (e.g. development time, fecundity, longevity) should be measured in conjunction with gene expression to fully understand how transcriptional plasticity influences diet breadth. Gene expression differences without host-plant associated fitness differences may indicate a less significant role for transcriptional plasticity in variation among insect species in their host plant associations, *e.g.*compared to top-down effects of predators (Bernays & Graham, 1988). A number of different, specific questions can be addressed using various experimental frameworks (Box 3), however, the broad, focal questions are often governed by the specific study system, *e.g.*, speciation in *Rhagoletis* spp. (Ragland et al., 2015), host plant switching in *Drosophila mettleri* populations (K. Hoang et al., 2015), or evolution of sequestration using toxic plant-herbivore systems (Birnbaum, Rinker, Gerardo, & Abbot, 2017).

Box 3. Experimental frameworks to address the evolution of diet breadth using transcriptomics.

Herbivore transcriptomic studies can be designed to answer a number of questions (outlined below) whose answers shed light onto ecological and evolutionary aspects of insect diet breadth. Clearly, experiments can be designed to answer multiple questions, and experiments should be designed to include dietary manipulations (1-5) in a comparative framework (6-8).

Specific questions that can be addressed- experiments with dietary manipulations:

1. What is the transcriptomic response to specific plant defenses (including host plant-artificial diet comparisons)?
2. What is the transcriptomic response to an alternate/ novel host plant?
3. Are there expression differences between ancestral and diverged plant hosts?
4. Do different abiotic and biotic stresses induce similar or different transcriptional responses?
5. What are the transcriptomic changes over time during a host plant shift or challenge?

Specific questions that can be addressed- experiments comparing insects:

6. What are the transcriptomic differences between biotypes/ populations associated with different host plants or that vary in virulence to plants?
7. What are the transcriptomic differences between species associated with different host plants?
8. How do transcriptomes differ between species that range from specialist to generalist?

We conducted a comprehensive review of studies applying whole transcriptomic analyses to answer questions surrounding insect adaptations towards host plants. We used the Web of Science database and search parameters (last search date Jan. 24, 2017): (plant* OR herbiv* OR host OR leaf OR leaves OR root* OR gall* OR stem OR agricul* OR crop) AND (transcripto* OR RNA-seq OR microarr*) AND (insect* OR larva* OR pest). The resulting list was manually refined to include only papers with a full transcriptome (cDNA library Sanger sequencing, microarray, or RNAseq) analysis and either a dietary manipulation that is relevant to host plant adaptation or a relevant comparison of species or biotypes that represent the spectrum of generalists to specialists. Studies focused on specific gene families, immune responses, or responses to insecticides were excluded. Table 1 lists references used in this review alongside the specific questions they address (Box 3) and the differentially expressed genes identified by the authors.

The patterns found thus far

To harness the power of transcriptomics to understand the role of gene expression plasticity in the evolution of herbivore dietary specialization, comparisons between specialists and generalists are required. We found that fewer than 10% of the herbivore transcriptome studies identified included gene expression data comparing species or biotypes varying in dietary specialization (Fig. 2; (Govind et al., 2010; Pearce et al., 2017; Roy et al., 2016; Schweizer, Heidel-Fischer, Vogel, & Reymond, 2017; Shiao et al., 2015; Silva-Brandão et al., 2017; Thorpe, Cock, & Bos, 2016; W.-Q. Xia, Wang, Liang, Liu, & Wang, 2017; Jin Zhang et al., 2015a)), and

four of these studies (Govind et al., 2010; Roy et al., 2016; Schweizer et al., 2017; Silva-Brandão et al., 2017) also examine gene expression on variable diets. Shiao et al. (2015), Thorpe et al. (2016), and Zhang et al. (2015) do not include variations in diet but provide chemosensory and salivary candidate genes important in the adaptation of generalists.

As discussed above, generalist herbivores are expected to exhibit greater transcriptional plasticity when feeding on host plants with increased defenses. In studies examining differential expression between generalist and specialist species (all using Lepidopteran species), all found that generalists demonstrate greater expression plasticity compared to specialists (Govind et al., 2010; Roy et al., 2016; Schweizer et al., 2017). When feeding on plants silenced for various defenses, a generalist herbivore, *Heliothis virescens*, differentially expresses a greater number of genes than the specialist herbivore, including a large number of canonical detoxification genes (Govind et al., 2010; Schweizer et al., 2017). Moreover, generalists differentially expressed similar genes in response to multiple, selectively silenced plant defenses in contrast to the diet-specific response observed in the specialist *Manduca sexta* (Govind et al., 2010). The transcriptomic response of specialists in these comparative studies is more varied. In Govind et al. (2010), compared to generalist herbivores, the specialist demonstrated differential expression of a smaller total number of genes but more involved in secondary metabolism (*e.g.* detoxification, transporters) most of which were downregulated. In contrast, Schweizer et al. (2017) found that specialist *Pieris brassicae* expresses very few detoxification genes compared to the generalist, but an equal number of genes were down- and up-regulated. Roy et al. (2016) compared gene expression in a broad generalist species, *Spodoptera littoralis*, with two biotypes, non-adapted and maize-adapted, of a less polyphagous species, *Spodoptera frugiperda*. They found that when feeding on a challenging maize diet as compared to artificial diet, expression plasticity decreased with increasing dietary specialization. The generalist, *S. littoralis*, differentially expressed a greater percentage of transcripts and experienced overall reduced fitness when feeding on maize. The generalist, *S. littoralis*, also generally differentially expressed a greater percentage of genes involved in digestion, while the less polyphagous, maize-adapted *S. frugiperda*, demonstrated greater differential expression of detoxification and transporter genes.

Overall, these results are in accordance with theory and indicate that when compared to specialists, generalists exhibit greater transcriptional plasticity to tolerate variable plant defenses. However, general patterns have not yet emerged in studies comparing transcriptomic responses in specialist gene expression (Table 1). While these studies lend support to existing theories, we must highlight the relative dearth of studies directly comparing transcriptional plasticity between specialists and generalists across a range of host plants. Further studies are needed encompassing a greater diversity of insects in more diverse systems.

Given that variation in plant secondary metabolite defenses is a primary barrier against novel plant use, it is important to understand the transcriptomic responses involved in adaptation towards plants varying in specific defenses. Around 30% of studies examined responses in herbivores feeding on such plants and an additional 19% of studies include gene expression of herbivores feeding on artificial diets varying in specific plant defensive chemicals. In general, these studies report differential expression of canonical detoxification genes, proteases, transporters, cuticle-related genes, and metabolic genes. GO enrichment analyses commonly showed enrichment of metabolic processes, oxidoreductase activity, hydrolase activity, catalytic activity, transporter activity, metal ion binding, and structural constituent of the cuticle (Table 1).
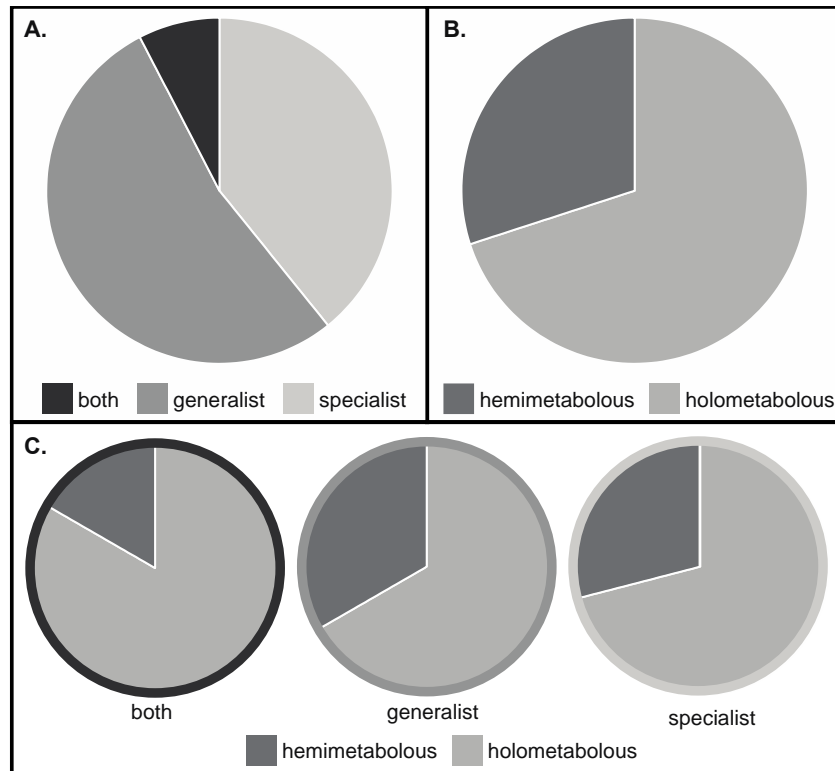
Figure 2. The distribution of transcriptomic studies identified in this review. **(A)** Dietary breadth- the proportion of studies investigating transcriptomic responses in generalist-only, specialist-only, or both generalist and specialist taxa. **(B)** Life cycle- the proportion of studies investigating transcriptomic responses in either holometabolous or hemimetabolous taxa. **(C)** The proportion of studies investigating either holo- or hemi- metabolous taxa for each group of diet breadth, both (generalist and specialist), generalist-only, and specialist-only.

To fully understand the role of gene expression plasticity in the evolution of diet breadth, gene expression must be evaluated after adaptation over many generations and ideally compared at multiple time points during adaptation on a novel host plant. Only approximately 20% of studies examined gene expression after multiple generations and just two of these studies, both investigating *Tetranychus urticae*, included expression both early and late in adaptation on alternate host plants (Dermauw et al., 2013; Wybouw et al., 2015). In contrast with theory, Dermauw et al. (2013) found that the polyphagous two-spotted spider mite, *T. urticae*, differentially expresses a greater number of transcripts after long-term adaptation (5 generations) on a challenging host plant as compared to transcription 2h and 12h after shifting host plants. Overall, there was little overlap in differential expression between short- and long-term responses, but many genes involved in shared responses were characterized as canonical detoxification genes (CCEs, P450s, GSTs, ABC transporters). Intradiol ring-cleavage dioxygenases, P450s, lipocalins, major facilitator superfamily genes, and transcription factors, among other gene families, were dynamically expressed in spider mites during adaptation to new host plants (Dermauw et al., 2013).

Few studies have directly examined the role of transcriptional plasticity in the evolution of diet breadth in insects, but Hoang et al. (2015) provide a relevant analysis. By comparing gene expression reaction norms (Fig. 1) in two diverged populations of *D. mettleri* on native and alternate host plants, they were able to characterize evolutionary divergence in gene expression between populations. The vast majority of differentially expressed genes were plastic, with no divergence between populations. Only four genes demonstrated expression patterns following genetic assimilation, whereby initially plastic genes (ancestral population) become constitutively expressed regardless of environment (diverged population), and these were

enriched for amino acid catabolism. A greater number of genes (65) demonstrated plasticity patterns fitting the Baldwin effect, and these were enriched for functions in glycan degradation, galactose metabolism, and pentose and glucuronate interconversions. When considering both environment-independent and environment-dependent responses, a relatively small number of genes were differentially expressed between *D. mettleri* populations (319 genes; 2.6%). The majority of these genes were differentially expressed between populations independent of environment, *i.e.* larval host diet, and these genes were enriched for GO processes involved in drug metabolism, starch/ sugar metabolism, and chemosensory perception. Of the remaining environment-dependent differentially expressed genes, interestingly, more genes were differentially expressed between populations when reared on the ancestral compared to derived host plant diet. In the set of differentially expressed genes when reared on the ancestral host, genes were enriched for functions associated with amino acid metabolism, proteasome, and chitin. This pattern suggests the importance of basic protein metabolism and the role of cuticle restructuring underlying population differences associated with the ancestral host. On the derived host, differentially expressed genes were enriched for only drug metabolism; these genes may underlie population differences after a host plant switch. Thus, a small number of plastic genes with key functions can underlie the differential adaptation of populations to alternate host plants.

The vast majority of studies included here investigated a limited subset of the possible questions (Box 3) that need to be addressed to fully apply transcriptomics towards our understanding of the evolution of host plant specialization. Moreover, the most powerful transcriptomic studies consider more than one of the outlined questions and include multiple comparisons on both the plant and insect side, however, only a quarter of the studies included in this review met that requirement.

Remaining knowledge gaps, and what transcriptomes can and cannot tell us about the evolution of diet breadth

Few studies directly compare transcriptional responses between groups that vary in host plant specialization (8/79), and most studies evaluate responses in generalist (42/79) and holometabolous insects (55/79). To gain a full understanding of the evolution of diet breadth, we need to understand how gene expression changes over the course of host plant adaptation. This includes an understanding of transcriptional changes early during interactions with a novel host plant compared to transcription after long-term adaptation to a new host plant as well as a comprehensive analysis of change in gene expression across different life stages in insects. There is a clear bias in the literature towards studies evaluating responses to varying plant diets over a short time period or within a single generation (75%), and less than 16% of the studies included in this review compare transcription between multiple life stages.

While transcriptomes are valuable for identifying candidate processes and mechanisms important in the evolution of dietary specialization and diversification, these studies are most powerful when combined with other organismal or ecological data or experiments. Important clues about the strength of trade-offs influencing plasticity can be gained through transcriptome experiments incorporating life history data and measures of fitness in alternate environments. To fully understand the role of transcriptional plasticity in patterns of diet breadth, direct transcriptomic comparisons of diverged populations with different diet breadths, or between generalists and specialists, are necessary.

Synthesis & future directions

Understanding the genome-wide patterns of the effects of selective history on adaptation to environmental challenges in insect herbivores addresses long-standing questions in evolutionary biology, and sheds light on our understanding of life history evolution, co-evolution between plants and insects, and ultimately the mechanisms that generate diversification. Numerous transcriptomic studies thus far have identified the important role of genes involved in metabolic processes, detoxification, and components of the cuticle in insect adaptations to host plants, but direct studies of the role of transcriptional plasticity in the

evolution of diet breath are limited. Studies comparing biotypes or species with varying host ranges or level of specialization are especially critical to understand patterns of variation in gene expression that may limit host range or facilitate host plant switches.

However, while patterns of differential expression can allow hypotheses to be generated about the role of transcriptional plasticity and of specific genes or gene families in adaptation, transcriptomics is most powerful when combined with methods that can determine the mechanisms underlying differences in gene expression. Network analyses can be used to group sets of genes with common expression patterns that are potentially co-regulated. Methods to evaluate changes in transcription factors, promoter sequences, or epigenetic mechanisms that govern differences in gene expression are ultimately required to comprehensively understand how variations in gene expression influence the evolution of host plant associations in herbivorous insects.

The effort to understand the evolution of specialization and diet breadth has informed our understanding of many topics in ecology and evolution, from the factors that regulate populations to co-evolution and speciation. The consequences of co-evolution between plants and insect herbivores are reflected in their genomes and the traits they express for exploitation and defense. With successes in next-generation sequencing, the challenge is to understand complex mechanisms that operate at the level of cells and molecules, operating over whole genomes and over developmental stages.

	Question Type (Box 3)													
Insect Species	**G**	**S**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**FD**	**Differentially Expressed Categories**	**Type**	**Citation**
Manduca sexta, Heliothis virescens	■		■							■		primary metabolism, digestion, detoxification, transport	microarray	Govind et al., 2010
Heliothis virescens, Pieris brassicae	■									■	■	detoxification, storage, cuticle	RNAseq[f]	Schweizer, et al., 2017
Spodoptera littoralis, S. frugiperda	■		■								■	metabolism, detoxification, digestion, transport, defense, cell signalling	RNAseq[f]	Roy et al., 2016
Trichoplusia ni				■								detoxification, digestion	RNAseq[d]	Herde & Howe, 2014
Diuraphis noxia								■	■		■	detoxification, digestion	RNAseq[j]	Anathakrishnan et al., 2014
Anoplophora glabripennis			■									detoxification, digestion, stress response, interactions with midgut microbes	RNAseq[e]	Mason et al., 2016
Bemisia tabaci			■								■	primary metabolism, detoxification, transport	microarray	Alon et al., 2012
Callosobruchus maculatus			■									primary metabolism, detoxification, digestion, development, defense	microarray	Guo et al., 2012
Callosobruchus maculatus			■									digestion, detoxification, immunity, stress response	microarray	Moon et al., 2004
Callosobruchus maculatus			■									primary metabolism, transport	microarray	Wang et al., 2015
Dendroctonus ponderosae			■									digestion, detoxification, immunity, stress response, reproduction	RNAseq[e]	Robert et al., 2013
Diabrotica undecimpunctata howardi			■								■	primary metabolism, digestion, peritrophic membrane/ cuticle	microarray	Liu et al., 2004
Helicoverpa armigera			■								■	detoxification *focus on detox and P450s, full GO only in suppl.	microarray	Tao et al., 2012
Helicoverpa armigera			■									primary metabolism, detoxification, cell adhesion, energy	microarray	Celorio-Mancera et al., 2011
Helicoverpa armigera			■								■	primary metabolism, detoxification, digestion, stress response, immunity	microarray	Kuwar et al., 2015
Helicoverpa zea			■									primary metabolism, detoxification, stress response, immunity	microarray	Jing et al., 2012
Locusta migratoria			■									digestion, detoxification, immunity, stress response, peritrophic membrane	microarray	Spit et al., 2016
Myzus persicae			■								■	digestion, detoxification	microarray	Ramsey et al., 2007
Sitobion avenae			■								■	digestion	RNAseq	Zhang et al., 2013
Spodoptera frugiperda			■									primary metabolism, proteolysis	RNAseq[a]	Fescemeyer et al., 2013
Spodoptera frugiperda			■									digestion *focus on proteinases	cDNAseq[A]	Briosci et al., 2007
Tetranychus urticae			■									detoxification *no full discussion of DE	microarray	Zhurov et al., 2014

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Question Type (Box 3)

Insect Species	G	S	1	2	3	4	5	6	7	8	FD	Differentially Expressed Categories	Type	Citation
Tupiocoris notatus	■		■									primary metabolism, detoxification	RNAseq[e]	Crava et al., 2016
Tetranychus urticae	■			■		■	■					detoxification, transport, transcription factors	microarray	Dermauw et al., 2013
Tetranychus urticae	■			■			■					detoxification, transport, stress response	microarray	Wybouw et al., 2015
Chilo suppressalis	■			■								primary metabolism, detoxification, digestion, transport, growth	RNAseq[e]	Zhong et al., 2017
Cryptotermes domesticus	■			■							■	primary metabolism, detoxification, intracellular components	RNAseq[e]	Wu et al., 2016
Helicoverpa armigera	■			■							■	primary metabolism, detoxification	microarray	Celorio-Mancera et al., 2012
Helicoverpa armigera	■			■								detoxification, digestion	RNAseq	Pearce et al., 2017
Hyalopterus persikonus	■			■								stress response, ribosome	RNAseq[e]	Cui et al., 2016
Myzus persicae	■			■							■	detoxification	cDNAseq[B]	Ramsey et al., 2014
Myzus persicae	■			■							■	detoxification, immunity, cuticle	RNAseq[e]	Mathers et al., 2017
Oedaleus asiaticus	■			■								primary metabolism, stress response, chitin	RNAseq	Qin et al., 2017
Oedaleus asiaticus	■			■								primary metabolism, detoxification, stress response, cuticle, DNA replication	RNAseq[e]	Huang et al., 2017
Paratachardina pseudolobata	■			■								transport, gene expression regulation	RNAseq[g]	Christodoulides et al., 2017
Reticulitermes flavipes	■			■	■						■	primary metabolism, detoxification, digestion	microarray	Raychoudhury et al., 2013
Trichoplusia ni	■			■	■		■	■			■	detoxification, digestion, splicesome pathway	RNAseq[f]	Rivera-Vega et al., 2017
Polygonia c-album	■				■			■				primary metabolism, detoxification, digestion, transport, transcription factors, cuticle	RNAseq[e]	Celorio-Mancera et al., 2013
Spodoptera frugiperda					■	■		■				detoxification, binding, cuticle	RNAseq[f]	Silva-Brandão et al., 2017
Bemisia tabaci		■			■			■				immunity	RNAseq[d]	Xie et al., 2014
Vanessa cardui		■			■			■			■	primary metabolism, detoxification, digestion	RNAseq[e]	Celorio-Mancera et al., 2016
Bemisia tabaci		■									■	detoxification *no full discussion of DE	microarray	Kliot et al., 2014
Bemisia tabaci		■									■	primary metabolism, digestion, muscle contraction	RNAseq[e]	Xia et al., 2017
Aphis glycines		■										digestion	RNAseq[e]	Enders et al., 2015
Ostrinia nubilalis		■										detoxification, digestion, transport, chitin	microarray	Yao et al., 2017
Aphis glycines		■										detoxification, digestion, stress response, effectors	RNAseq[d]	Bansal et al., 2014
Diabrotica virgifera virgifera		■										detoxification, immunity	RNAseq[c]	Miller & Zhao, 2014
Leptinotarsa decemlineata		■									■	primary metabolism, digestion	microarray	Petek et al., 2012

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	Question Type (Box 3)													
Insect Species	**G**	**S**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**FD**	**Differentially Expressed Categories**	**Type**	**Citation**
Scaptomyza flava	■	■	■								■	stress response, cuticle, development	RNAseq[d,i]	Whiteman et al., 2012
Drosophila mojavensis		■		■		■					■	digestion, detoxification, chitin, energy, tRNA function	microarray	Etges et al., 2016
Drosophila mojavensis		■		■			■					primary metabolism, digestion, detoxification, energy	microarray	Etges et al., 2016
Drosophila mojavensis		■		■			■	■				primary metabolism, development, cuticle	microarray	Etges et al., 2014
Acyrthosiphon pisum		■		■				■				primary metabolism, digestion, cuticle/ chitin	RNAseq[e]	Eyres et al., 2016
Drosophila mettleri		■		■								primary metabolism, detoxification, cuticle, proteasome	RNAseq[e]	Hoang et al., 2015
Pieris rapae		■		■							■	primary metabolism, transport, stress response	RNAseq[e]	Sikkink et al., 2017
Rhagoletis pomonella, R. zephyria		■		■					■		■	primary metabolism, detoxification, storage	microarray	Ragland et al., 2015
Aphis nerii		■		■								detoxification, cuticle	RNAseq[f]	Birnbaum et al., 2017
Heliconius melpomene		■		■							■	primary metabolism, detoxification, digestion, transport, peritrophic matrix	RNAseq[e]	Yu et al., 2016
Manduca sexta		■		■							■	detoxification, transport, transcription factors, cuticle	RNAseq[f]	Koenig et al., 2015
Drosophila mojavensis		■			■	■		■				environmental sensing, cuticle	microarray	Rajpurohit et al., 2013
Drosophila mojavensis		■			■							detoxification, digestion, energy	microarray	Etges et al., 2016
Diabrotica virgifera virgifera		■									■	primary metabolism, transport, immunity	RNAseq[e]	Chu et al., 2015
Drosophila buzzatii		■						■			■	detoxification, stress response	RNAseq[e]	De Panis et al., 2016
Drosophila mojavensis		■			■							primary metabolism, immunity, methylation	RNAseq[b]	Smith et al., 2013
Drosophila mojavensis		■			■							detoxification, energy, cuticle, mRNA binding	microarray	Matzkin et al., 2012
Drosophila mojavensis		■			■							primary metabolism, detoxification, immunity, cuticle	microarray	Matzkin et al., 2006
Aphis gossypii		■						■				primary metabolism, response to stress	RNAseq	Li et al., 2013
Daktulosphaira vitifoliae		■						■				transport, cuticle, nucleosome, mitosis	RNAseq[e]	Rispe et al., 2016
Rhagoletis pomonella		■										primary metabolism, response to stress *no real analysis of DE	RNAseq[h]	Schwarz et al., 2009
Pyrrhalta maculicollis, P. aenescens		■							■			odorant binding proteins, odorant receptors *antennae	RNAseq[f]	Zhang et al., 2016

Table 1. List of studies included in this review. Insect species names are listed and studies are grouped based on the focal organisms' diet breadth (specialist [S] or generalist [G]) and then by the specific questions addressed in the studies (Box 3). Studies that included data on fitness (e.g. development time, fecundity, survival) in alternative environments are also indicated [FD]. The broad groups of differentially expressed genes identified by the authors are listed. If the specific platform for cDNAseq or RNAseq studies was listed in the Methods section of the study, it is indicated by the following superscript symbols: cDNAseq- A) ABI 3700, B) ABI 3730XL; RNAseq- a) ABI SOLiD 3 Plus, b) ABI SOLiD 4, c) Illumina GA, d) Illumina GA II, e) Illumina HiSeq2000, f) Illumina HiSeq2500, g) Illumina HiSeq3000, h) Roche 454, i) Roche 454 GSFLX, j) Sanger

CHAPTER III

INSECT ADAPTATIONS TOWARDS PLANT TOXINS IN MILKWEED-HERBIVORE SYSTEMS

Author Contributions: Stephanie S.L. Birnbaum wrote this Chapter and created the Figure. Patrick Abbot edited the manuscript. This chapter is published: Birnbaum, S. S. L. & Abbot, P. Insect adaptations toward plant toxins in milkweed-herbivores systems - a review. Entomologia Experimentalis et Applicata 58, 579–10 (2018).

Abstract

Studies of plant defenses and insect herbivores have been important in the development of our understanding of coevolution and specialization. Milkweed-herbivore systems have been a model for studying plant secondary chemistry defense evolution, insect adaptations to that chemistry, and coevolution between toxic plants and their herbivores for over a century, yet we are only beginning to unravel the multitude of adaptations required for insect specialization on milkweed plants. We review the empirical evidence for specialist insect adaptations towards milkweed toxins, coevolution between insects and milkweed plants, and canonical paradigms for sequestration and highlight areas for further research. By comparing research performed with diverse milkweed insects, we discuss the imperative to comprehensively study adaptations and specialization in divergent insect taxa.

Introduction

Studies of plant-herbivore interactions have long focused on the important role of the plant 'chemical arms race' in driving patterns of coevolution between partners. Plant-insect interactions are common models for studying patterns of coevolution and the evolution of specialized defenses, however, there is an imbalance in our understanding of the molecular mechanisms involved in these processes. Defense mechanisms are better characterized in plants, and a comparable understanding of how insects defend against these chemicals is only now emerging. The genetic and molecular mechanisms of insect tolerance and resistance to host plant secondary compounds have been described in a few systems, but we lack a comprehensive understanding of the interplay between the mechanisms underlying insect adaptations to host plants and their interactions with insect development, reproduction, and immunity (Heckel, 2014; Simon et al., 2015). In effect, our understanding of the coevolution of plants and insects remains incomplete.

Insects can mitigate the effects of plant defenses behaviorally by how, when or where they feed on their host plants. Beyond behavioral adaptations, herbivores can also secrete salivary effectors, either through saliva, regurgitant, or microbes, that reduce or evade plant defenses (Felton, Chung, & Hernandez, 2014). However, it is the post-ingestive mechanisms of herbivorous insects that have given rise to the most active area of inquiry on insect counter-defenses (Simon et al., 2015). Work over the past several decades has shown that herbivorous insects exhibit two general categories of molecular adaptation to plant chemical defenses. Insect proteins targeted by plant defenses can be desensitized via structural changes associated with mutations that alter the conformation of active sites on the proteins themselves. Secondly, insects express metabolic adaptations that result in the modification of ingested plant chemicals that either render them less toxic (phase I mechanisms), easier to transport or excrete (phase II & III mechanisms), or functionally advantageous to the insects themselves (sequestration) (Dobler, Petschenka, & Pankoke, 2011). Aside from defenses derived from insects themselves, gut microbes can play additional roles in detoxifying dietary plant compounds (Hammer & Bowers, 2015; Henry et al., 2013; Sugio, Dubreuil, Giron, & Simon, 2015). In some insect groups, obligate microbial

symbionts are indispensable partners that provide nutritional or other services that enable host plant utilization (Sugio et al., 2015; Giron et al. 2017).

When functional information is available on the biochemical targets of plant defenses, it has been possible to identify the target proteins in insects that either confer resistance, or else facilitate adaptation when associated with mutational alterations. However, in most cases, either sufficient functional information is lacking to fully identify the reciprocal insect adaptations, or more commonly, colonization of host plants is facilitated by generalized metabolic adaptations, in which P450s, esterases, glutathione-S-transferases, and other expressed proteins are orchestrated in complex networks of molecular interactions (Heckel, 2014; Simon et al., 2015). Increasingly, approaches to discovering such adaptations are using gene expression assays to reveal the signatures of metabolic defenses (Vogel, Musser, & Paz Celorio-Mancera, 2014). Below, we use the interactions between milkweeds and their herbivores to illustrate the knowns and the known-unknowns about how insect herbivores persist on highly defended host plants. We then turn to a specialist aphid on milkweed, *Aphis nerii*, to illustrate some of the opportunities for discovery that lie beyond the herbivores that are most commonly studied on milkweed.

Milkweed-herbivore perspectives on insect adaptations to plant secondary metabolites

Insecticides and plant chemical defenses target a common set of post-ingestive pathways in insects, and studies of the resistance to insecticides have provided insights into mechanisms for detoxification (Després et al., 2014; Miresmailli & Isman, 2014). Detoxification mechanisms are canonically divided into three phases: Phase I) introduction of reactive and polar groups into substrates through oxidation, reduction, or hydrolysis; Phase II) conjugation of metabolites with other compounds to create more polar or more easily excretable molecules; Phase III) transport and elimination of compounds.

Canonical phase I enzymes include cytochrome P450s, broad substrate enzymes that catalyze monooxygenase reactions, and choline/ carboxylesterases (CCEs), hydrolases that convert carboxylic esters to more soluble products. Described Phase II detoxification enzymes include glutathione-S-transferases (GSTs), which conjugate substrates with reduced glutathione, and uridine diphosphate (UDP)-glycosyltransferases (UGTs), which help to generate water soluble products that are more easily excreted. Phase III enzymes include ATP-binding cassette (ABC) transporters and products of multidrug resistance proteins such as p-glycoprotein efflux carriers. Through these processes, insects are able to actively protect sensitive tissues and excrete toxins (Chahine & O'Donnell, 2011). In addition to enzymatic modifications and degradation, many species evolve 'target-site modifications' at the binding location of toxins to protect themselves from insecticides or secondary host plant compounds (N. Liu, 2015; Ujvari et al., 2015; Zhen, Aardema, Medina, Schumer, & Andolfatto, 2012). Aside from directly degrading or avoiding secondary metabolites, some insects use host plant toxins for their own benefit and actively sequester, or accumulate and store, host plant toxins, presumably to provide protection from predators or parasites. Remarkably, we know very little about the genes involved in sequestration (Petschenka & Agrawal, 2016). In most cases, insects express multiple adaptations for exploiting host plants, and outside of model systems for studying how insects exploit plants, we know surprisingly little about the diversity of mechanisms insects express, or the synergies and trade-offs involved between multiple traits for exploiting host plants (Després et al., 2014; Simon et al., 2015).

Milkweed plant-herbivore systems, made famous by the unpalatable monarch butterfly (*Danaus* spp.), have been models in chemical ecology and plant-insect coevolution for more than sixty years [reviewed in (A. A. Agrawal, Petschenka, Bingham, Weber, & Rasmann, 2012; Malcolm, 1994)]. Plants in the family Apocynaceae have evolved specialized chemical defenses including cardenolides, which are members of the cardiac glycoside family and are a group of diverse steroids derived from terpenoids (A. A. Agrawal et al., 2012; Dobler et al., 2011). Cardenolides bind to the α-subunit of the Na$^+$/K$^+$ ATPase, disrupting ion translocation that drives secondary transport mechanisms and the generation of action potentials in nerve cells. Variation in cardenolide toxicity derives from differences in total concentrations and in chemical polarity altered by various glycoside groups that attach to the core steroid nucleus and five-membered lactone group

(Horisberger, 2004). While a single milkweed plant species can produce a range of cardenolides, most plants usually favor the production of either nonpolar or polar compounds and avoid midpolarity cardenolides. More toxic species that produce higher concentrations of cardenolides also tend to produce a higher proportion of nonpolar compounds, increasing their diffusion potential across membranes and their toxic effects on herbivores (Rasmann & Agrawal, 2011). Milkweed species vary in other characteristics including the production of latex, trichomes, and phenolics, however, the assignment of plant toxicity based on cardenolide concentration and composition is widely accepted (A. A. Agrawal et al., 2012; A. A. Agrawal, Fishbein, Halitschke, Hastings, et al., 2009a; A. A. Agrawal, Salminen, & Fishbein, 2009b).

Cardenolides are thus remarkably effective against herbivores, and only limited mechanisms of cardenolide tolerance have been described in insects (Fig. 3). Principal among these are specific amino acid substitutions in the Na$^+$/K$^+$ ATPase α-subunit that decrease binding of a polar cardenolide, ouabain. Such 'target site insensitivity', which protects the Na$^+$/K$^+$ pumps from the inhibitory effects of cardenolides, has evolved convergently in diverse insect taxa in the Lepidoptera, Heteroptera, Coleoptera, Diptera, and Hymenoptera (Dobler, Dalla, Wagschal, & Agrawal, 2012; Dobler, Petschenka, Wagschal, & Flacht, 2015; Zhen et al., 2012). However, not all cardenolide adapted insects possess these adaptive mutations. Two lepidopteran species, *Daphnis nerii* (cardenolide adapted) and *Manduca sexta* (not cardenolide adapted, *e.g.* doesn't feed on cardenolide-containing plants), lack the protective Na$^+$/K$^+$ ATPase α-subunit substitutions, and express efflux carrier p-glycoproteins that protect sensitive nerve tissues against cardenolides (Petschenka & Dobler, 2009; Petschenka, Pick, Wagschal, & Dobler, 2013b). Using RNAi and mutant stock lines, multidrug transporters and organic anion transporting polypeptides have also been shown to be important in protecting *Drosophila melanogaster* from dietary cardenolides (Groen et al., 2017; Torrie et al., 2004). Analyses comparing cardenolide uptake and composition across different tissues in various cardenolide adapted insect species suggest both passive and selective uptake models of both nonpolar and polar cardenolide compounds (Brower, McEvoy, Williamson, & Flannery, 1972; Detzel & Wink, 1995; Duffey, Blum, & Isman, 1978; Frick & Wink, 1995; Nickisch-Rosenegk, Detzel, Wink, & Schneider, 1990; Scudder & Meredith, 1982; Yoder, Leonard, & Lerner, 1976). Moreover, even in species with target site insensitivity, cardenolide metabolism and sequestration require Phase I-III detoxification mechanisms, but the molecular mechanisms underlying these processes have not yet been fully described (Dobler et al., 2011; Petschenka & Agrawal, 2016).
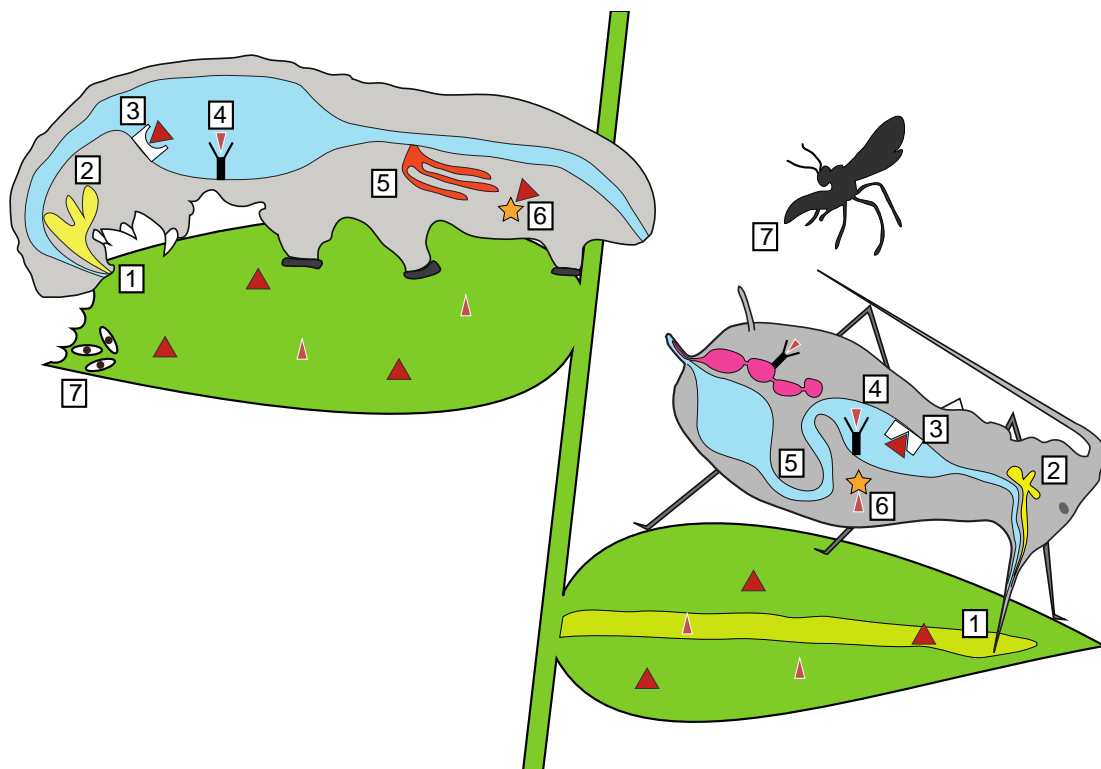
Figure 3. Comparison of the ways in which different milkweed specialists interact with milkweed cardenolides. Monarch larva, *D. plexippus* (left) and the milkweed aphid, *A. nerii* (right); cardenolides are represented as red triangles (polar cardenolides as darker equilateral triangles, nonpolar cardenolides as lighter isosceles triangles). Monarch larvae feed on milkweed plant tissues through five larval stages. *A. nerii* feed on plant phloem through the entirety of their life. *1. Behavior. Host plant choice.* All insects use chemosensation (olfaction and taste) to locate appropriate host plants (Simon et al., 2015), but the perception of cardenolides by insects is unclear (A. A. Agrawal et al., 2012). Adult monarchs tend to oviposit on low to moderate cardenolide host plants (Oyeyele & Zalucki, 1990; Zalucki, Brower, & Malcolm, 1990), likely to balance the need for beneficial sequestration and the cost of cardenolide toxicity (A. A. Agrawal et al., 2012; Malcolm, 1994). *Host plant feeding*. Monarch larvae cut distal veins prior to feeding to avoid the latex produced by milkweeds (Dussourd & Eisner, 1987; Zalucki, Brower, & Alonso M, 2001). *A. nerii* feed on sap from phloem sieve tubes they access with minimal penetration of other cells, *e.g.* epidermis, parenchyma tissues, and like other aphids, are stealthy feeders (Botha, Evert, & Walmsley, 1975a; Botha, Malcolm, & Evert, 1977). *2. Salivary gland components.* Oral secretions and salivary components provide important early important defenses and aid in host plant adaptation (Simon et al., 2015). Salivary oral effectors that suppress the production of plant defenses have been identified in other lepidopterans (Musser et al., 2002; Schmelz et al., 2012), but given the induction of cardenolides after monarch feeding (Rasmann & Agrawal, 2011; Rasmann, Agrawal, Cook, & Erwin, 2009), monarch salivary components are unlikely to directly suppress cardenolides. Aphid saliva contains proteins that inactivate plant phytochemicals and prevent plant defenses that deter aphid feeding (Elzinga, de Vos, & Jander, 2014; Miles, 1999; Will, Tjallingii, Thönnessen, & van Bel, 2007). *A. nerii* cause no change or even a reduction in plant cardenolides (Mooney, Jones, & Agrawal, 2008; Zehnder & Hunter, 2007b). *3. Target site modifications.* Monarchs have evolved amino acid substitutions in their Na^+/K^+ ATPases at the binding site of cardenolides which provide protection from toxicity (Dobler et al., 2012). *A. nerii* do not possess protective substitutions (Zhen et al., 2012), and must rely on cardenolide modification, degradation, transport, and excretion. *4. The insect gut as a barrier.* Milkweed plants produce a diversity of cardenolides that range in polarity, and more nonpolar cardenolides are more toxic as they can passively cross membranes (A. A. Agrawal et al., 2012). Thus, the insect gut can provide a barrier against cardenolide toxicity by preventing movement of nonpolar compounds. Efflux carriers prevent nonpolar cardenolide movement to sensitive neural tissues in lepidopteran species, and may act in gut tissues as well (Petschenka, Pick, Wagschal, & Dobler, 2013b). Efflux carriers or other proteins are also likely important in *A. nerii* gut tissues or other sensitive tissues such as developing embryos (shown in pink). *5. Detoxification.* Genes involved in cardenolide degradation or detoxification have not been described in cardenolide-adapted insects (A. A. Agrawal et al., 2012). Reduction in cardenolide toxicity can occur via disruption or modification of the cardenolide binding site through hydrolysis, oxidation, or reduction. Conjugation of cardenolides with other substrates may also increase cardenolide polarity and aid in transport and excretion. Like all lepidopterans, monarchs possess Malpighian tubules which likely play a primary role in cardenolide detoxification. In contrast, *A.nerii* lack Malpighian tubules, and thus, their gut tissues may play important roles in detoxification (Jing, White, Yang, & Douglas, 2015). *6. Sequestration.* Both monarchs and *A. nerii* are aposematic and sequester cardenolides. Monarch larvae actively sequester polar cardenolides into their integument (Frick & Wink, 1995; Malcolm, Cockrell, & Brower, 1989; Seiber, Tuskes, Brower, & Nelson, 1980). In contrast to active sequestration in monarchs, *A. nerii* are thought to passively sequester cardenolides. *A. nerii* retain a subset of primarily nonpolar cardenolides from their host plants (Malcolm, 1990; Züst & Agrawal, 2015), but may modify cardenolides post-ingestion. *7. Interactions with higher trophic levels.* Insects are thought to feed on toxic host plants because of the protection they gain from parasites or predators. Parasites may be inhibited directly by cardenolides or indirectly by cardenolide detoxification or metabolic by-products. Further, cardenolides may alter immune processes, affecting the outcome of infection. Monarchs on host plants with moderate levels of increased cardenolides have increased longevity and decreased spore loads when infected with a protozoan parasite (Gowler, Leon, Hunter, & de Roode, 2015; Sternberg et al., 2012). In aphids, host plant species has an effect on parasitoid mummy formation and adult emergence (Colvin & Yeargan, 2013; Helms, Connelly, & Hunter, 2004), but the direct and/ or indirect effect of cardenolides on aphid parasitoids or aphid immunity have not been investigated. As with

monarchs, the basis of host-plant mediated protection of *A. nerii* against parasitoids remains uncharacterized. (Figure adapted from (Simon et al., 2015).)

Evidence of coevolution between milkweed plant hosts and herbivores

Milkweed-herbivore systems have been identified as models of coevolution for more than sixty years (Ehrlich & Raven, 1964), yet evidence of reciprocal, escalatory changes between plant and herbivore partners remains scarce (Futuyma & Agrawal, 2009). Coevolution can be pairwise or diffuse, acting through multiple community members or populations, and requires evolutionary change in at least two interacting populations (Janzen, 1980). Milkweed plants produce an array of defenses including cardenolides, latex, trichomes, and phenolics. One prediction about the coevolution of milkweeds and herbivores is that milkweed defenses have escalated in response to herbivory over evolutionary time, such that more derived taxa exhibit greater defenses (Agrawal & Fishbein 2008). However, phylogenetic analyses revealed that only phenolic production increases with *Asclepias* diversification (A. A. Agrawal, Salminen, & Fishbein, 2009b), and, in general, milkweed defenses decrease as species diversify (A. A. Agrawal, Fishbein, Halitschke, Hastings, et al., 2009a). As for herbivores, there is evidence of convergent evolution with diverse insects evolving identical modes of target site insensitivity although this alone is not evidence of coevolution (Dobler et al., 2012; Zhen et al., 2012). The best herbivore example of milkweed plant-herbivore coevolution is found in Danaini butterflies which demonstrate stepwise evolution of increasingly resistant Na^+/K^+ ATPases (Petschenka, Fandrich, Sander, Wagschal, Boppré, et al., 2013a), but evidence of coevolutionary changes in other herbivore species is lacking.

While a reduction in defenses over the course of milkweed adaptive radiation may seem paradoxical in a story of coevolution, Agrawal et al. (2009) suggest that this reduction in defensive investment accompanied by increased herbivory tolerance may be driven by the successful colonization of milkweed plants by specialist insects (A. A. Agrawal & Fishbein, 2008). In addition to variation in investment towards constitutive defenses, milkweed species can vary in their herbivore-induced defenses. Moreover, the induction of cardenolides varies between herbivore species, with most leaf chewing herbivores eliciting increased cardenolide production. However, sap-sucking herbivores, such as aphids, appear to have minimal or density-dependent effects on cardenolide production, all of which are milkweed species specific (A. A. Agrawal et al., 2012). Overall, if coevolution has acted on milkweed species and their community of herbivores, it has likely been diffuse, acting through many herbivore species. Furthermore, interactions between toxic host plants and herbivores are typically connected to higher trophic levels, *e.g.* parasitoids or predators, and the coevolutionary history of plants and their insects is undoubtedly impacted by these factors (Petschenka & Agrawal, 2016).

Ecological consequences of detoxification and sequestration

The mechanisms insects use to detoxify and sequester toxins from host plants, as described above, are generally assumed to be costly. These costs may be manifested in the form of trade-offs impacting behavior, reproduction, survival, or immunity (Després, David, & Gallet, 2007; Schwenke, Lazzaro, & Wolfner, 2016; Stearns, 1989). Costs of insecticide resistance, such as reduced survival, fecundity, or energy reserves, have been demonstrated in several insect species (Carrière, Deland, Roff, & Vincent, 1994; J. R. Gordon, Potter, & Haynes, 2015; Rivero, Magaud, Nicot, & Vézilier, 2011). However, insecticide resistance has also been associated with increases in fitness, evidenced by increased immunity against pathogens or decreased generation time (Agnew, Berticat, Bedhomme, & Sidobre, 2004; J. R. Gordon et al., 2015). Likewise, costs and benefits should be expected from insect resistance to secondary metabolites, but the direction and magnitude of the effect is dependent on the ecological context and the specific mechanism of resistance employed (Després et al., 2007).

It has long been assumed that specialist insects incur the potential costs of feeding on toxic plants for the benefits gained under certain ecological conditions, such as exposure to parasites or predators, yet, we are only beginning to unravel the mechanisms underlying these complex interactions (Forister et al., 2012). Studies investigating cardenolide-adapted insects have demonstrated the unpalatability and toxic effects of monarch

butterflies (*Danaus plexippus*) to bird predators (Brower & Moffitt, 1974; van Zandt Brower, 1958), milkweed bugs (*Oncopeltus fasciatus*) to mantid predators (Berenbaum & Miliczky, 1984; Paradise & Stamp, 1991), and milkweed aphids (*Aphis nerii*) to spiders (Malcolm, 1989; Petschenka, Bramer, Pankoke, & Dobler, 2011), however, monarch larvae and milkweed aphids are predated upon by a diversity of insects in the field (A. A. Agrawal et al., 2012). Parasitoids have reduced emergence from cardenolide-adapted aphids as compared to other aphid species (Desneux, Barta, Hoelmer, Hopper, & Heimpel, 2009) and milkweed host plant species, presumably driven by differences in cardenolides, has an effect on parasitoid success in milkweed aphids (Colvin & Yeargan, 2013), but the basis of cardenolide protection against parasitoids is unknown. While milkweed insects appear to be partially protected from generalist predators by cardenolides, predators can exhibit feeding behaviors to reduce milkweed insect toxicity (Mebs, Wunder, Pogoda, & Toennes, 2017; Rafter, Agrawal, & Preisser, 2013), and protection by cardenolides may not extend to specialist predators or parasites (Ode, 2006).

The most extensive studies of cardenolide protection against higher trophic levels have been done investigating the effects of milkweed species and cardenolides on an obligate protozoan parasite (*Ophryocystis elektroscirrha*) of monarchs (de Roode, Pedersen, Hunter, & Altizer, 2008; Gowler et al., 2015; Myers & Cory, 2015; Sternberg et al., 2012). Host plants with intermediate levels of cardenolide provide the highest levels of fitness for infected monarch larvae and adults (de Roode et al., 2008; Sternberg et al., 2012), and infected adult butterflies can preferentially lay eggs on higher cardenolide plants providing protection to their offspring (Lefèvre, Oliver, Hunter, & de Roode, 2010; Lefévre et al., 2011). Milkweed host plants vary in other characteristics besides cardenolides (A. A. Agrawal & Fishbein, 2006), but the direct manipulation of dietary cardenolides also demonstrates a relationship between increased cardenolides and increased fitness of infected larvae (Gowler et al., 2015). Parasite spores are transmitted when they are ingested by feeding larvae and parasites may be directly impacted by dietary cardenolides in the gut, however, parasites penetrate the gut wall, replicate in hypodermal cells, and eventually mature in the hemolymph where cardenolides are transient (de Roode et al., 2008; Frick & Wink, 1995). While these studies suggest a role of cardenolides in protecting monarchs against infection, the mechanistic basis of cardenolide protection in monarchs and the effects of cardenolides on insect immunity are unknown.

Aphids and milkweeds- values of the system

Aphids are plant phloem-feeding insects of the order Hemiptera and have become model systems for studies of insect adaptations towards and specialization on host plant species (Kamphuis, Zulak, Gao, Anderson, & Singh, 2013; Knolhoff & Heckel, 2014). *Aphis nerii* Boyer de Fonscolombe, milkweed-oleander aphids, commonly feed on toxic oleander and milkweed plant species. They are aposematic (brightly colored), which is a common indication of sequestration and a trait unique to *A. nerii* amongst milkweed specialist aphids, and are thought to be obligately parthenogenic (J. S. Harrison & Mondor, 2011). While other aphid species are also specialized on milkweed (*A. asclepiadis* and *Myzocallis asclepiadis*), previous studies have found that *A. nerii* outcompete these species on shared host plants, suggesting that *A. nerii* possess superior mechanisms to circumvent host plant toxicity (R. A. Smith, Mooney, & Agrawal, 2008; Züst & Agrawal, 2015).

Despite *A. nerii*'s ability to thrive on milkweed and oleander in the United States (Hall & Ehler, 1980; R. A. Smith et al., 2008), there is evidence that milkweed plant species can have an impact on *A. nerii* life history traits (Zehnder & Hunter, 2007a). *A. nerii* possess sensitive Na^+/K^+ ATPases and are thought to passively sequester cardenolides (Zhen et al., 2012; Züst & Agrawal, 2015). Thus, they likely possess a different set of adaptations for cardenolide tolerance than the better-studied lepidopteran species (Fig. 3). Moreover, aphids are phloem feeders and hemimetabolous, unlike the leaf chewing holometabolous Lepidoptera, and feed on their host plant for the entirety of their lifetime. Furthermore, aphids lack Malpighian tubules, the primary insect organ involved in excretion, osmoregulation, and immunity. A recent study comparing gene expression between pea aphid (*Acyrthosiphon pisum*) gut tissues and *Drosophila melanogaster* Malpighian tubules found that over 50% of the genes expressed in *D. melanogaster* Malpighian tubules had expressed homologues in the aphid guts, especially those involved in metabolic processes (Jing et al., 2015).

This suggests that the aphid gut may serve additional functions compared to other insect guts, especially in processes involving metabolism and excretion. Thus, *A. nerii* is an ideal, unique model to investigate questions of novel insect adaptations to host plant secondary compounds and the benefits and costs associated with host plant specialization.

Milkweed-herbivore systems are models for sequestration and tritrophic interactions (Petschenka & Agrawal, 2016), and aphids have been well studied for their interactions with parasitoids (Stary 1970). In support of toxic milkweed plants providing protection from higher tropic levels, previous studies demonstrated reduced performance of generalist predators and parasitoids towards *A. nerii* compared to other aphids that do not sequester plant toxins (Desneux et al., 2009; Omkar & Mishra, 2005). Furthermore, milkweed plant species can have an impact on *A. nerii* interactions with parasitoids suggesting that there may be a direct or indirect effect of cardenolides on parasitoids (Colvin & Yeargan, 2013; Helms et al., 2004), but these studies used host plants with relatively low cardenolide concentrations. Thus, the effects of cardenolides on aphid parasitoids or immunity remain uncharacterized.

Previous studies indicate unknown mechanisms of passive sequestration in *A. nerii*; they have shown that *A. nerii* tend to retain nonpolar cardenolides from host plants and metabolize cardenolides post-ingestion (Malcolm, 1990; Züst & Agrawal, 2015). The benefits associated with cardenolides are dependent on many factors, *e.g.* host plant species or the presence of predators or parasites, and cardenolide tolerance and sequestration mechanisms are likely to be costly, either in the form of life history trade-offs, *e.g.* slower development, reduced fecundity, or decreased survival, or reduced capacity to mount an immune response (Després et al., 2007; Lindstedt, Talsma, Ihalainen, Lindström, & Mappes, 2010; Schwenke et al., 2016; Smilanich, Dyer, Chambers, & Bowers, 2009; Stearns, 1989). However, clear examples of trade-offs in detoxification, life history traits, and immunity and descriptions of genes contributing to these trade-offs are lacking. While there are many hypotheses for mechanisms of cardenolide tolerance and sequestration in *A. nerii*, future genomic and transcriptomic studies are needed to identify the genes responsible for *A. nerii*'s mastery of milkweed plants.

How do aphid generalists and specialists differ in their interactions with cardenolides?

Most herbivorous insects specialize on one or a small group of plants (monophagous, or various degrees of oligophagous), but some are generalists (polyphagous). The remarkable diversity of insect herbivores is thought to derive from how diet breadth interacts with diversification, with differences in plant phytochemicals acting as some of the primary drivers in insect specialization. Coevolution and trade-offs in defenses are leading hypotheses explaining why insects specialize to narrow host plant ranges, but the mechanisms that link diet breadth to ecological specialization are only beginning to be understood (Forister et al., 2012).

The ability of a range of generalist to specialist aphid species to feed on milkweed plants provides a unique opportunity to investigate several leading questions. A recent study examined aphid growth and cardenolide sequestration in four aphid species [*Myzus persicae* (polyphagous generalist, >400 species from 40 families), *A. nerii* (oligophagous, broad specialist, >50 species from 13 families), *A. asclepiadis* (oligophagous, narrow specialist, <10 hosts from one family), *Myzocallis asclepiadis* (monophagous specialist)] feeding on *A. syriaca*, a relatively low cardenolide host plant (Züst & Agrawal, 2015). When feeding on *A. syriaca*, *A. nerii* had the highest growth rate, *M. persicae* grew the second most rapidly, then *A. asclepiadis*, and *M. asclepiadis*, the most specialized species, grew most slowly. All aphid species excreted a range of cardenolides, and the two *Aphis* species had the highest concentration of cardenolides in their excretions. The amount of cardenolides sequestered generally increased with increasing dietary specialization, and all aphid species retained a subset of just three cardenolides, with the exception of *A. nerii*. *A. nerii* bodies also contained a polar cardenolide that was not present in host plant leaves, indicative of post-ingestion modification of cardenolides. Notably, *A. nerii* is the only aposematic species of the four studied.

The differing growth rates and levels of cardenolide sequestration in aphids (Züst & Agrawal, 2015) indicate differing mechanisms for cardenolide detoxification and tolerance, however, these processes have not

been investigated in aphids. *A. nerii*'s sensitive Na$^+$/K$^+$ ATPase sequence is identical to that of the pea aphid (Zhen et al., 2012), but the Na$^+$/K$^+$ ATPase sequences of other aphid species have not been identified. Comparative genomic and transcriptomic studies are necessary to identify the convergent and divergent processes employed by aphids to feed on toxic milkweed plants.

Summary

　　Observations of host plant secondary chemistry-herbivore interactions have provided the biological framework of coevolutionary theory for over half a century (Ehrlich & Raven, 1964). However, even in the current "-omics" age, we still have not identified the molecular processes underlying many canonical coevolutionary paradigms. Coevolution implies specialization, but we do not have a comprehensive understanding of the molecular mechanisms governing highly specialized species interactions. Furthermore, specialization assumes trade-offs with other processes, however, we still do not know specifically how tolerance, detoxification, and sequestration processes interact with molecular processes involved in development and immunity. The identification of novel molecular mechanisms herbivores use to tolerate, detoxify, and sequester host plant compounds will provide important missing information in the fields of evolution and ecology (Després et al., 2007; Heckel, 2014; Simon et al., 2015). Moreover, studying adaptations in divergent taxa such as hemipterans and lepidopterans will allow the identification of convergent and divergent processes involved in solving the problem of feeding on toxic plant secondary metabolites. Further, many aphid species are common agricultural pests and have evolved mechanisms of resistance to insecticides, and an understanding of how aphids detoxify host plant compounds may facilitate better predictions about when evolution to xenobiotics should be expected (Bass et al., 2014; Russell et al., 2011).

EFFICIENT METHODS FOR MAINTAINING BIOLOGICAL CULTURES AND MEASURING GENE EXPRESSION IN APHIS NERII: A NON-MODEL SYSTEM FOR PLANT-INSECT INTERACTIONS

Abstract

Aphids are excellent experimental models for a variety of biological questions ranging from the evolution of symbioses and the development of polyphenisms to questions surrounding insect's interactions with their host plants. Genomic resources are available for a number of aphid species, and with advances in the next-generation sequencing, transcriptomic studies are being extended to non-model organisms that lack published genomes. Furthermore, aphid cultures can be collected from the field and reared in the laboratory for the use in organismal and molecular experiments to bridge the gap between ecological and genetic studies. Last, many aphids are able to be maintained in the laboratory on their preferred host plants in perpetual, parthenogenic life cycles allowing for comparisons of asexually reproducing genotypes. *Aphis nerii*, the milkweed-oleander aphid, provides one such model to study insect interactions with toxic plants using both organismal and molecular experiments. Methods for the generation and maintenance of plant and aphid cultures in the greenhouse and laboratory, DNA and RNA extractions, microsatellite analysis, *de novo* transcriptome assembly and annotation, transcriptome differential expression analysis, and qPCR verification of differentially expressed genes are outlined and discussed here.

Introduction

Aphids are small, hemimetabolous insects that specialize on diverse plant families worldwide. They are distinctive for a number of features, most notably their complex life cycles involving cyclical parthenogenesis and discrete polyphenisms, and their obligate nutritional symbioses with bacterial or yeast endosymbionts that supply nutrients missing from their diet of plant sap (Brisson & Stern, 2006). While most aphids are host plant specialists, some generalist species are important crop pests, inflicting considerable economic damage on crops either directly or via the pathogens and viruses they vector (Dixon, 1985). The publication of the first aphid genome in 2010, the pea aphid *Acyrthosiphon pisum* (Consortium, 2010), marked an important milestone in the study of aphid biology, because it provided the genomic resources for addressing questions about insect adaptations to herbivorous lifestyles, including those that might lead to better control strategies (Srinivasan & Brisson, 2012). Since that time, additional genomic resources have accumulated with the publication of an annotated genome for the soybean aphid *Aphis glycines* (Wenger et al., 2017), and publicly-available whole genome resources for another three-aphid species (*Myzus cerasi* (black cherry aphid), *Myzus persicae* (peach-potato aphid), *Rhopalosiphum padi* (bird cherry-oat aphid) (bipaa.genouest.org/is/aphidbase/). Valuable *de novo* transcriptomic resources are available as well for a number of other aphid species (*e.g. Aphis gossypii (*cotton aphid) (Zhao-Qun Li et al., 2013), *Sitobion avenae* (grain aphid) (D. Wang, Liu, Jones, Bruce, & Xia, 2014), *Cinara pinitabulaeformis* (pine aphid) (S. Wu et al., 2017), *Aphis nerii* (milkweed-oleander aphid) (Birnbaum et al., 2017)).

Aphids have also made lasting contributions to our understanding of plant-insect interactions and the ecology of life on plants (Dixon, 2005). One area where aphids have made particularly important contributions is in our understanding of the chemical ecology of host plant interactions. Herbivorous insects express diverse adaptations for overcoming plant defenses, and some even co-opt plant defenses for their own benefit (Goggin, 2007; Webster, 2012; Will, Furch, & Zimmermann, 2013). For example, the milkweed-oleander aphid, *Aphis nerii*, is a bright yellow, invasive aphid found in temperate and tropical regions worldwide that specializes on plants in the milkweed family (Apocynaceae). Plants in the family Apocynaceae have evolved diverse chemical defenses, including milky latex and cardiac glycosides known as cardenolides, that bind the cation carrier Na,K-ATPase and are effective deterrents to generalist herbivores (A. A. Agrawal et al., 2012; Dobler et al., 2011). Milkweed specialists express various modes of resistance to cardenolides, and some selectively or passively accumulate or modify cardenolides in their tissues as a means to deter predation or for other benefits (Opitz & Müller, 2009). *A. nerii* is thought to sequester cardenolides in this way, although the mechanisms and functional benefits remain unclear (Birnbaum et al., 2017; Birnbaum & Abbot, 2018).

Given the genomic resources at hand, *A. nerii* provides an excellent experimental model for the study of the molecular and genetic mechanisms involved in the chemo-ecological interactions between toxic host plants and their specialist herbivores. It is worth noting that, while some of the earliest studies of *A. nerii* focused on sequestration of cardenolides (Rothschild, Euw, & Reichstein, 1970), since that time, studies of *A. nerii* have provided insights into a broad set of evolutionary and ecological questions, including the genetic structure of invasive insects (J. S. Harrison & Mondor, 2011) and the interplay between bottom-up and top-down regulation on herbivore density (Mooney, Halitschke, Kessler, & Agrawal, 2010). *A. nerii* is thus a good candidate as an experimental model for an especially broad set of studies of insect-plant interactions. Critical to the success of any study with *A. nerii* is careful culture of aphid populations, which includes culture of plants on which the aphids depend, as well as efficient generation of high-quality -omic data. Our goal is to guide the reader through both. Outlined below are step-by-step methods for the generation and maintenance of plant and aphid cultures in the greenhouse and laboratory, DNA and RNA extractions, microsatellite analysis, *de novo* transcriptome assembly and annotation, transcriptome differential expression analysis, and qPCR verification of differentially expressed genes. While these methods are written for *A. nerii*, the general culturing, extraction, and analysis methods can extend to variety of aphid species.

Protocol

Plant cultures (Protocol 1)

1.1. Purchase seeds from any commercial vendor or collect from mature plants in the field.

Note: This protocol is suitable for most commercially available milkweed species (*e.g., Asclepias incarnata*, *A. syriaca*, *A. curassavica*, *Gomphocarpus physocarpus*). Some seeds may need to be cold-stratified, and instructions from the seed supplier should be checked.

1.2. Plant seeds in a fine germinating soil (60-70% fine peat moss, perlite, vermiculite, limestone).

1.2.1. Fill a standard seedling tray with germination mix soil; ensuring that the soil reaches the top of the wells. In each well, make an indentation to create a hole in the soil about a 3 cm deep.

1.2.2. Place one seed in each hole and water very well with a watering can such that the soil covers the seeds and is saturated.

1.2.3. Grow seeds in a greenhouse (see conditions below, 1.5).

1.2.4. Water regularly, daily to every-other-day; enough to maintain moderate soil moisture.

1.3. When the seedlings have grown their first set of full leaves, repot seedlings in a general potting mix (50-60% peat moss, bark, and limestone) (Fig. 4A).

1.3.1. Use 4-inch round pots that fit with a tight seal with the cup cages (see below). Fill with general potting soil

up to about 5 cm below the rim.

1.4.2. Create a hole in the soil deep enough to reach the bottom of the pot.

1.4.3. With your hand, gently scoop the mature seedling from its well and place it deep inside the hole in the 4-inch pot. Cover the seedling with soil. Water very well.

1.4.4. Grow plants in the greenhouse and water regularly, daily to every other day; enough to maintain moderate soil moisture.

1.5. Greenhouse conditions.

1.5.1. Set greenhouse thermostats to maintain daytime temperatures between 18-28 °C and nighttime temperatures between 16-22 °C using the manufacturer's instructions.

1.5.2. During winter months when the days are shorter, supplement daylight with 600 W high pressure sodium bulbs (12 hr, 8am-8pm).

1.6. Control unwanted pests (*e.g.* thrips, aphids) with a foliar organic soap solution, however, use these products with caution.

1.6.1. Make the soap solution according to the manufacturer's recommendation and apply using a spray bottle.

1.6.2. Leave the soap on the plants for 4-24 h. Gently rinse the plants with water to remove the soap 4-24 h post-application and rinse them with water a second time prior to use with laboratory aphid cultures.

1.7. Culture the average aphid population on plants that have grown at least 3-4 sets of full leaves and are at least 10 cm tall (Fig. 4B).
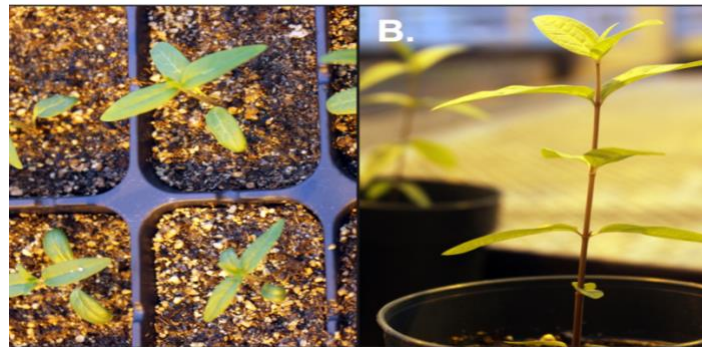
Figure 4. Representative plants for aphid cultures. **A.** Seedlings can be repotted after they have developed their first full set of true leaves. **B.** Plants can be used for aphid cultures when they have developed 3-4 sets of true leaves.

Aphid cultures (Protocol 2)

2.1. Start laboratory aphid populations from an existing lab isoclonal population or start from the field-collected aphids following the directions below.

2.1.1. When starting a laboratory population from an existing lab isoclonal population, transfer aphids as described in 2.3.1-2.3.3.

2.2. When starting new isoclonal, field-collected aphid populations, place a single, reproducing, adult aphid on a suitable host plant as maintained in Step 1.7.

Note: Populations may be started from winged (alate) or unwinged (apterous) adults (Figs. 6A, C).

2.2.1. Manually inspect plants from the greenhouse for unwanted pests prior to use with laboratory aphids. Freeze any plants with unwanted aphids. If desired, thrips or other pests can be removed with an ethanol vacuum flask.

Note: Be sure to rinse plants that have been treated with soap prior to use as described in Step 1.6.2.

2.2.2. Safely transfer a single adult aphid using a paintbrush or a mouth pipette created with 3/16" ID x 1/4" OD plastic tubing, a 1,000 µL pipette tip, and a 2 µL pipette tip (Fig. 5A).

2.2.3. Securely cover plants with aphids with a cup cage created with a plastic cup with the top cut off and covered with a fine mesh and secured with tape (Fig. 5B).

2.2.4. Place aphid-infested plants in a tray and keep in an environmental chamber (16L:8D, 22 °C, 70% humidity).

2.3. To maintain stock populations, transfer aphids to fresh, new plants weekly (2.2.1-2.2.3).

2.3.1. Safely transfer 1-3 2nd or 3rd instar nymphs and 1 adult-aged aphids using a mouth pipette (Figures 2A, 3).

Note: Stocks are best maintained by transferring unwinged individuals.

2.3.2. Securely cover aphid-infested plants with a cup cage created with a plastic cup with the top cut off and covered with a fine mesh and secured with tape.

2.3.3. Place plants in a tray and keep aphids in an environmental chamber (16L:8D, 22 °C, 70% humidity).

2.3.4. Alternatively, if desired and if the host plant is of decent quality, use an ethanol vacuum flask to reduce populations leaving only one reproducing adult and two to three 2nd or 3rd instar nymphs.

2.4. To create same age populations for use in experiments, place up to five adults (preferably unwinged) from the stock population onto a new host plant.

2.4.1. Remove the adults 24 h later.

2.4.2. About 5-7 days later, once the F_1 offspring have matured to adulthood, place up to five unwinged F_1 adults on a new host plant. Remove the adults 24 hours later.

2.4.3. Once the F_2 population has matured to adulthood, this population is ready to be used in experiments. This process ensures that the experimental population is roughly the same age and are born of roughly same age mothers.

2.5. Genotypic differences between field-caught isoclonal lines should be confirmed using microsatellite genotyping (described below, Sections 3 & 4).
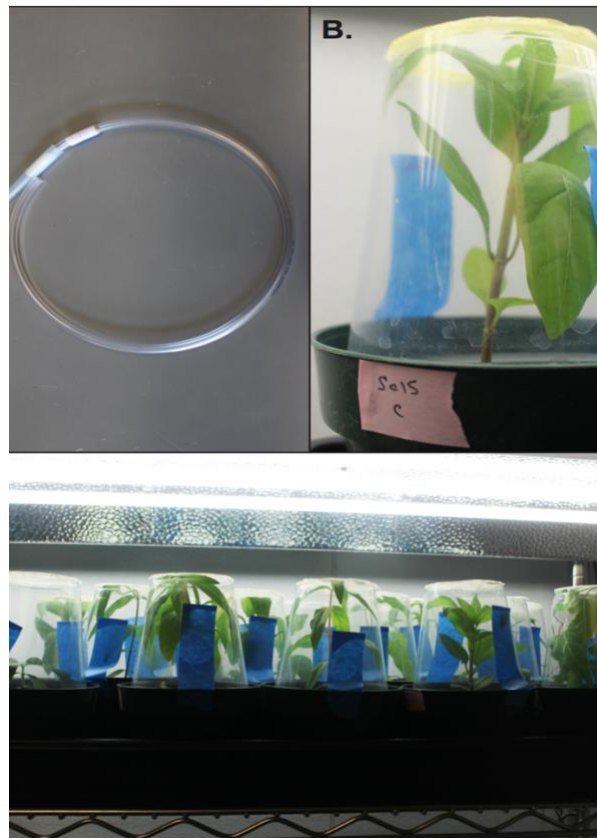
Figure 5. Examples of tools used for culturing aphids. **A.** Mouth pipettes can be created using 3/16" ID x 1/4" OD plastic tubing, a 1000 µL pipette tip, and a 200 µL pipette tip. **B, C.** Use cup cages (clear Solo cup with top cut off and secured with fine mesh) to securely fit over the top of 4 in. pots used for aphid cultures. This allows for ample light and ventilation to create a suitable environment for the aphids and plant, and keeps the aphids contained.

DNA extraction (Protocol 3)

3.1. Preparation.

3.1.1. Use sterile techniques to prepare 1 L lysis buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris (pH 9.1), 0.05 M EDTA, 0.05% SDS).

3.1.2. Warm the heating block or water bath to 65 °C.

3.2. Tissue homogenization and lysis.

3.2.1. Place aphid near bottom of a sterile, 1.5 mL microcentrifuge tube.

3.2.2. Place sterile pestle in the tube with aphid and immerse the bottom of the tube in liquid nitrogen.

Note: Optimal tissue disintegration is achieved when the aphid is positioned between the pestle and side of the tube.

3.2.3. Grind aphid with pestle to initially lyse cells.

3.2.4. For a single adult aphid, use 200 µL (split into 2 x 100 µL aliquots) of lysis buffer. Add first aliquot to grind and resuspend crushed aphid until sample is visibly disintegrated, then use the second aliquot to wash off pestle.

3.2.5. Incubate the crushed aphids in lysis buffer at 65 °C in water bath or heat block for 30 min.

3.3. DNA precipitation.

3.3.1. While the tube is warm, add 14 μL of 8 M KOAc. Invert tube to mix.

3.3.2. Store sample on ice for 30 min.

Note: The protocol can be paused here, and samples can be stored at -20 °C for up to 24 hours.

3.3.3. Centrifuge at 13,000 xg for 15 minutes at room temperature.

3.3.4. Transfer supernatant to new 1.5 ml tube with a pipette. BE CAREFUL not to remove any of the pelleted debris.

3.3.5. To improve DNA pellet visualization, add 2 μL glycogen (20 μg/ml) to the supernatant. This step may be omitted for larger samples.

3.3.6. Add 200 μL of cold 100% molecular grade ethanol to the supernatant. Invert tubes to mix and leave at room temperature for at least 15 minutes.

Note: The protocol can be paused here, and samples can be stored at -20 °C for up to 24 hours.

3.3.7. Centrifuge at 13,000 xg for 15 minutes at room temperature.

3.3.8. Remove ethanol by pipetting.

3.4. DNA wash and elution.

3.4.1. Add 200 μL of cold 70% molecular grade ethanol and flick the tube to resuspend and wash the pellet.

3.4.2. Centrifuge at 13,000 x g for 5 minutes.

3.4.3. While visualizing the pellet, carefully remove ethanol by pipetting and add 200 μL of cold 100% molecular grade ethanol.

3.4.4. Centrifuge at 13,000 x g for 5 minutes.

3.4.5. While visualizing the pellet, carefully remove ethanol by pipetting.

Note: Repeat the 100-70-100 ethanol wash if necessary.

3.4.6. Air dry the pellets for 5-10 minutes with tube laying horizontal and open on a Kimwipe™.

3.4.7. Resuspend DNA pellet in 80 μL of low TE (10 mM Tris-HCl, 0.1 mM EDTA).

3.4.8. Quantify resuspended DNA using a spectrophotometer.

3.4.9. Store at 4 °C.

Microsatellite PCR and sequencing for aphid genotyping (Protocol 4)

4.1. Order the appropriate F and R primers for microsatellite sequencing (Table 2 (J. S. Harrison & Mondor, 2011)).

Note: Reverse primer sequences should be modified with 5'-6-FAM or 5'-5-HEX fluorescent labels to allow for multiplexed samples for microsatellite sequencing.

4.2. Perform PCR with single aphid DNA samples (described in Section 3) and fluorescently labeled microsatellite primers.

4.2.1. Mix PCR reactions according to the manufacturer's protocol (0.2 μM each F/R primer, 2.5 mM $MgCl_2$, 50-200 ng DNA template).

4.2.2. Use the following thermocycler settings: initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 30 sec, 58 °C for 35 sec, 72 °C for 45 sec, and a final elongation step at 72 °C for 10 min.

4.3. PCR samples with different fluorescent tags can be combined to reduce the number of samples sequenced, and samples can be microsatellite sequenced at a genotyping facility.

4.4. Analyze the .fsa raw sample files using microsatellite analysis software.

Primer Name	Direction	Sequence (5'-3')
Ago24_F	forward	TTTTCCCGGCACACCGAGT
Ago24_R	reverse	GCCAAACTTTACACCCCGC
Ago 53_F	forward	TGACGAACGTGGTTAGTCGT
Ago 53_R	reverse	GGCATAACGTCCTAGTCACA
Ago 59_F	forward	GCGAGTGGTATTCGCTTAGT
Ago 59_R	reverse	GTTACCCTCGACGATTGCGT
Ago 66_F	forward	TCGGTTTGGCAACGTCGGGC
Ago 66_R	reverse	GACTAGGGAGATGCCGGCGA
Ago 69_F	forward	CGACTCAGCCCCGAGATTT
Ago 69_R	reverse	ATACAAGCAAACATAGACGGAA
Ago 84_F	forward	GACAGTGGTGAGGTTTCAA
Ago 84_R	reverse	ACTGGCGTTACCTTGTCTA
Ago 89_F	forward	GAACAGTGCTCGCAGTCTAT
Ago 89_R	reverse	GACAGCGTAAACATCGCGGT
Ago 126_F	forward	GGTACATTCGTGTCGATTT
Ago 126_R	reverse	TAAACGAAAAAACCACGTAC

Table 2. Microsatellite primer sequences used to genotype *Aphis nerii* (J. S. Harrison & Mondor, 2011).

RNA extraction for RNAseq (Protocol 5)

5.1. Collect aphid samples for RNA extraction in 1.5 ml RNase/ DNase-free tubes and immediate freeze in liquid nitrogen.

Note: If the following steps are not performed immediately, the samples can be stored at -80 °C.

5.2. Tissue homogenization.

5.2.1. With a sterile pestle in tube with aphid, freeze in liquid nitrogen for 10-15 seconds, until the sample stops sizzling.

Note: Optimal tissue disintegration is achieved when the aphid is positioned between the pestle and side of the tube.

5.2.2. Crush aphid well with the pestle as described in step 3.2.

5.2.3. In the fume hood, add 800 µl of guanidinium thiocyanate-phenol-chloroform extraction reagent to sample (1-5 adult aphids). Homogenize samples more with pestle and dispose of the pestle.

5.3. Phase separation.

Note: All steps should be performed in a fume hood.

5.3.1. Incubate the homogenized samples for 5 min at room temperature.

5.3.2. Add 160 µl of chloroform to sample. Shake by hand for 15 s.

5.3.3. Incubate for 2-3 min at room temperature.

5.3.4. Centrifuge for 15 min at 12,000 xg at 4 °C.

Note: Following centrifugation, the mixture separates into 3 layers: a lower, red phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The RNA remains exclusively in the aqueous phase. The volume of the aqueous will be ~480 µL.

5.4. RNA precipitation.

5.4.1. In a fumehood, transfer the aqueous phase to a fresh, RNase-free tube. Do not disturb the intermediate phase.

5.4.2. Precipitate the RNA by adding 400 µl of isopropanol and incubate the sample at -20 °C for 10 min.

Note: The protocol can be paused here, and samples can be stored at -20 °C for up to 24 hours.

5.4.3. Centrifuge for 10 min at 12,000 x g at 4 °C.

5.5. RNA wash and elution.

5.5.1. Remove the supernatant; watch for the RNA pellet.

5.5.2. Wash the RNA pellet with 1 ml of 75% ethanol in DEPC-treated water. Mix by low vortexing.

5.5.3. Centrifuge for 5 min at 7,500 xg at 4 °C.

5.5.4. Repeat steps 5.5.1 – 5.5.3 to help remove phenol contaminants.

5.5.5. Remove the supernatant and air dry the pellet for 5-10 min with tube laying horizontal and open on a sterile bench. Do not let the RNA pellet dry completely.

5.5.6. Dissolve the RNA pellet in 30 µl of RNase-free or DEPC-treated water. Gently pipette up and down to mix. Incubate at 55-60 °C for 10-15 min.

RNAseq de novo *transcriptome assembly, annotation, and differential expression analysis (Protocol 6)*

6.1. Analyze RNA sample concentration and quality using a Bioanalyzer.

Note: A Bioanalyzer is preferable to analysis with a spectrophotometer because it provides a more accurate and sensitive measure of RNA concentration and quality.

6.1.1. If samples are of suitable quality ($\geq$ 250 ng total, RIN (RNA Integrity Number) $\geq$ 5), perform RNA sequencing.

Note: Importantly, because this sequencing data will be used for both expression profiling and *de novo* transcriptome assembly, more read depth will result in a higher quality transcriptome. For a reasonably comprehensive assembly using Illumina sequencing technology, 100-200 million 100bp, paired end reads would be a recommended starting point.

Note: Total mRNA library preparation and RNA sequencing were performed by a sequencing facility.

6.2. Check the quality of reads using Fast QC (Andrews, 2010).

6.3. Combine all sample reads and assemble the transcriptome *de novo* using Trinity (Grabherr et al., 2011; Haas et al., 2013) (Trimmomatic quality filtering enabled).

6.4. Refine the assembly.

6.4.2. Use Transdecoder to identify open reading frames (ORFs) that are a minimum of 100 amino acids in length (transdecoder.sf.net).

6.4.3. Perform homology searches of the translated ORFs against Pfam (Finn et al., 2016) and UniProt (The UniProt Consortium, 2017) databases using BLASTP (M. Johnson et al., 2008) and HMMER (hmmer.org), respectively.

6.4.4. Remove bacterial transcripts (any translated sequence whose best BLAST hit was to a bacterial gene with a bit score of over 300 and a minimum amino acid sequence identity of 50%).

6.4.5. Collapse any complete, translated ORFs that are at least 99% identical at the amino acid level using CD-HIT (Fu, Niu, Zhu, Wu, & Li, 2012).

6.4.6. Collapse the remaining, incomplete ORFs that are at least 95% identical at the nucleotide level using CD-HIT (Fu et al., 2012).

6.4.7. Assign the remaining nucleotide sequences with unique, species-specific identifiers (*e.g.* APHNE 0001)

6.5. Assess the completeness of the refined assembly, using BUSCO (http://busco.ezlab.org/) and the Arthropoda gene dataset (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015).

6.6. Transcriptome annotation.

6.6.1. First, annotate the refined transcriptome using HMMER (hmmer.org) against the Pfam database (Finn et al., 2016).

6.6.2. Second, annotate the transcriptome using BLASTP against the UniProt database (M. Johnson et al., 2008; The UniProt Consortium, 2017).

6.6.3. Third, annotate the transcriptome using BLASTP against the coding sequences of selected insects with published, annotated genomes.

6.6.4. Last, annotate the transcriptome using BLASTP against the pea aphid protein database only.

6.6.5. Use Trinotate to generate GO annotations from UniProt accessions.

6.6.6. Use Trinotate to organize all the annotation results into a SQLite database and generate an annotation report.

6.7. Differential expression analysis.

Note: Using the refined transcriptome as a reference, align and quantify each library separately.

6.7.1. Use Trimmomatic to quality-filter and trim original read files (Bolger, Lohse, & Usadel, 2014).

Note: If performing this step subsequent to a Trinity assembly, you may instead use the Trimmomatic output from that step.

6.7.2. Perform local alignments for each sample using Bowtie2 (Langmead & Salzberg, 2012).

6.7.3. Extract read counts from each sample individually using SAMtools (H Li et al., 2009).

6.7.4. Calculate differential expression between samples of interest using DESeq2 with default parameters and a parametric fit (Love, Huber, & Anders, 2014b).

qPCR verification of differentially expressed genes (Protocol 7)

Note: If users are interested in differentially expressed genes from their RNAseq experiments, the following protocol can be used to verify patterns of differential expression.

7.1. Generate RNA samples as described above (Section 5).

7.2. Quantitate RNA extractions using a spectrophotometer to ensure quality and obtain concentration.

7.3. Synthesize cDNA samples using a First-Strand Synthesis kit according to the manufacturer's protocol.

7.4. Determine primer efficiencies for genes of interest to ensure accurate two-fold PCR amplification.

7.4.1. Based on original RNA concentrations, perform serial dilutions (10^1) to obtain 3 cDNA concentrations.

7.4.2. Using a quantitative PCR master mix, mix triplicate qPCR reactions according to the manufacturer's protocol using three primer concentrations (*e.g.* 100 nM, 200 nM, 300 nM) with three serially diluted cDNA concentrations (*e.g.* 0.1 ng/µl, 10 ng/µl, 100 ng/µl).

7.4.3. For each target gene, calculate the slope (m) of the line created using the mean C_t values for each sample as the dependent variables and the log (cDNA concentration) as the independent variables (three points total).

7.4.4. Use the following equation to calculate the primer efficiency (E) where m is the slope calculated in 7.4.3:

$E = 10^{\wedge}(-1/m)$

Note: Primer efficiencies between 90-110% are suitable for analyses. This process ensures equal amplification of all genes included in the calculations.

7.5. Use the $\Delta\Delta C_t$ method with a housekeeping gene to quantify differential expression for genes of interest (Rieu & Powers, 2009).

Representative Results

Plant cultures

Seeds will take approximately two to four weeks, depending on the season, to grow large enough to repot (Fig. 4A). Repotted seedlings will take another two to four weeks to grow to an optimal size for aphid cultures (Fig. 4B).

Aphid cultures

Adult *A. nerii* are distinguished by some darkened cauda and may be unwinged (apterous, Figs. 6A, B) or winged (alate, Figs. 6C, D). Developing wing pads become visible when nymphs reach the third instar (Figs. 6E, F). Stock cultures are best maintained by transferring one to three mid-instar and one adult-aged unwinged aphids; this ensures a healthy, mixed age population. Populations to be used for experiments should be cultured using unwinged aphids as described above (2.4). One *A. nerii* adult can produce 3-10 offspring per day, dependent on the host plant and age of the aphid (Birnbaum et al., 2017).
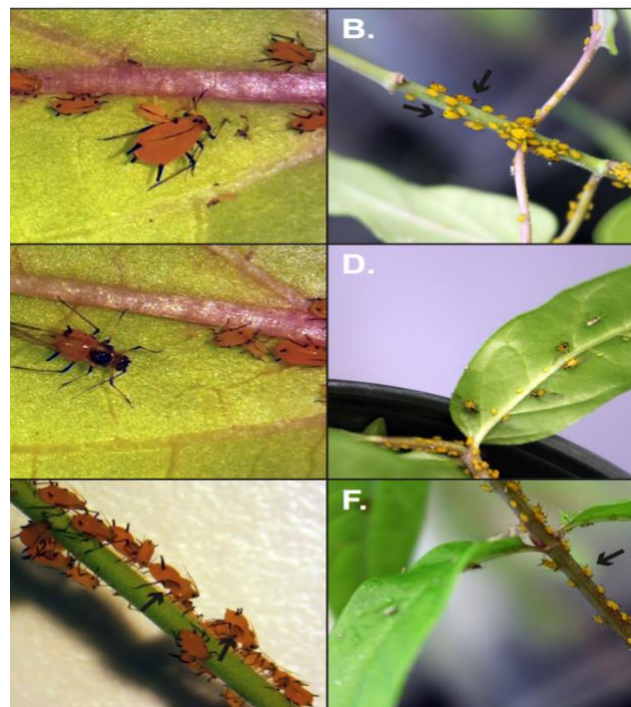
Figure 6. Representative adult and nymph *Aphis nerii*. **A, B.** Apterous (unwinged) adult *A. nerii* are identified by darkened cauda at their posterior end. **C, D.** Alate (winged) adults are identified by fully developed wings and darkened cauda at their posterior. **E, F.** Developing *A. nerii* nymphs go through four instar stages and developing wing pads become apparent during the third instar stage.

DNA and RNA extractions

Single, adult *A. nerii* will yield approximately 100 – 200 ng/µl DNA (80 µl elution; Fig. 7A) and 150 – 300 ng/µl RNA (30 µl elution; Fig. 7B). Representative microsatellite peaks are shown in Figure 8. Representative relative expression of a candidate gene under three conditions (control, Treatment 1, Treatment 2) are calculated in Table 3 and shown in Figure 9.
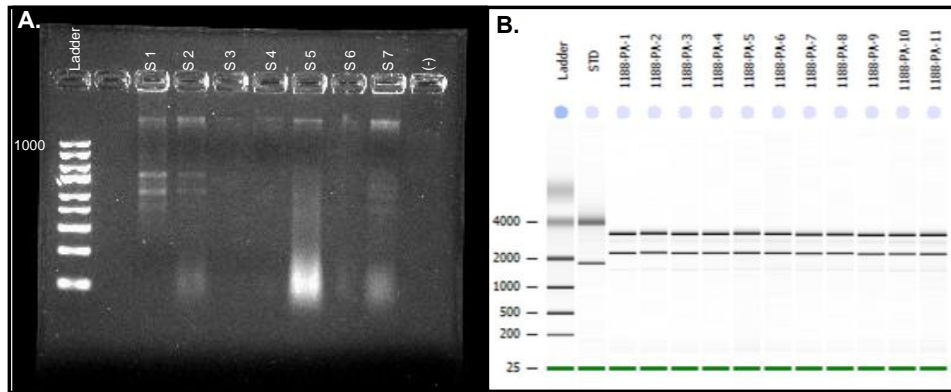
Figure 7. Representative gels. **A.** DNA extractions (1kb ladder). Seven *A. nerii* DNA extractions are visualized in lanes 3-9. Negative control is in lane 10. **B.** RNA extractions. Eleven *A. nerii* RNA extractions are visualized in lanes 3-13.

Figure 8. Representative microsatellite peaks. 6-FAM-tagged peaks are visualized in blue. LIZ-500 ladder is shown in orange.
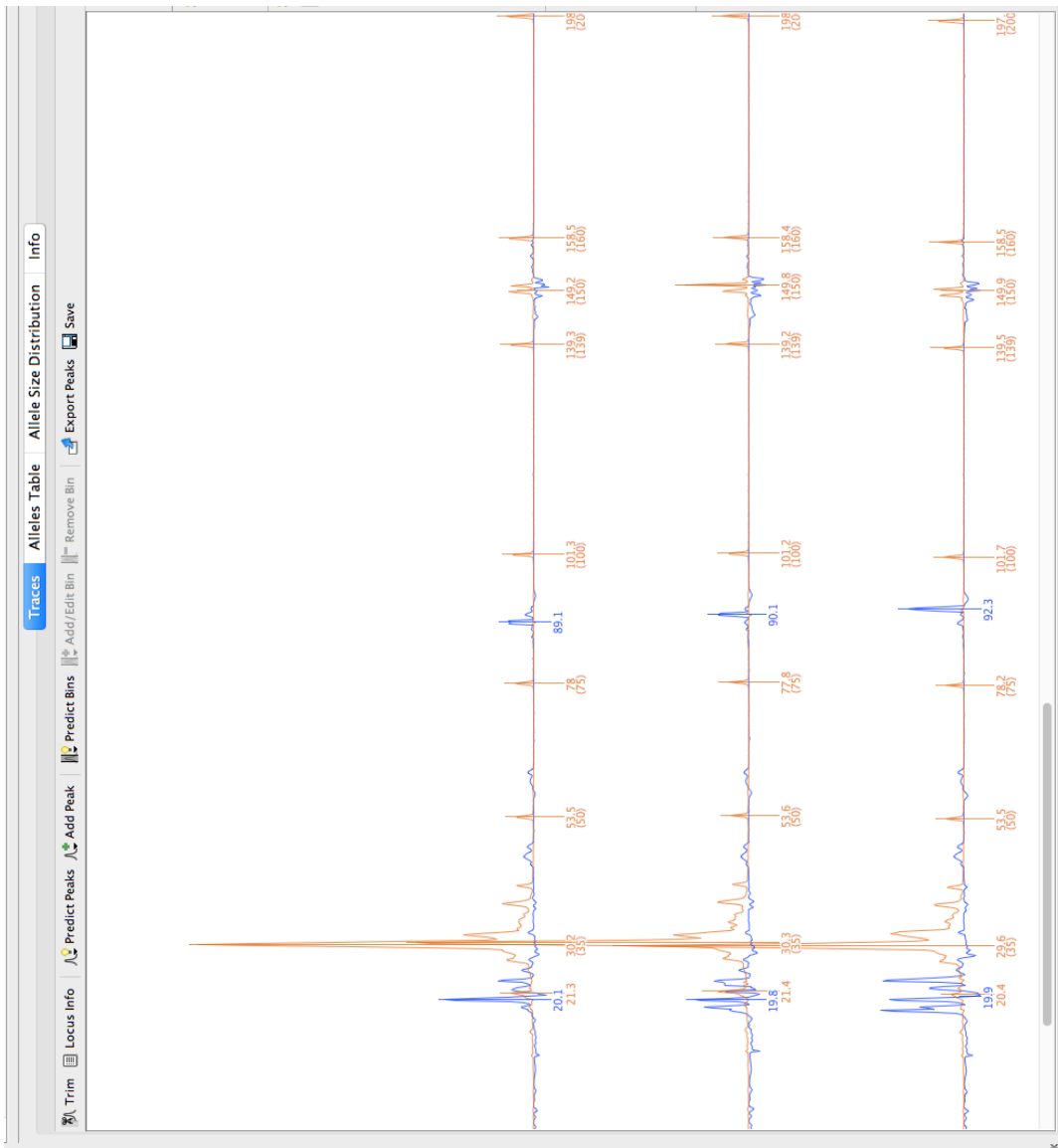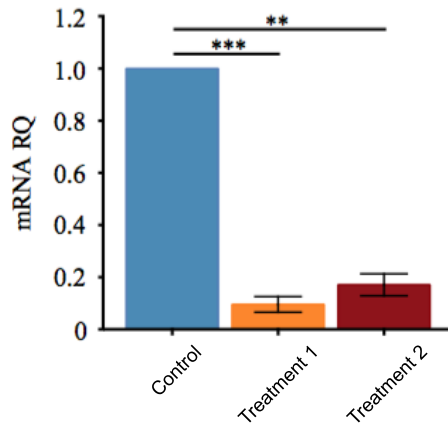
Figure 9. qPCR verification of a differentially expressed gene. Representative mRNA relative quantity (RQ) expression (calculated using the ΔΔCt method, Table 3) shown for a candidate gene of interest under three conditions: control, treatment 1, treatment 2. Graph shows decreased expression of candidate gene under treatments 1 and 2 compared to the control (Table 3) and display the mean SEM fold difference in average mRNA levels (two biological replicates of five adult pooled aphids each; two technical replicates each). Bars and stars represent significant differences in gene expression when compared to A. incarnata (Tukey's multiple comparisons test; ** = $p < .005$, *** = $p < .0005$)

Target	Sample	Ct Mean	Ct Std. Dev	ΔCt	avg. ΔCt	ΔΔCt	RQ=2^(-ΔΔCt)	RQ SEM
ef1a	1.1	22.59	0					
ef1a	1.2	20.31	0					
ef1a	1.3	20.36	0.226					
ef1a	1.4	20.27	0.036					
ef1a	1.5	20.55	0.003					
ef1a	1.6	20.52	0.245					
ef1a	2.1	20.49	0.082					
ef1a	2.2	19.86	0.033					
ef1a	2.3	20.19	0.037					
ef1a	2.4	19.67	0.058					
ef1a	2.5	20.25	0.188					
ef1a	2.6	18.16	0.089					
ef1a	3.1	20.93	0.157					
ef1a	3.2	20.22	0.003					
ef1a	3.3	20.44	0.039					
ef1a	3.4	20.91	0.559					
ef1a	3.5	20.63	0.017					
ef1a	3.6	20.3	0.135					
gene of interest	1.1	24.6	0.173	2.01		0	1	
gene of interest	1.2	24.25	0.019	3.94	2.975	0	1	0
gene of interest	1.3	24.79	0.04	4.43		0	1	
gene of interest	1.4	25.23	0.285	4.96	4.695	0	1	0
gene of interest	1.5	24.6	0.103	4.05		0	1	
gene of interest	1.6	25.08	0.033	4.56	4.305	0	1	0
gene of interest	2.1	27.52	0.155	7.03		5.019033762	0.03084042	
gene of interest	2.2	27.23	0.061	7.37	7.2	3.428355679	0.092888533	0.031024057
gene of interest	2.3	27.18	0.058	6.99		2.56158174	0.169389724	
gene of interest	2.4	27.45	0	7.78	7.385	2.820764967	0.141535419	0.013927153
gene of interest	2.5	27.44	0.032	7.19		3.138956897	0.113521944	
gene of interest	2.6	28	0	9.84	8.515	5.284272079	0.025661119	0.043930413
gene of interest	3.1	27.23	0.143	6.3		4.292437371	0.051032588	
gene of interest	3.2	27.05	0.088	6.83	6.565	2.891234282	0.134788164	0.041877788
gene of interest	3.3	27.45	0.109	7.01		2.578145722	0.167456035	
gene of interest	3.4	27.58	0.019	6.67	6.84	1.709038085	0.305863936	0.069203951
gene of interest	3.5	27.06	0.067	6.43		2.384498984	0.191511246	
gene of interest	3.6	27.36	0	7.06	6.745	2.513723938	0.175103043	0.008204101

Table 3. Calculations for qPCR $\Delta\Delta C_t$ verification of candidate gene. Candidate gene expression is calculated relative to ef1a (Fig. 9). Samples 1.1-1.6 represent six biological replicates under the control treatment; samples 2.1-2.6 represent six biological replicates under Treatment 1; samples 3.1-3.6 represent six biological replicates under Treatment 2. C_t Std. Dev. is calculated from three technical replicates.

Discussion

As a specialist on highly defended plants in the dogbane and milkweed family, it has long been recognized that the aposematic *A nerii* can provide insights into the patterns and mechanisms of resistance to plant defenses, and particularly chemical sequestration (Birnbaum & Abbot, 2018; Malcolm, 1990). A number of genomic resources have recently emerged for *A. nerii* (Birnbaum et al., 2017), offering new opportunities for ecological and functional genomic studies that use *A. nerii* as a model. We outline basic protocols in aphid and plant culture, and molecular/genomic techniques, with the assumption that future work on this species will likely involve studies that utilize genomic and functional ecological approaches. Many open questions remain

about the mechanisms and significance of cardenolide detoxification and sequestration in *A. nerii*. Techniques such as RNAi for expression knockdown or gene editing approaches will prove valuable in this regard.

One of the challenges in culturing aphids is in their prodigious capacities for reproduction and dispersal. These traits, which directly relate to why they are serious crop pests, mean that aphid cultures require almost daily attention, as well as extreme care if isogenic lines are required for experiments. The reader should carefully note the steps described in section 2 of the protocol. The reader will find that techniques described above, including those for generating data for the analysis of gene expression, while similar to general protocols for aphid rearing and molecular analysis, provide a specific step-by-step guide to generating sufficient biological material for *A. nerii* for a diverse set of molecular and ecological applications.

To this end, if functional or ecological genomic studies are on the horizon for *A. nerii*, these will need to be coupled with live cultures to fully capitalize on the experimental opportunities these offer. Insect herbivores live in complex communities on their host plants, and both intraspecific interactions (A. A. Agrawal, Underwood, & Stinchcombe, 2004; Zehnder & Hunter, 2007a) as well as interspecific interactions (Hartbauer, 2010) shape the ultimate response of *A. nerii* to their host plants. The host plants on which *A. nerii* specialize represent a diverse set of plants that express divergent life history strategies (A. A. Agrawal et al., 2012; Mooney et al., 2010), underscoring the importance of coupling purely genomic or physiological approaches with experimental manipulations that account for naturally-occurring variation in *A. nerii* communities. The methods outlined here are starting points for a functional and ecological genomic perspective on *A. nerii* and its interactions with toxic host plants.

CHAPTER V

TRANSCRIPTIONAL PROFILE AND DIFFERENTIAL FITNESS ACROSS A TOXICITY GRADIENT IN A SPECIALIST MILKWEED INSECT

Abstract

Interactions between plants and herbivorous insects have been models for theories of specialization and coevolution for over a century. Phytochemicals govern many aspects of these interactions and have fostered the evolution of adaptations by insects to tolerate or even specialize on plant defensive chemistry. While genomic approaches are providing new insights into the genes and mechanisms insect specialists employ to tolerate plant secondary metabolites, open questions remain about the evolution and conservation of insect counter-defenses, how insects respond to the diversity of defenses mounted by their host plants, and the costs and benefits of resistance and tolerance to plant defenses in natural ecological communities. Using a milkweed-specialist aphid (*Aphis nerii*) model, we test the effects of host plant species with increased toxicity, driven primarily by increased secondary metabolites, on aphid life history traits and whole-body gene expression. We show that more toxic plant species have a negative effect on aphid development and lifetime fecundity. When feeding on more toxic host plants with higher levels of secondary metabolites, aphids differentially express a narrow, targeted set of genes, including those involved in canonical detoxification processes (e.g., cytochrome P450s, hydrolases, UDP-glucuronosyltransferases, and ABC transporters). These results indicate that *A. nerii* marshal a variety of metabolic detoxification mechanisms to circumvent milkweed toxicity and facilitate host plant specialization, yet, despite these detoxification mechanisms, aphids experience reduced fitness when feeding on more toxic host plants.

Introduction

Plants and their herbivorous insects account for more than half of the species described and have long been models for the study of coevolution and the mechanisms that generate biological diversity (Futuyma & Agrawal, 2009). While some insect species are polyphagous generalists and can feed on diverse, chemically distinct plant families, the vast majority of insect species are specialists feeding on one or a few plant species or families characterized by particular phytochemicals (Forister et al., 2015; Futuyma & Moreno, 1988). Theories for the evolution of specialization to a narrow set of host plants have centered on coevolution, trade-offs, and the interactions between genotypes and environments that emerge during reciprocal adaptation between plants and insects (Forister et al., 2012), yet the mechanisms that drive ecological specialization are only beginning to be understood (Vamosi, Armbruster, & Renner, 2014).

Studies of plant secondary chemistry and insect herbivores are critical in the development of our understanding of coevolution and specialization. Milkweed-herbivore systems have been a model for studying

coevolution of insect adaptations to plant secondary chemistry and insect adaptations to that chemistry for more than fifty years (reviewed in (Malcolm 1994)). However, aside from a small number of genes (Dobler et al., 2012; Petschenka, Pick, Wagschal, & Dobler, 2013b), relatively little is known about the genes underlying specialist insects' adaptations to milkweeds (A. A. Agrawal et al., 2012). Milkweed (Apocynaceae) is a diverse plant family characterized by the production of toxic secondary metabolites known as cardenolides. Cardenolides are members of the cardiac glycoside family of steroidal compounds and contribute to the toxicity of milkweed plants for most animals. Cardenolide toxicity derives primarily from their ability to disrupt electrochemical gradients by inhibition of the Na^+/K^+ ATPase. Milkweed species vary in the concentration and composition of polar and apolar cardenolides; apolar cardenolides cross membranes more readily and are more difficult to excrete, and more toxic species with greater cardenolide concentrations typically have more apolar compounds (A. A. Agrawal et al., 2012; Rasmann & Agrawal, 2011). A relatively small but diverse group of insects have evolved to obligately feed and live on milkweed species, employing various strategies to avoid cardenolide toxicity and minimize their negative impacts, including metabolism, excretion, and sequestration (A. A. Agrawal et al., 2012). For clarification, we define *resistance* as any mechanism that insects employ to avoid or reduce the toxicity of plants (Després et al., 2007; H. T. Gordon, 1961), and *tolerance* as the reduction in the detrimental effects of plant defenses after they are encountered (H. T. Gordon, 1961). These definitions are roughly consistent with how the concepts are applied in the plant and pathogen literature wherein resistance refers to mechanisms that directly act against plant defenses and tolerance refers to reductions in the negative effects of plant defenses (Restif & Koella, 2004). We also define *metabolic defense* as a mechanism of resistance involving the biotransformation of plant toxins (Brattsten 1979) and *sequestration* as the specific and selective uptake, transport, and accumulation of plant toxins resulting in tolerance of plant phytochemicals (Erb, Erb, Robert, & Robert, 2016).

Some milkweed feeders, including monarch butterflies (*Danaus plexippus*) and milkweed-oleander aphids (*Aphis nerii*), are aposematic and sequester cardenolides in tissues, presumably for protection from predators or parasitoids (Malcolm, 1990; Petschenka & Agrawal, 2016). A primary mechanism for managing the harmful effects of cardenolides is insensitivity of cardenolides' Na^+/K^+ ATPase target site via specific substitutions that lower binding affinity. Target site insensitivity has convergently evolved in at least five orders of milkweed specialists (Dobler et al., 2012; Petschenka & Dobler, 2009; Zhen et al., 2012). However, milkweed specialists tend to vary in the relative resistance of Na^+/K^+ ATPase to cardenolides, and some sequestering species, such as *A. nerii*, lack the substitutions that confer insensitivity (Zhen et al., 2012). Such species are thought to express alternative mechanisms for resisting or tolerating cardenolides, such as protection of sensitive tissues by specialized proteins or metabolic defenses involving the breakdown of cardenolides into less toxic or more readily transported or excreted forms (A. A. Agrawal et al., 2012). Because specialization to host plant chemistry involves or even requires multiple, possibly synergistic mechanisms (Simon *et al.* 2015), genome-level responses of herbivores to milkweeds have the potential to provide novel insights into the diverse and intertwined mechanisms at work in host plant specialization.

Aphis nerii (Hemiptera: Aphididae) is a polyphagous specialist that feeds on more than 50 species of milkweed and oleander plants (Blackman & Eastop 1984; Stoetzel 1990; (Züst & Agrawal, 2016). Previous studies in the field have shown that *A. nerii* naturally colonize many different oleander and milkweed species spanning a range of toxicity (Botha, Evert, & Walmsley, 1975b; Botha et al., 1977; Groeters, 1989; Rothschild et al., 1970), and studies in both the laboratory and field demonstrate that *A. nerii* population growth can vary across different milkweed plant species. These differences in population growth are presumably due to differences in cardenolide toxicity (A. A. Agrawal, 2004; de Roode, Rarick, Mongue, Gerardo, & Hunter, 2011; Mooney et al., 2010), although milkweed species also vary in latex, trichome, and phenolic defenses (A. A. Agrawal & Fishbein, 2008; A. A. Agrawal, Salminen, & Fishbein, 2009b). While the suite of defenses produced by milkweed species are complex, phylogenetic patterns indicate that, rather than escalation in defenses as milkweeds and their herbivores radiate (Ehrlich & Raven, 1964), total investment in defenses was greatest early in the radiation of milkweed species (*Gomphocarpus* spp. and *Asclepias* spp.) (A. A. Agrawal & Fishbein, 2008; A. A. Agrawal, Fishbein, Halitschke, Hastings, et al., 2009a), and only phenolic defenses increase over evolutionary time as cardenolide and latex production declines (A. A. Agrawal, Fishbein, Halitschke, Hastings,

et al., 2009a; A. A. Agrawal, Salminen, & Fishbein, 2009b). Thus, in general, more ancient milkweed species, *e.g. Gomphocarpus* spp., are considered more toxic, and we interpret the effects of milkweed toxicity variation in light of the multitude of defenses produced by milkweeds with particular focus on cardenolide defenses.

The adaptations underlying *A. nerii*'s ability to feed on milkweeds that dramatically vary in toxicity and the impact of these detoxifying strategies on *A. nerii* fitness are unknown. Modification through hydrolysis or oxidation of cardenolide molecules to disrupt specific binding to the Na^+/K^+ ATPase is hypothesized to be an important general mechanism in insect tolerance and resistance (A. A. Agrawal et al., 2012; Repke, 1985). Milkweed host plants produce a range of cardenolide molecules, and there is evidence that *A. nerii* sequesters a fraction of mostly non-polar molecules at relatively low levels in their body tissues and modify cardenolides post-ingestion (Malcolm 1990; Zust & Agrawal 2015). Resistance to and tolerance of cardenolides likely requires multiple mechanisms involving behavioral avoidance, modification of cardenolides to render them less toxic, protection of sensitive tissues from cardenolides, and efficient excretion of cardenolides and their metabolic byproducts (A. A. Agrawal et al., 2012; Petschenka & Agrawal, 2016; Petschenka, Pick, Wagschal, & Dobler, 2013b). Metabolism and excretion of cardenolides, both forms of resistance, may involve canonical pathways implicated in host plant secondary metabolite and xenobiotic degradation, such as cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and ATP-binding cassette (ABC) transporters (Dermauw et al., 2013; Erb et al., 2016; Heckel, 2014).

Here, we use milkweed plant species that encompass a broad range of cardenolide toxicity in the Apocynaceae family to characterize responses of *A. nerii* to milkweed toxicity variation (A. A. Agrawal, Fishbein, Halitschke, Hastings, et al., 2009a). *Asclepias incarnata*, *A. curassavica*, and *Gomphocarpus physocarpus* milkweed species naturally vary in cardenolide concentration from low to high, respectively (Gowler et al., 2015; Rasmann & Agrawal, 2011). Despite these differences in cardenolides, the plants do not appear to have substantial nutritional differences for aphids, and variation in phloem nutrient content is thought to be minimal (Gowler et al., 2015; Martel & Malcolm, 2004; Pringle, Novo, Ableson, Barbehenn, & Vannette, 2014). We investigated the effects of plant variation in secondary metabolites on several measures of insect fitness and on whole transcriptome differential gene expression. We hypothesized that increased milkweed toxicity is costly, due to either direct toxic effects of host plants or to potentially costly mechanisms used to resist or tolerate increased milkweed toxins, and thus would result in lower *A. nerii* fitness when reared on plants with higher toxicity. We expected that differential gene expression by *A. nerii* across host plants would reflect the mechanisms involved in cardenolide resistance and tolerance. Our results confirmed the costly effects of increased host plant toxicity on *A. nerii* performance and revealed an extremely targeted transcriptional response involving differential expression of canonical detoxification genes (P450s, GSTs, UDP-glucoronosyltransferases, multidrug resistance-associated genes, ABC transporters) and genes promoting hydrolysis. These results show that *A. nerii* differentially express a variety of metabolic detoxification mechanisms aiding in the circumvention of milkweed secondary compounds, thereby facilitating specialization on these toxic plants. Despite these strategies of counter defense to plant toxicity, increased detoxification and host plant toxicity are associated with negative effects on development and fecundity.

Materials and methods

Plants and Aphis nerii *stock lines*

Seeds of *Asclepias incarnata*, *A. curassavica*, and *Gomphocarpus physocarpus* were obtained commercially (Joyful Butterfly, Blackstock, SC, US). Seeds were grown in the Vanderbilt University greenhouse. They were first planted in a fine germination mix (Sungro Propagation Mix), and after 2-3 weeks, they were repotted in four inch pots filled with a general use potting soil (Sungro 3B Mix).

Two clones of *Aphis nerii* were collected in the spring of 2014 from Atlanta, GA (GH14) and Miami, FL (MIA14). A third *A. nerii* clone was collected in the summer of 2015 from Nashville, TN (Sc15). The fourth clone was collected from Minnesota and was received from the University of Minnesota in 2011 (UMN). Aphids were acclimated to new host plant species for at least four generations prior to experimentation.

For genotyping, DNA was extracted from a single adult aphid from each clonal line using a standard lysis buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M TRIS (pH 9.1), 0.05 M EDTA, 0.05% SDS) and ethanol precipitation. Individual aphids were genotyped using five previously published microsatellite loci: Ago24, Ago53, Ago59, Ago66, and Ago69 (J. S. Harrison & Mondor, 2011; Vanlerberghe-Masutti, Chavigny, & Fuller, 1999).

A. nerii laboratory fitness experiment

To investigate the effects of increased host plant toxicity on *A. nerii* fitness, we measured development time, lifetime fecundity, and survival across host plants in two laboratory fitness experiments, completed in June-July 2015 and October-November 2015. For the first experiment, clones GH14 and MIA14 were reared on three host plant species of varying toxicity: *A. incarnata* (low), *A. curassavica* (medium), and *G. physocarpus* (high). As described in the results below, because this first experiment suggested an effect of aphid genotype on host plant performance, we added a second experiment in which a third *A. nerii* clone was added (GH14, MIA14, and Sc15) and measured fitness on the two host plants representing the most extreme differences in cardenolide concentration (*A. incarnata* and *G. physocarpus*). Thus, the first experiment involved two aphid clones on three host plants, and the second involved three aphid clones on two host plants.

To obtain same age populations of aphids for experimental monitoring, four healthy reproducing aphids were placed on each of five plants per treatment. Aphids were allowed to reproduce for 24 hours, then the mother aphid was removed and the remaining offspring populations were reduced to 15-20 aphids per plant (~75-100 aphids per treatment). Aphids were monitored each day to quantify the time to adulthood. Once aphids matured to adults, they were distributed to new plants to reduce the population size; each plant housed about five adults. Adult aphids were monitored daily for fecundity and survival until all aphids were dead; offspring were removed each day.

All data were analyzed using the statistical package R v3.2.1 (R Core Team). Laboratory replicate experiments were analyzed separately because they utilized a different number of aphid genotypes and host plant species. The effects of host plant species and *A. nerii* genotype on development time to adulthood were evaluated using a two-way ANOVA on ranked data as the distribution of residuals deviated from normality; data distribution and the distribution of residuals were evaluated using Shapiro-Wilk tests. We used a restricted maximum likelihood (REML) approach to fit a linear mixed effects model to the lifetime fecundity data, with plant pot as a random factor, using the lme4 package in R. Survival analysis was performed using the survival package in R. For the first experiment, survival analysis used a Cox proportional hazards model (coxph), with no censoring (data were tested to confirm they fit model assumptions using the coxzph function). The second experiment did not fit the Cox proportional hazards model assumptions, so parametric models were built using the survreg function. We compared several distributions (exponential, logistic, lognormal, loglogistic, Weibull, Gaussian) and chose the best fit model based on the lowest AIC value, which takes model complexity into account.

RNA extraction and sample preparation for sequencing

Transcriptional responses were measured on aphids mirroring the second experimental design (three clones on two host plant species). Three *A. nerii* genotypes (GH14, MIA14, UMN) were reared on *A. incarnata* and *G. physocarpus* for at least four generations; unwinged, single adult aphids were crushed in liquid nitrogen, homogenized in Trizol, and incubated at room temperature for 5 minutes. Total RNA from the six samples was extracted using chloroform, and the RNA was precipitated from the aqueous layer using isopropanol. The RNA pellet was additionally washed with 75% ethanol in DEPC-treated water and dissolved in DEPC-treated water. RNA quality and quantity were assessed using a Nanodrop spectrophotometer and Bioanalyzer 2100. Total RNA was sent to the Hussman Institute for Human Genomics (University of Miami Miller School of Medicine)

for library preparation and paired end sequencing on the Illumina HiSeq 2000/2500 platform; raw reads were also used in a separate publication (Duncan, Feng, Nguyen, & Wilson, 2016).

de novo *transcriptome assembly and refinement*

Reads from all six individual samples were quality checked (FastQC; (Andrews 2010)) and no systemic problems were detected; sequence quality was consistently high and levels of Illumina contaminants were low. Reads from all samples were then pooled and *de novo* assembly was preformed using Trinity (v2.1.1; (Grabherr et al., 2011)). Trinity was run with default settings and only transcripts of 200 bp or longer were reported. Quality filtering was performed within Trinity using the trimmomatic option, and reads were assembled in paired end mode. Transdecoder (v2.0.1; http://transdecoder.sf.net) was used to identify open reading frames (ORFs) that were a minimum of 100aa in length. Homology searches of the translated ORFs were performed against PFAM and Uniprot using local BLASTP (version 2.2.26+; (Altschul et al., 1997)) and HMMER, respectively. These search results established minimal retention criteria for ORFs to be carried forward.

This set of ORF-containing nucleotide sequences was then further refined to remove likely contaminants as well as to collapse highly similar isoforms around a single consensus sequence. First, we removed any translated coding sequence whose best BLAST hit (Uniprot) was to a bacterial gene, received a bit score of over 300 and displayed a minimum amino acid sequence identity of 50%. Then, complete, translated ORFs that were at least 99% identical at the amino acid level were collapsed to a single sequence using CD-Hit (Fu et al. 2012). Finally, CD-Hit was run on the remaining, incomplete ORFs (5- and 3-prime fragments) to collapse sequences that were at least 95% identical at the nucleotide level. The nucleotide sequences remaining following these steps were then each assigned APHNE identifiers; each APHNE designation represents a complete or partial protein coding, nucleotide sequence. This refined *A. nerii* transcriptome was carried forward through all subsequent analysis.

Transcriptome completeness

To assess the completeness of the assembly, the translated nucleotide sequences of the refined transcriptome were analyzed using BUSCO (Benchmarking Universal Single-Copy Orthologs) against an arthropod gene set (BUSCO, Arthropda obd9; (Simão et al., 2015)).

Transcriptome annotation

All assembled nucleotide sequences from the raw Trinity assembly were first annotated using BLASTX against the Uniprot protein database (Haas et al, in prep). The refined transcriptome (as designated by APHNE identifiers) was then further annotated by each of four homology searches preformed on the translated nucleotide sequences using the following databases: 1) Pfam (HMMER), 2) Uniprot proteins (BLASTP), 3) the coding sequences of six insect taxa (*Acyrthosiphon pisum, Bombyx mori, Drosophila melanogaster, Nasonia vitripennis, Rhodnius prolixus, Tribolium castaneum* (BLASTP)), and 4) pea aphid (*A. pisum*) proteins only (aphidbase_2.1b_pep; BLASTP). GO annotations were then generated using Trinotate (http://trinotate.github.io/) from Uniprot accessions. Finally, Trinotate was used to organize these results into a SQLite database and to generate an annotation report.

Differential expression calculation

The refined transcriptome was used as a reference for the alignment and quantitation for each of the six individual samples. Read files were quality filtered and trimmed (Trimmomatic; (Bolger et al., 2014)) to remove Illumina adapters and poor quality reads. Reads were then aligned with Bowtie2 (v 2.2.5) using the local alignment option in paired end mode. Bowtie2 alignment rate ranged between 74% and 79% for all

samples. Uniquely aligned read counts were extracted from the six individual alignments (SAMtools; (H Li et al., 2009)), and differential expression was calculated between the low- and high-toxic conditions with DESeq2 using default parameters with a parametric fit (Love, Huber, & Anders, 2014a). For the differential expression design matrix, the three genotypes were treated as replicates, which were then conditioned on the two levels of host toxicity. P-values within DEseq2 were adjusted by the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995).

Functional enrichment analyses

To test if differentially expressed genes were enriched for particular functional categories, functional enrichment tests were performed using the go-basic.obo and the R function dhyper (R Core Team). We compared the GO terms of significantly differentially expressed ($padj < 0.05$) *A. nerii* genes (109) against those of the entire *A. nerii* transcriptome (12,162 genes). Statistical significance was adjusted for multiple comparisons using the Benjamini & Hochberg (BH) method in the R function p.adjust (Benjamini & Hochberg, 1995).

qPCR verification of differentially expressed genes putatively involved in milkweed toxicity adaptation

Candidate genes were selected from those with putative functions in milkweed adaptation that were significantly differentially expressed ($padj < 0.05$, fold change ≥ 1.5) between host plant species. Genes with annotated protein functions involved in canonical detoxification processes, hydrolysis, stress, immunity, and development were selected for qPCR analysis to verify the differential expression observed in the transcriptome. To confirm transcriptional differential expression patterns observed between *A. incarnata* and *G. physocarpus*, three *A. nerii* genotypes (GH14, MIA14, Sc15) were reared on three host plants varying in toxicity: *A. incarnata*, *A. curassavica*, and *G. physocarpus*. To understand how differences in gene expression may correlate with differences in fitness between genotypes, as described in the results below, genotypes were treated separately in qPCR analyses and pooled adult samples were treated as biological replicates. Same age adult aphids were harvested for RNA extraction; five aphids were pooled for one biological replicate and two biological replicates per genotype were used for qPCR analysis of 16 candidate genes; qPCR primers were designed using Primer3 (Table 4). Total RNA was extracted as described above. cDNA samples were prepared using oligo dT primers (Superscript III, Invitrogen) and primer efficiencies were tested following previously published protocols (Altincicek, Kovacs, & Gerardo, 2012).

Expression of 16 genes of interest was standardized relative to the endogenous control gene ef1-α using the ΔΔCT method, and gene expression on *A. curassavica* and *G. physocarpus* are displayed relative to expression on *A. incarnata*. When testing if a gene is differentially regulated between *A. nerii* genotypes or between host plant treatments, a two factor ANOVA was performed, using Prism7 (GraphPad Software, La Jolla, CA, USA), on $\log_2$ transformation of relative abundance values ($\log_2(\Delta CT)$) with two pooled biological samples per treatment as replicates (Rieu & Powers, 2009). If there was no significant effect of genotype on gene expression, data for all three genotypes were combined for graphical presentation.

Transcript	Protein	F Sequence	R Sequence
APHNE16988	Glutathione S-transferase	GCAGGTAGCGATGATTGGGA	AGTATCCGTCGTTTTCACCCA
APHNE01273	Probable cytochrome P450 6a14	AAAGGCAAAGCGACCTAACG	ACAAATCCTGGGTCCATCGC
APHNE18214	Probable cytochrome P450 6a13	GGACACGATCAAGAACGACG	AGTTTCAACACCCAAGGGCA
APHNE11549	UDP-glucuronosyltransferase 2B16	GGGCAAAAGCCACTGGAATG	GGCCTCCATCATATGTCGCT
APHNE16403	UDP-glucuronosyltransferase 2B33	CGTGGATCGCTACTCCTGTT	CCGTCGAAGAACGGTGTGAA
APHNE19721	Pancreatic lipase-related protein 2	GAGATAGTCCCGACGGTCAAC	TCTCCAATCCACACAAATCGC
APHNE15804	Legumain	AAAGCGACCCGAGTAACACG	GCACACCAGGTTCGGGATTT
APHNE10801	Cyanogenic beta-glucosidase	AGCGTATCATTGAATTTACCGAGG	TCCATCGAATGTAGCTTGCAA
APHNE05988	Dehydrogenase/reductase SDR family member 11	CAAGTTGGTTCAATGCGGCG	CACGGCTTGTTCGTCTGTCA
APHNE08505	Cathepsin B	CCACCTTGCCCTTATGACGA	CGATGCTTCAATTGGTCCGT
APHNE05369	Major facilitator superfamily domain-containing protein 6	GTTGGTAGGCGCTGTCTTCT	GCGCTGTTTCATCTCGTTCG
APHNE16941	Juvenile hormone acid O-methyltransferase	TGCAGTGGCCAGAGTATTACG	ATCAACACCAACCAACTGATTGAT
APHNE13342	Esterase FE4	GCAAACATGCTAGGATGCCC	CGGCTAATTCAACGGTTGGAC
APHNE13309	Uncharacterized family 31 glucosidase KIAA1161	TGGTGGTTCCGAAACGACTT	ACACATTTGACCCGGTCGTT
APHNE05270	Larval cuticle protein A3A	CAGCGTTGAATCCGTTGACG	AAGTGCGTGACGGTGATGTA
APHNE15220	ACYPI009002-PA	GTCCAACTGCCAACTACCCA	TCGACCGTTACTGTTCACACC

Table 4. List of primer sequences used for qPCR analyses.

Results

A. nerii laboratory fitness experiments

Previous research investigating genetic diversity between *A. nerii* populations collected from Georgia and California indicated extremely low genetic diversity (J. S. Harrison & Mondor, 2011). However, the three *A. nerii* clones used in the fitness experiments here differed at three of the five loci that were genotyped (data not shown here).

Laboratory fitness experiments. Development time to adulthood

In the first experiment using three milkweed host plant species and two *A. nerii* genotypes, host plant and aphid genotype had significant effects on *A. nerii* development time to adulthood (Fig. 10a, b; host plant- $F_{2,1472} = 5.36$, p = 0.0048; genotype- $F_{1,1472} = 9.26$, p = 0.0024). Aphids reared on *A. incarnata*, the low cardenolide species, matured more quickly than those reared on *A. curassavica* and *G. physocarpus*. Overall, on day 5, about 30% more *A. nerii* living on *A. incarnata* had matured to adults compared to *A. nerii* on *A. curassavica* and *G. physocarpus*. In the second experiment using two host plant species and three aphid genotypes, host plant and genotype again had significant effects on development (Fig. 11a-c; host plant- $F_{1,1878} = 37.95$, p = $8.87*10^{-10}$; genotype- $F_{2,1878} = 3.84$, p = 0.022). Again, aphids reared on *A. incarnata* (low) matured more quickly than those reared on *G. physocarpus* (high), and genotypes differed in their maturation times. Overall, on day 5, about 43% more *A. nerii* living on *A. incarnata* had matured to adults compared to *A. nerii* on higher toxicity host plants. Based on these data, it appears that one cost of living on increased toxicity host plants for *A. nerii* is a slower rate of development.

Figure 10. Effects of milkweed host plant species with variable cardenolide concentrations on *A. nerii* fitness in the laboratory- experiment 1. **A, B.** *A. nerii* (**A,** GH14; **B,** MIA14) reared on low cardenolide host plants, *A. incarnata*, matured to adulthood more quickly than those reared on higher cardenolide host plants, *A. curassavica* and *G. physocarpus*. **C, D.** *A. nerii* reared on low cardenolide host plants had higher fecundity than those reared on higher cardenolide host plants. **E.** *A. nerii* longevity was extended when reared on higher cardenolide host plants. There was no statistical difference between genotypes, so results were combined.
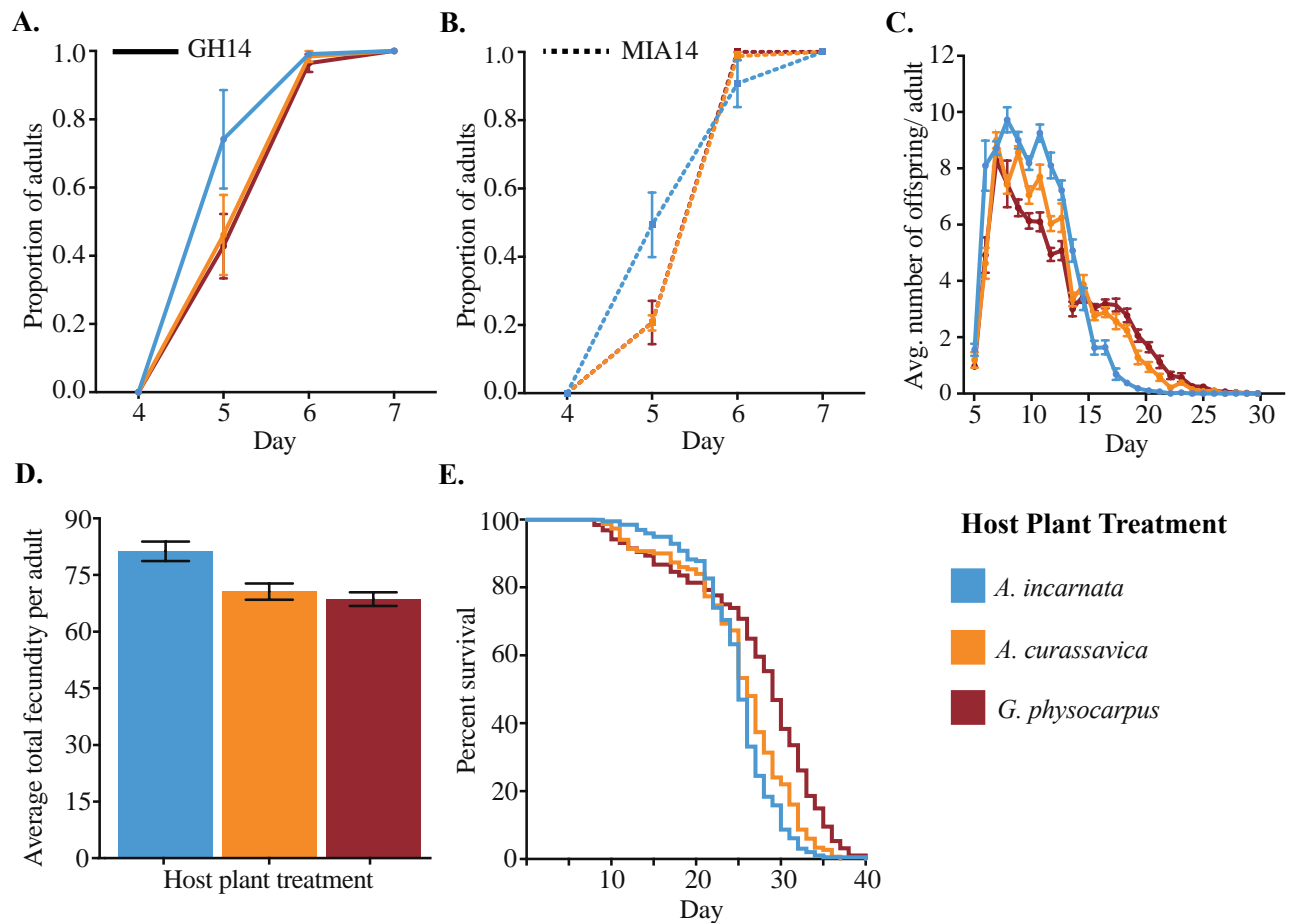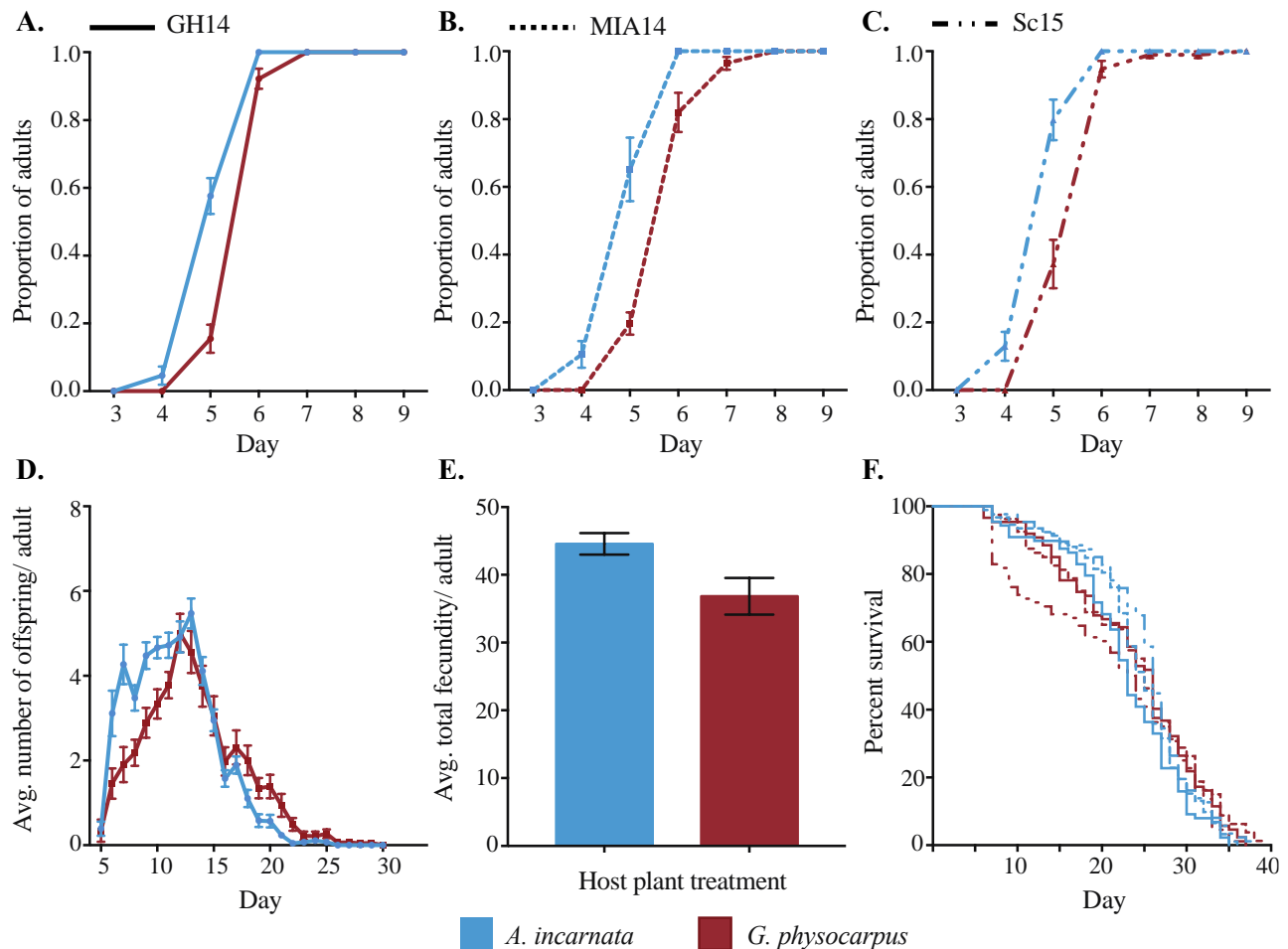
Figure 11. Effects of milkweed host plant species with variable cardenolide concentrations on *A. nerii* fitness in the laboratory- experiment 2. **A-C.** *A. nerii* (*A,* GH14; *B,* MIA14; *C.* Sc15) reared on low cardenolide host plants, *A. incarnata*, matured to adulthood more quickly than those reared on high cardenolide host plants, *G. physocarpus*. **D, E.** *A. nerii* reared on low cardenolide host plants had marginally higher fecundity than those reared on higher cardenolide host plants. **F.** Host plant did not have a significant effect on *A. nerii* longevity.

Laboratory fitness experiments. Fecundity and survival

Host plant species had a significant effect on lifetime fecundity in the first experiment, but there were no differences in fecundity between aphid genotypes (Fig. 10c, d; lme: host plant- $\chi^2(2) = 9.1$, p = 0.01; genotype- $\chi^2(1) = 0.16$, p = 0.69). By day 12, *A. nerii* living on the more toxic *A. curassavica* and *G. physocarpus* had on average 21% and 30% fewer offspring per adult, respectively, compared to those living on *A. incarnata*. Overall, *A. nerii* reared on higher cardenolide plants (*A. curassavica* and *G. physocarpus*) had 13% and 16% fewer offspring, respectively, compared to *A. nerii* reared on *A. incarnata*. The second experiment reinforced the first, with *A. nerii* experiencing reduced fecundity on higher toxicity host plants. By day 12, *A. nerii* living on *G. physocarpus* had an average of 33% fewer offspring per adult compared to those reared on *A. incarnata*. *A. nerii* reared on *A. incarnata* had overall about 18% more offspring compared to those reared on *G. physocarpus*, although the trend was non-significant (Fig. 11d, e; lme: host plant- $\chi^2(1) = 3.4$, p = 0.06; genotype- $\chi^2(2) = 1.0$, p = 0.60). Overall, when living on increased toxicity host plants relative to *A. incarnata*, *A. nerii* experience a marked reduction in fecundity that emerges early in adult lifespan.

For survival, in the first experiment, host plant species had a significant effect on *A. nerii* lifetime survival which was consistent across the three aphid genotypes (Fig. 10e; cox ph model; host plant- $\chi^2(2) =$

55.6, p < 1*10^{-13}; genotype- $\chi^2(1) = 0.02$, p = 0.88; interaction- $\chi^2(2) = 5.85$, p = 0.05). By day 15, there was a reduction in survival of aphids reared on the more toxic plants relative to *A. incarnata*, (Fig 10e). In the second experiment, survival did not vary based on host plant species or *A. nerii* genotype (Fig. 11f; survreg model, Gaussian distribution; host plant- $d_i(1) = 2.74$, p = 0.098; genotype-. $d_i(2) = 1.22$, p = 0.54; interaction- $d_i(2) = 10.36$, p = 0.0056). In the first experiment, there was evidence of greater longevity on more highly defended plants, perhaps indicating that one consequence of greater fecundity and faster development on less toxic host plants is a reduction in overall longevity (Fig. 10e). However, we interpret this result with caution, both because the effect was not replicated in the second experiment (Fig 11f), and because it is unlikely aphids in natural conditions would live as long as the length of these laboratory experiments. Likewise, we interpret with caution the significant interaction between aphid genotype and host plant species in the second experiment, as this trend was non-significant in the first experiment. Such an interaction indicates that the effects of host plants on *A. nerii* survival can be modulated by aphid genotype, suggestive of the possibility of genetically-based variation in *A. nerii* performance across its host range (Via, 1991; Vorburger & Ramsauer, 2008). Moreover, we evaluated only three genotypes across three host plants and measured performance in laboratory conditions, limiting the confidence that we can place in our evidence of host x genotype interactions in *A. nerii*.

Transcriptome analysis and differential expression

The *de novo* transcriptome assembly of all six individual samples resulted in 129,023 putative transcripts of at least 200 bp. A total of 33,288 coding sequences (CDS) were extracted, and after duplicates and bacterial transcripts were removed, the final transcriptome assembly included 20,312 non-redundant ORFs of 100 amino acids or longer. Each sample library mapped back to the full assembly with 74-79% overall alignment rate. Overall assembly statistics are shown in Table 5. BUSCO analysis indicated that the *A. nerii* transcriptome includes 1,020 complete and 14 fragmentary BUSCOs, indicating that the assembly is 97% complete based on expression of expected insect genes.

Transcriptome Summary Statistics	
Total no. transcipts	129,023
Median transcript length	370
Avg. transcript length	813.58
N50 (all transcripts)	1217
Transcript %GC	31.23
Total no. CDS	33,288
Median CDS length	750
Avg. CDS length	1135.3
N50 (all CDS)	1605
CDS %GC	38.23

Table 5. Transcriptome assembly summary statistics.

Irrespective of host plant, *A. nerii* expressed several members of key canonical detoxification gene families, including cytochrome P450s (CYPs or P450s), UDP-glucuronosyltransferases (UGTs), and ABC transporters, representing the spectrum of Phase I to Phase III detoxification (Fig. 12). CYPs are a broad family of genes that perform essential physiological functions but are also primary Phase I detoxifying molecules and are capable of metabolizing a wide range of substrates (Feyereisen, 2006). *A. nerii* expressed 78 P450 transcripts annotated to 20 genes representing the four clades of insect CYPs. The most frequently expressed families were those involved in primary xenobiotic metabolism, CYP4 and CYP6. UGTs are Phase II

detoxification proteins known to modify a wide range of substrates to produce more polar molecules (S.-J. Ahn, Vogel, & Heckel, 2012; Rowland, Miners, & Mackenzie, 2013). *A. nerii* expressed 64 UGT transcripts annotated to 26 different proteins. Furthermore, ABC transporters, one of the largest transporter families with described functions in xenobiotic transport and resistance (Dermauw & Van Leeuwen, 2014), were also widely expressed in the *A. nerii* transcriptome (81 ABC transporter transcripts representing 23 genes in seven families).

Hierarchical clustering across the six *A. nerii* transcriptome libraries indicated a stronger effect of host plant than genotype on gene expression (Fig. 13). When comparing *A. nerii* gene expression on *A. incarnata* (low) versus *G. physocarpus* (high) at the 2-fold level, only 0.21% genes were significantly differentially expressed (Fig. 14; $\log_2FC \geq 1$, padj < 0.05; 26 genes upregulated, 16 genes downregulated when on *G. physocarpus*). At a lower 1.5-fold threshold for differential expression, 0.95% of transcripts were significantly differentially expressed ($\log_2FC \geq 0.58$, padj < 0.05; 64 upregulated, 129 downregulated) across host plants. Regardless of fold change, only 1.11% of the *A. nerii* transcriptome was significantly differentially expressed (padj < 0.05; 73 upregulated, 152 downregulated) between host plant species. Overall, *A. nerii* downregulated a greater number of transcripts when feeding on more toxic host plant species (Fig. 15).

A GO enrichment test comparing *A. nerii* genes that are differentially expressed between host plants revealed significant enrichment of ten molecular function and four biological process GO categories (Table 6). Five of these enriched GO terms correspond to genes involved in the catalysis of hydrolysis of sugar residues (GO:0004553, GO:0004558, GO:0015926, GO:0016798, GO:0090599; 28 genes), and one GO term corresponds to the catalysis of oxidation-reduction reactions (GO:0050664; 3 genes). Both hydrolysis and oxidation-reduction are hypothesized to be important in degrading cardenolide molecules (A. A. Agrawal et al., 2012). One of the first barriers to cardenolide toxicity may involve restriction of cardenolide movement from the gut to the hemocoel, and two GO terms involved in cuticle structure and development (GO:0008365, GO:0042302; 12 genes) were enriched in the differentially expressed gene set.

The top 25 differentially expressed *A. nerii* genes are listed in Table 7; 60% of these are involved in hydrolysis, transport, or detoxification. Of the top differentially expressed genes ($\log_2FC \geq 1$, padj < 0.05), about 40% (17/42) have annotations associated with hydrolysis, transport, or other functions putatively associated with detoxification of plant allelochemicals (Taylor & Feyereisen, 1996). Over a quarter (28.57%, 12/42) of the most strongly differentially expressed genes are unannotated or have unknown functions. Canonical detoxification genes including glutathione S-transferase, esterase FE4, and two multidrug resistance-associated proteins were expressed at higher levels in *A. nerii* feeding on the more toxic *G. physocarpus*. Three different UDP-glucuronosyltransferases (2B16, 2B23, 2B33) were also upregulated in *A. nerii* on the higher toxicity host plants. An ABC transporter G family member 20 was downregulated in *A. nerii* feeding on *G. physocarpus*, and two cytochrome P450s, 6a13 and 6a14, transcripts were differentially up- and down-regulated, respectively, when feeding on *G. physocarpus*. Aphids lack Malpighian tubules, the primary detoxification organ in insects, and a recent study identified genes expressed in *A. pisum* gut tissues that are homologous to detoxification genes expressed in *D. melanogaster* Malpighian tubules (Jing et al., 2015); however, none of these genes were differentially expressed in *A. nerii* reared on *A. incarnata* compared to *G. physocarpus*.
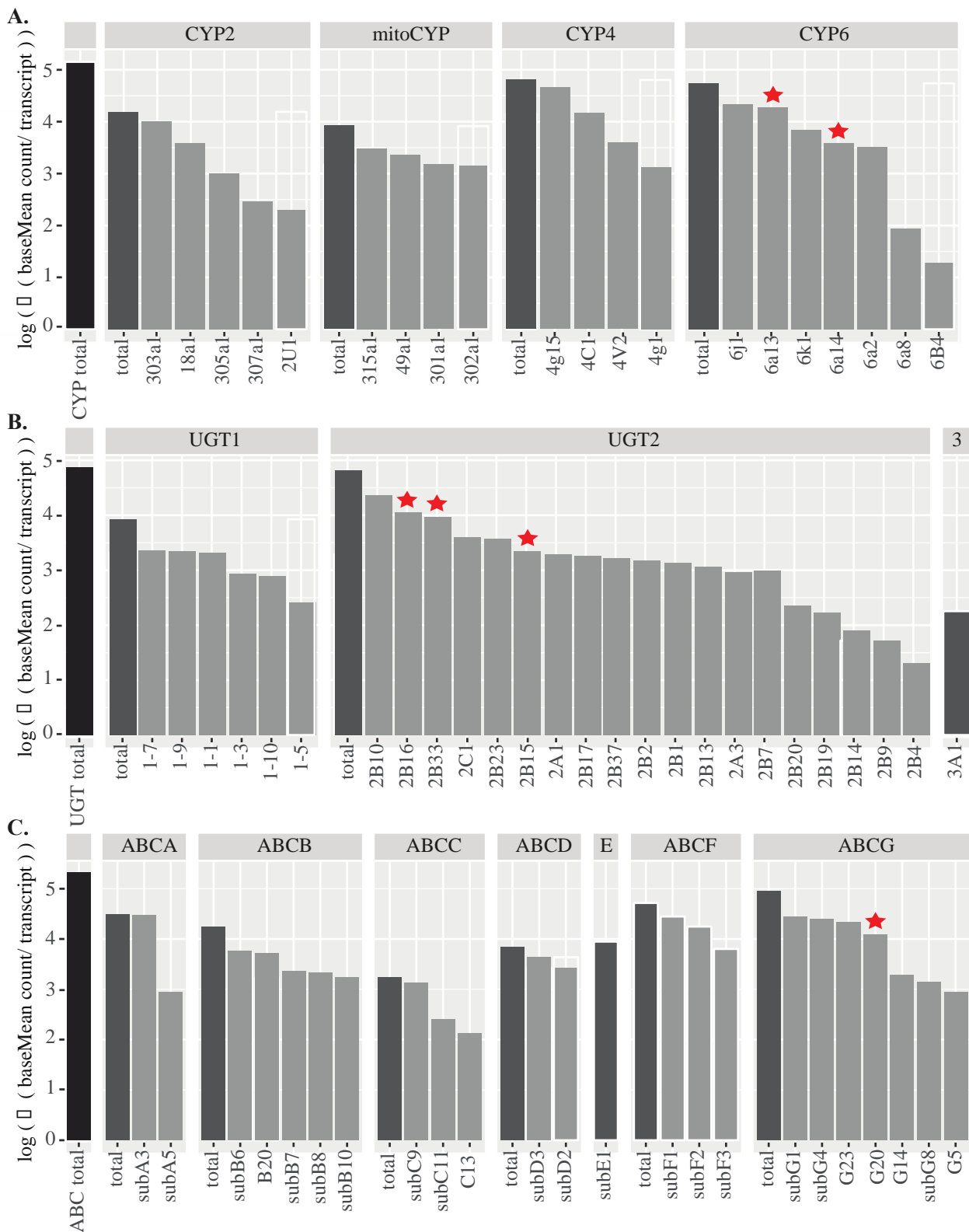
Figure 12. Expression of canonical detoxification genes. Graphs show the log transformed sum of the mean read counts of *A. nerii* transcripts, regardless of host plant. Stars indicate genes which had at least one transcript differentially expressed in *A. nerii* reared on *A. incarnata* compared to *G. physocarpus*. **A.** Expression of Cytochrome P450 genes. *A. nerii* expressed 78 CYP transcripts representing 20 genes in four families. **B.** Expression of UDP-glucuronosyltransferase genes. *A. nerii* expressed 64 UDP- glucuronosyltransferase

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transcripts representing 26 genes in three families. **C.** Expression of ABC transporter genes. *A. nerii* expressed 81 ABC transporter transcripts representing 23 genes in seven families.
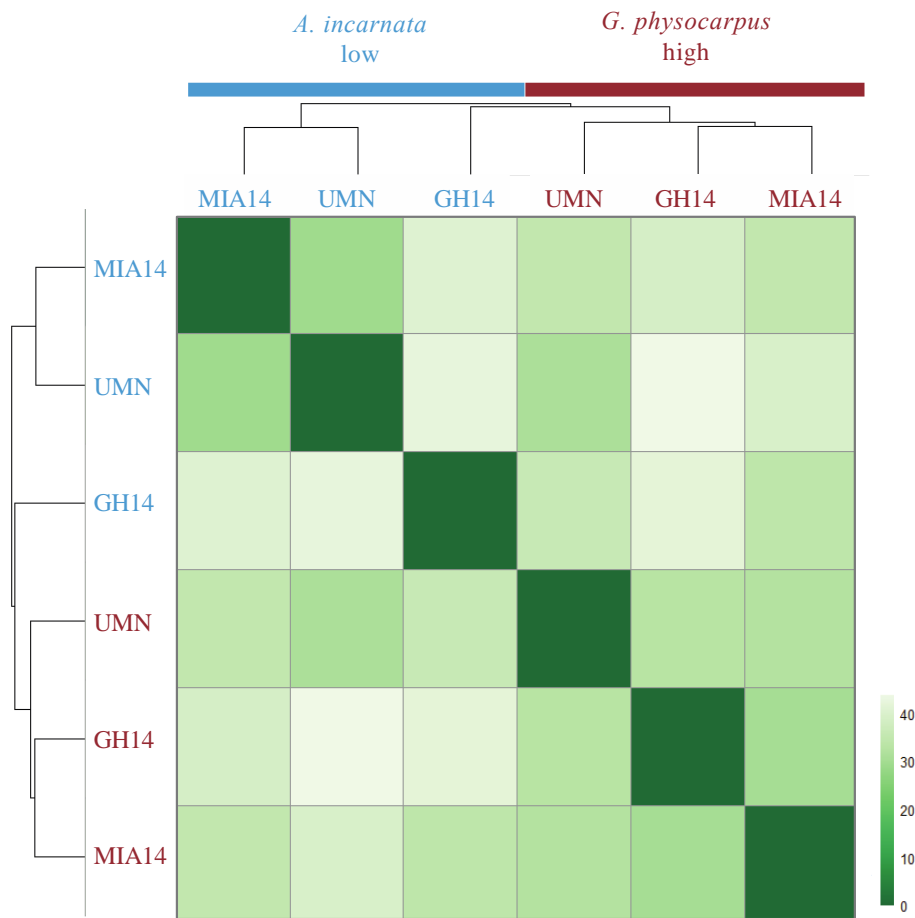
Figure 13. Sample clustering of the six sequenced samples for three different *A. nerii* genotypes (MIA14, GH14, and UMN). The heat map displays the sample-to-sample Euclidean distance of the rlog-transformed count data (DEseq2). The dendrogram shows hierarchical clustering of the samples based upon sample-to-sample distances.
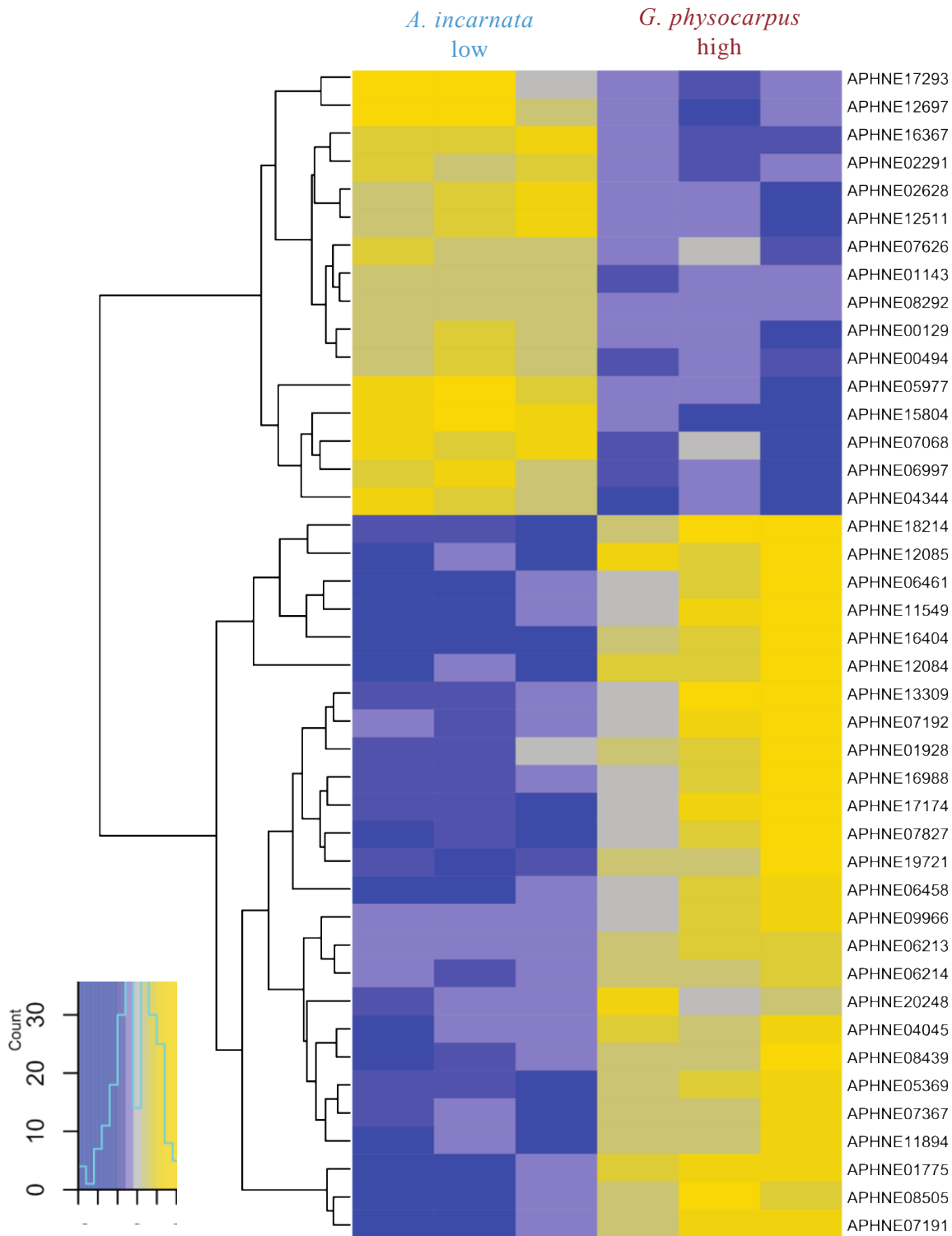
Figure 14. Clustering and heatmap analysis of per-sample abundance for all highly significantly differentially expressed transcripts (log$_2$FC $\geq$ 1, padj < 0.05) in the six sequenced samples (MIA14, GH14, UMN genotypes on *A. incarnata* and *G. physocarpus*). Two clusters were detected, one due to upregulation of genes when *A. nerii* were reared on low toxicity plants (top clade) and the other due to upregulation of genes when reared on high toxicity plants (bottom clade). Color intensities reflect Z-scores of DESeq2 variance stabilizing transformed read counts. Complete linkage clustering is performed on Z-score normalized read counts for each gene.
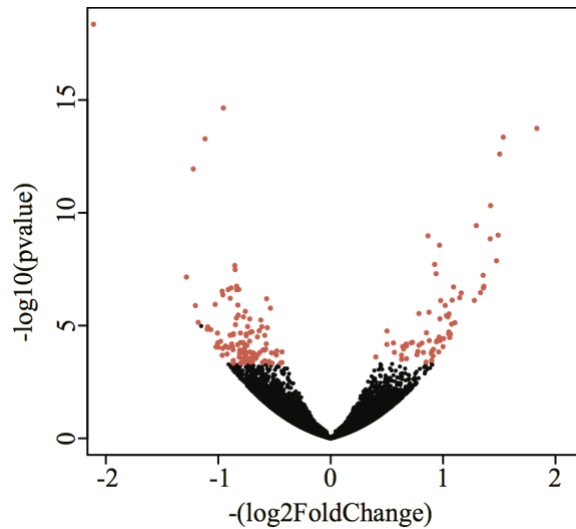
Figure 15. Volcano plot for all APHNE gene IDs. Horizontal axis shows the log$_2$ fold change observed in the DESeq2 fitted model between *A. neriii* samples exposed to low (*A. incarnata*, left) or high cardenolide (*G. physocarpus,* right) host plants. A greater number of genes are downregulated when *A. nerii* are exposed to high cardenolide host plants. The vertical axis reports adjusted p-values. Genes highlighted in red have an FDR < 0.05.

GO term	Ontology	Description	Number in input	Number in ref	adj. p-value
GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds	7	119	0.01383577
GO:0004558	F	alpha-1,4-glucosidase activity	4	15	0.00227312
GO:0005198	F	structural molecule activity	15	410	0.00227312
GO:0005509	F	calcium ion binding	11	358	0.04526743
GO:0008365	P	adult chitin-based cuticle development	2	2	0.01277833
GO:0015926	F	glucosidase activity	5	28	0.00227312
GO:0016798	F	hydrolase activity, acting on glycosyl bonds	8	131	0.00492737
GO:0019221	P	cytokine-mediated signaling pathway	4	38	0.04526743
GO:0019374	P	galactolipid metabolic process	4	14	0.00227312
GO:0032450	F	maltose alpha-glucosidase activity	4	15	0.00227312
GO:0042302	F	structural constituent of cuticle	10	70	$9.83*10^{-7}$
GO:0050664	F	oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	3	8	0.00744781
GO:0050665	P	hydrogen peroxide biosynthetic process	2	2	0.01277833
GO:0090599	F	alpha-glucosidase activity	4	17	0.00335053

Table 6. List of significant functionally enriched GO terms. All *A. nerii* differentially expressed transcripts with GO terms (109 genes) were compared to the annotated *A. nerii* transcriptome (12,162 genes). Ontology: P = metabolic process, F = molecular function.

Transcript	log2FC	adj. p-value	Protein	Function
APHNE15804	-2.105	$4.789*10^{-16}$	Legumain	hydrolysis
APHNE18214	1.845	$2.171*10^{-11}$	Probable cytochrome P450 6a13	detoxification/ redox
APHNE01775	1.547	$2.171*10^{-11}$	Pancreatic lipase-related protein 2	hydrolysis; digestion
APHNE12085	1.490	$8.183*10^{-7}$	Maltase 2	hydrolysis
APHNE11549	1.445	1.484*10-5	UDP-glucuronosyltransferase 2B16	detoxification/ conjugation
APHNE12084	1.439	4.319*10-6	Maltase 2	hydrolysis
APHNE05369	1.430	9.706*10-10	Major facilitator superfamily domain-containing protein 6	immunity
APHNE08505	1.424	4.611*10-8	Cathepsin B	immunity
APHNE13309	1.416	6.440*10-7	Uncharacterized family 31 glucosidase KIAA1161	hydrolysis
APHNE19721	1.402	9.913*10-8	Pancreatic lipase-related protein 2	hydrolysis; digestion
APHNE07367	1.370	4.053*10-10	E3 ubiquitin-protein ligase IAP-3	transferase
APHNE16404	1.365	6.294*10-5	UDP-glucuronosyltransferase 2B23	detoxification/ conjugation
APHNE04344	-1.307	9.848*10-6	Unannotated	unknown
APHNE06214	1.258	5.414*10-5	Probable RNA-directed DNA polymerase from transposon BS	housekeeping
APHNE07191	1.224	3.120*10-5	Legumain	hydrolysis
APHNE06213	1.215	3.566*10-5	Probable RNA-directed DNA polymerase from transposon X-element	housekeeping
APHNE07192	1.193	6.958*10-5	Legumain	hydrolysis
APHNE04045	1.146	2.354*10-4	Unannotated	unknown
APHNE16367	-1.123	0.002608	Protein TAR1	housekeeping
APHNE20248	1.108	0.002647	Radical S-adenosyl methionine domain-containing protein 2	immunity
APHNE17174	1.083	0.002195	Probable multidrug resistance-associated protein lethal(2)03659	detoxification/ transport
APHNE06461	1.083	0.001978	Probable juvenile hormone-inducible protein 26	unknown
APHNE12697	-1.082	2.510*10-4	Unannotated	unknown
APHNE01928	1.078	0.003054	Inactive pancreatic lipase-related protein 1	hydrolysis; digestion
APHNE11894	1.077	0.0002393	Maltase 2	hydrolysis

Table 7. List of top differentially expressed genes when *A. nerii* are reared on *G. physocarpus* compared to *A. incarnata*. The 25 transcripts with the greatest, significant absolute log2FoldChange are listed. Differential expression was calculated between the high- and low-toxic conditions with DESeq2.

Many genes, such as P450s and ABC transporters, have pleiotropic effects on stress and detoxification responses (Vogel et al., 2014), and genes differentially expressed in *A. nerii* could also be indicative of a generalized stress response. However, no GO terms classified under stress responses are enriched in *A. nerii* feeding on higher toxicity milkweed plants (Table 6). The transcriptomic responses to plant defensive and abiotic stresses have been investigated in a few aphid species, and comparison of enriched GO terms in these studies with those enriched in *A. nerii* feeding on different toxicity milkweed hosts reveal similarities and differences. Few GO terms enriched in *A. nerii* feeding on higher toxicity host plants were similarly enriched in stressed aphids in other studies (Bansal et al., 2014; Enders et al., 2015; Fen Li et al., 2017a; Sinha, Chandran, Timm, Aguirre-Rojas, & Smith, 2016; Vellichirammal, Madayiputhiya, & Brisson, 2016). These include 'structural constituent of cuticle' (GO:0042302) and 'calcium ion binding' (GO:0005509); 'structural constituent of cuticle' was enriched in soybean aphids (A. glycines) exposed to abiotic and biotic stressors (Bansal et al., 2014; Enders et al., 2015) and 'calcium ion binding' was enriched in wheat aphids (Diuraphis noxia) exposed to aphid resistant wheat (Sinha et al., 2016). No overlapping GO terms were found between the *A. nerii* differentially expressed genes and differentially expressed genes in pea aphids (*Ac. pisum*) exposed to abiotic stressors (Vellichirammal et al., 2016) nor in cotton aphids (*A. gossyppii*) exposed to several different plant allelochemicals (Fen Li et al., 2017a). Several of these studies found enriched GO terms involved in hydrolase activity (Fen Li et al., 2017a; Sinha et al., 2016) and oxioreductase acitivity (Bansal et al., 2014;

Enders et al., 2015; Fen Li et al., 2017a; Sinha et al., 2016; Vellichirammal et al., 2016), but the specific activity of the enriched genes differs. Given that cardenolide molecules vary by glycoside structures attached to the core steroid molecule (Repke, 1985), it is intriguing that the GO terms involving hydrolase activity enriched in *A. nerii* feeding on higher toxicity milkweeds act on glycosyl compounds (GO:0004553, GO:0016798; Table 6).

To further investigate differential expression of genes putatively involved in *A. nerii*'s adaptation to milkweed plants, *A. nerii* were reared on host plants of varying toxicity and candidate genes selected from the RNAseq experiment (padj < 0.05, fold change ≥ 1.5) were measured using qPCR. Patterns of gene expression using qPCR confirmed patterns of differential expression observed in the RNAseq experiment for the majority of genes tested (Figs. 16, 17, Table 8). Host plant had a significant effect on gene expression for 12 genes tested and qPCR confirmed the direction of differential expression between *A. incarnata* and *G. physocarpus* observed in the RNAseq experiment (Fig. 16). Gene expression did not significantly vary based on host plant species on which the aphids were reared for four genes tested, however, differential expression directional trends were similar between the RNAseq experiment and qPCR experiments for these genes (Fig. 17b-d). When *A. nerii* were reared on host plants varying in toxicity (*A. incarnata* (low), *A. curassavica* (medium), and *G. physocarpus* (high)), qPCR verified significant differential expression of genes in several categories, including canonical detoxification molecules (Fig. 16a-e); genes putatively promoting the disruption of specific binding through hydrolysis or oxidation/ reduction of cardenolides (Fig. 16f-i); immunity (Fig. 16j, k); and development (Fig. 16l). *A. nerii* genotype had a significant effect on gene expression for two genes, an esterase FE4 and a glycoside hydrolase (Fig. 17a, b), and there was a significant interaction effect between host plant and genotype for two genes, UDP-glucoronosyltransferase 2B33 and juvenile hormone acid O-methyltransferase (Fig. 16e, l). For the intermediate toxicity *A. curassavica,* patterns of *A. nerii* gene expression were remarkably consistent across candidate genes, with expression patterns on *A. curassavica* either intermediate between lower and higher cardenolide host plants or matching that of the higher cardenolide host plant.
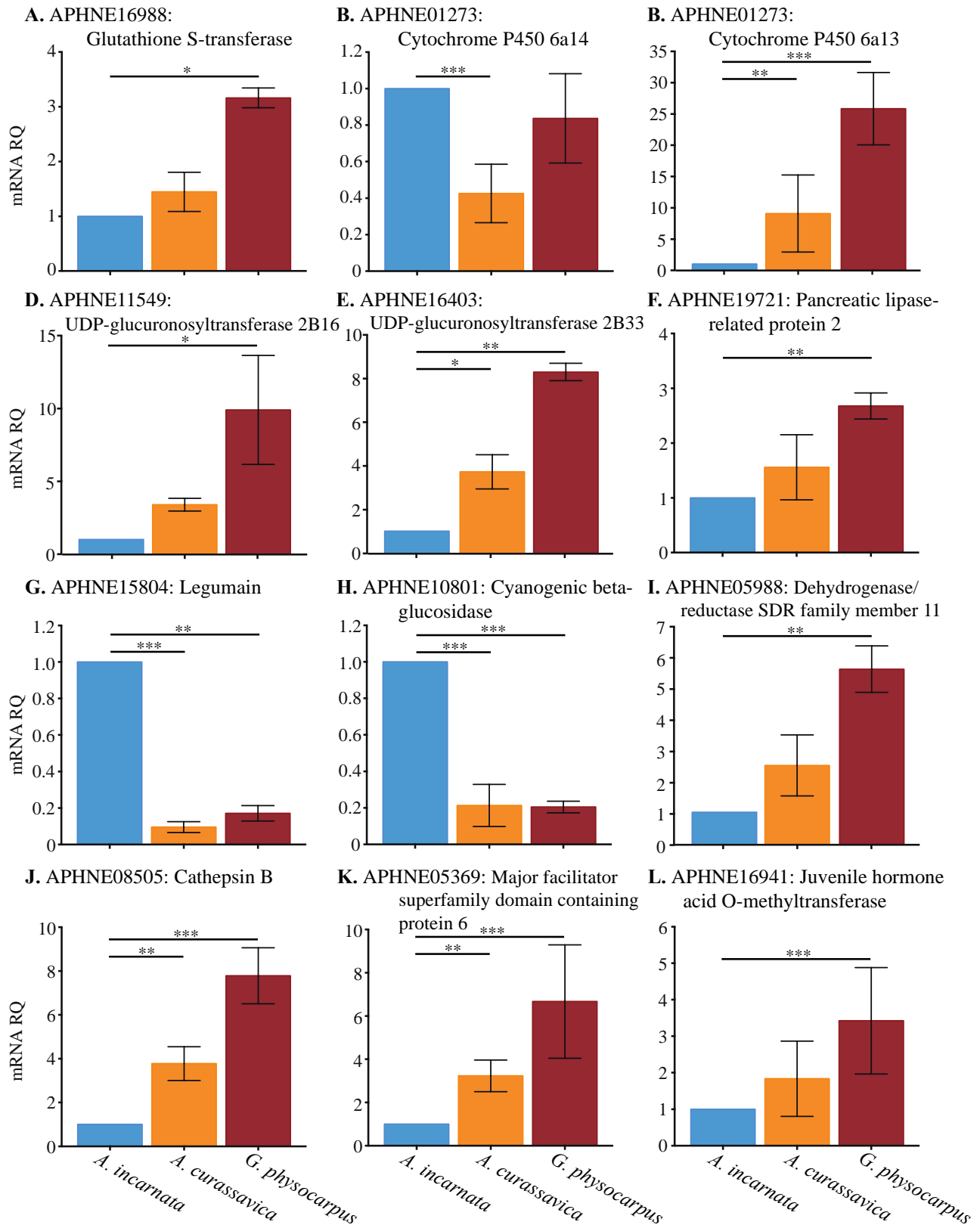
Figure 16. Quantitative RT-PCR analysis of candidate gene expression of *A nerii* reared on *A. curassavica* and *G. physocarpus* relative to expression when reared on *A. incarnata*. Trinity *A. nerii* transcript prefixes follow gene names. **A-E.** Canonical detoxification genes: Glutathione S-transferase (APHNE16988), Probable cytochrome P4506a14 (APHNE01273), Probable cytochrome P450 6a13 (APHNE18214), UDP-glucuronosyltransferase 2B16 (APHNE11549), UDP-glucuronosyltransferase 2B22 (APHNE16403). **F-H.**

Hydrolysis activity: Pancreatic lipase-related protein 2 (APHNE19721), Legumain (APHNE15804), Cyanogenic beta-glucosidase (APHNE10801). **I.** Oxioreductase activity: Dehydrogenase/ reductase SDR family member 11 (APHNE05988). **J, K.** Immunity genes: Cathepsin B (APHNE08505), Major facilitator superfamily domain containing protein 6 (APHNE05369). **L.** Development gene: Juvenile hormone acid O-methyltransferase (APHNE16941). Graphs display the mean +/- SEM fold difference in average mRNA levels (2 biological replicates of 5 adult pooled aphids each; 2 technical replicates each). Bars and stars represent significant differences in gene expression when compared to *A. incarnata* (Tukey's multiple comparisons test; * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$).
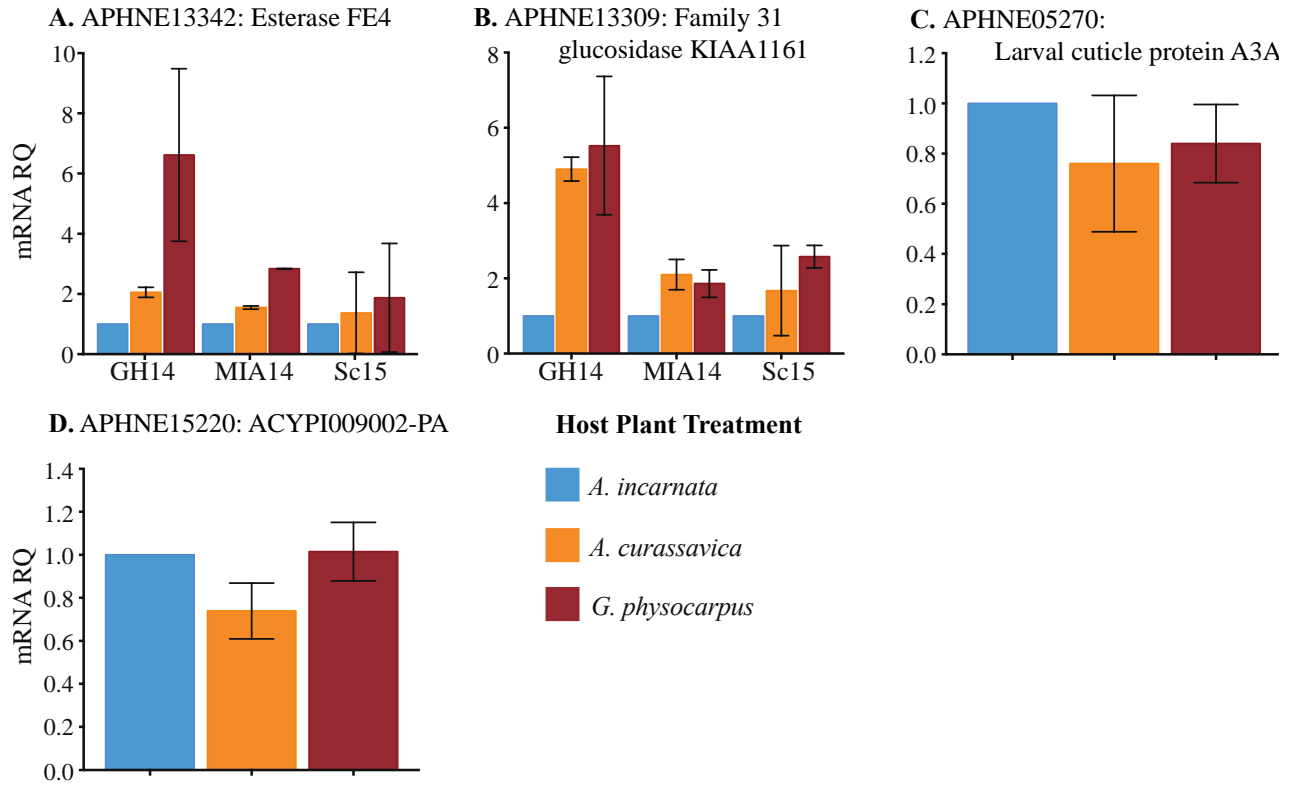
Figure 17. Quantitative RT-PCR analysis of candidate gene expression of *A nerii* reared on *A. curassavica* and *G. physocarpus* relative to expression when reared on *A. incarnata*. **A.** Canonical detoxification gene: Esterase FE4 (APHNE13342). **B.** Hydrolysis promoting gene: Family 31 glucosidase KIAA1161 (APHNE13309). **C.** Development gene: Larval cuticle protein A3A (APHNE05270). **D.** Uncharacterized gene: ACYPI009002-PA (APHNE15220). Graphs display the mean +/- SEM fold difference in average mRNA levels (2 biological replicates of 5 adult pooled aphids each; 2 technical replicates each).

Transcript	Host Plant	Genotype	Interaction
Figure 3			
A. APHNE16988	$F_{(2,9)}$ = 5.99 p = 0.0222	$F_{(2,9)}$ = 0.028, p = 0.9729	$F_{(4,9)}$ = 1.25, p = 0.3572
B. APHNE01273	$F_{(2,9)}$ = 22.7, p = 0.0003	$F_{(2,9)}$ = 0.025, p = 0.9753	$F_{(4,9)}$ = 2.22, p = 0.1472
C. APHNE18214	$F_{(2,9)}$ = 31.71, p < 0.0001	$F_{(2,9)}$ = 0.25, p = 0.7853	$F_{(4,9)}$ = 1.47, p = 0.2958
D. APHNE11549	$F_{(2,9)}$ = 6.59, p = 0.0173	$F_{(2,9)}$ = 0.44, p = 0.6540	$F_{(4,9)}$ = 0.97, p = 0.4687
E. APHNE16403	$F_{(2,9)}$ = 11.67, p = 0.0032	$F_{(2,9)}$ = 3.29, p = 0.0846	$F_{(4,9)}$ = 4.14, p = 0.0358
F. APHNE19721	$F_{(2,9)}$ = 9.96, p = 0.0052	$F_{(2,9)}$ = 0.60, p = 0.5686	$F_{(4,9)}$ = 0.56, p = 0.6961
G. APHNE15804	$F_{(2,9)}$ = 23.56, p = 0.0003	$F_{(2,9)}$ = 2.29, p = 0.1567	$F_{(4,9)}$ = 1.27, p = 0.3511
H. APHNE10801	$F_{(2,9)}$ = 32.53, p < 0.0001	$F_{(2,9)}$ = 1.50, p = 0.2734	$F_{(4,9)}$ = 0.88, p = 0.5140
I. APHNE05988	$F_{(2,9)}$ = 9.03, p = 0.0071	$F_{(2,9)}$ = 0.30, p = 0.7457	$F_{(4,9)}$ = 0.90, p = 0.5039
J. APHNE08505	$F_{(2,9)}$ = 32.93, p < 0.0001	$F_{(2,9)}$ = 0.56, p = 0.5880	$F_{(4,9)}$ = 0.32, p = 0.8563
K. APHNE05369	$F_{(2,8)}$ = 47.7, p < 0.0001	$F_{(2,8)}$ = 0.069, p = 0.9340	$F_{(4,8)}$ = 1.56, p = 0.2744
L. APHNE16941	$F_{(2,9)}$ = 19.99, p = 0.0005	$F_{(2,9)}$ = 0.086, p = 0.9179	$F_{(4,9)}$ = 5.95, p = 0.0127
Supplemental Figure 5			
A. APHNE13342	$F_{(2,9)}$ = 8.40, p = 0.0088	$F_{(2,9)}$ = 10.55, p = 0.0044	$F_{(4,9)}$ = 2.79, p = 0.0927
B. APHNE13309	$F_{(2,9)}$ = 0.94, p = 0.4252	$F_{(2,9)}$ = 7.20, p = 0.0136	$F_{(4,9)}$ = 1.62, p = 0.2502
C. APHNE05270	$F_{(2,9)}$ = 0.031, p = 0.9693	$F_{(2,9)}$ = 0.16, p = 0.8508	$F_{(4,9)}$ = 0.58, p = 0.6830
D. APHNE15220	$F_{(2,9)}$ = 1.78, p = 0.2233	$F_{(2,9)}$ = 1.40, p = 0.2963	$F_{(4,9)}$ = 1.26, p = 0.8969

Table 8. Statistical results from two factor ANOVA analyses completed to test the effects of milkweed host plant species and *A. nerii* genotype on *A. nerii* expression of key candidate genes. ANOVA tests were performed on log2 transformation of relative abundance values (log2(ΔCT)) in Prism7.

Discussion

Some insects that feed on milkweed and sequester cardenolides have converged on a common mechanism for managing the toxicity of cardenolides, involving protein-level changes that inhibit cardenolide binding to the Na^+/K^+ ATPase (*i.e.,* target site insensitivity (Dobler et al. 2012; Zhen et al.2012)). However, many insects specialize on milkweed but exhibit Na^+/K^+ ATPase sensitivity, indicating that alternative mechanisms are at work. In the present study, we asked whether variation in toxicity across milkweed host plants affects the performance of an aphid herbivore that specializes on them but lacks target site insensitivity, and used this variation to profile the global, genome-level response to host plant toxicity. *A. nerii* experience reduced fitness when feeding on plants with higher levels of cardenolides. While only a small number of genes were differentially expressed, these genes were enriched for functions related to detoxification. For *A. nerii*, which specializes on host plants that vary considerably in toxicity, a combination of inducible enzymatic and transport/excretory detoxification mechanisms appear to be particularly important.

Effect of increased host plant toxicity on insect fitness

A. nerii specializes on oleander and milkweed plant species which are characterized by their production of cardiac glycosides. Milkweed plants exhibit marked variation in the types, concentrations, and toxicity of cardenolides they characteristically produce and also vary in other physiological traits and chemical defenses

such as trichomes, latex, and phenolics (A. A. Agrawal, Fishbein, Halitschke, Hastings, et al., 2009a; Rasmann & Agrawal, 2011). Some studies have demonstrated no effect of cardenolides on *A. nerii* survival, fecundity, development, density, and growth rate, but these studies focused on low toxicity plant species or experimental applications to vary cardenolide production within a species (Mooney et al., 2008; 2010; Zehnder, Parris, & Hunter, 2007). Comparing milkweed species that vary more substantially in cardenolide toxicity, we found that host plants with increased and more non-polar cardenolides have negative effects on *A. nerii*, consistent with other studies that have demonstrated that plant species characterized by more toxic cardenolides are negatively associated with *A. nerii* population growth (A. A. Agrawal, 2004; Colvin, Snyder, & Thacker, 2013; de Roode et al., 2011).

While cardenolide variation is the most likely reason for the fitness effects seen here and in other studies (A. A. Agrawal, 2004; Colvin et al., 2013; de Roode et al., 2011), milkweeds utilize a suite of defenses and it is possible that other plant defenses could also play a role in influencing these insects' fitness. However, latex, trichomes and total phenolics do not correlate with cardenolides (A. A. Agrawal & Fishbein, 2006; A. A. Agrawal, Salminen, & Fishbein, 2009b). Nutritional differences between host plants could also play a role, however, a previous study reported that increased cardenolides have minimal effects on the aphids' ability to obtain normal nutrients; honeydew collected from *A. nerii* reared on *A. incarnata* and *A. curassavica* differed in cardenolides, but not total sugar or amino acid concentration (Pringle et al., 2014). This suggests that if these two hosts differ in their nutritional sufficiency for *A. nerii*, these effects are minor in comparison to differences in cardenolides. More likely, costs may be attributable to direct and/ or indirect effects of secondary metabolite toxicity, from direct effects of toxins with their target sites, reactive metabolic byproducts, or to the costs of increased resource allocation to detoxification itself. Future work could confirm this by adding synthesized cardenolides to host plants, though to our knowledge, such synthesis of milkweed chemicals has not been achieved to date.

Although specialist insects may thrive on their well-defended host plants (D. A. Peterson et al., 2015; Smilanich, Fincher, & Dyer, 2016), resistance and tolerance to natural or synthetic xenobiotics are expected to be associated with fitness costs, because responses to plant defenses require energy allocations or have pleiotropic effects that compromise other physiological processes (Coustau et al., 2000). Experiments on the specialist lepidopteran, *Danaus plexippus*, suggest negative effects of increased cardenolides on monarch larval survival and adult longevity (Lefèvre et al., 2010; Sternberg et al., 2012; L. Tao, Hoang, Hunter, & de Roode, 2016) driven by both the overall concentration and the amount of non-polar cardenolides present in host plants (L. Tao et al., 2016). Ultimately, whether or not costs are detectable depends upon both the ecological context as well as particulars of the molecular mechanisms involved: the degree to which, for example, enzymes involved in detoxification exhibit broadly activity across unrelated substrates (Khersonsky & Tawfik, 2010). Consequently, there is evidence of both costly (Cresswell, Merritt, & Martin, 1992; Rand et al., 2015) and cost-free detoxification of xenobiotics in insects (McCart, Buckling, & ffrench-Constant, 2005). In hemipterans, the fitness costs of detoxification of naturally-occurring xenobiotics have been most thoroughly characterized in dietary generalists, and overall, the pattern is consistent with herbivores generally: that both molecular and ecological specifics generate strong context dependency. Castenada et al. (Castaneda, Figueroa, Fuentes-Contreras, Niemeyer, & Nespolo, 2009; Castañeda, Figueroa, & Nespolo, 2010) found little evidence of costs in the cereal aphid *Sitobion avenae* for constitutive expression of detoxification enzymes acting on allelochemicals common to their host plants. By contrast, Kliot et al. (Kliot et al., 2014) showed that nicotine-resistant whitefly strains (*Bemisia tabaci*) on tobacco exhibit reduced fecundity relative to susceptible strains on low nicotine diets. While high levels of constitutively expressed enzymes must bear some costs, what is not clear yet is the extent to which inducible expression of detoxification enzymes themselves are costly, especially in the context of the evolution of diet breadth in insects (A. A. Agrawal, Vala, & Sabelis, 2002).

Further complicating the interpretation of how costs of milkweed toxicity are expressed in *A. nerii* is the fact that, unlike monarchs and other holometabolous insects that specialize on milkweed, the negative effects of host plant toxicity are incurred across all life stages of *A. nerii,* acting on multiple life history traits over their lifetime (*i.e.* development, lifetime fecundity). Sap-feeders interact with their host plants differently than chewing insects. Aphids feed on plant phloem by creating sieve tubes and avoid plant defenses to plug sieve

tubes by controlling calcium influx from the plant (Will, 2006), and overexpression of calcium signaling proteins in aphids has been implicated in tolerance to aphid-resistance plants (Sinha et al., 2016). The enrichment of differentially expressed genes involved in calcium ion binding (Table 2) suggests that *A. nerii* may also depend on strategies to deplete plant calcium to effectively feed on different milkweed species. Finally, the effects of increased cardenolides are dependent on the ecological context. Insects that feed on and sequester compounds from toxic host plants are assumed to do so for the protective benefits provided by the chemicals against predators and parasites (Després et al., 2007; Petschenka & Agrawal, 2016), however, studies investigating the effect of increased host plant toxicity demonstrate costs (Reudler, Lindstedt, Pakkanen, Lehtinen, & Mappes, 2015; Smilanich et al., 2009), benefits (Barthel et al., 2016; Lefèvre et al., 2010), and no effects (Cogni, Trigo, & Futuyma, 2012) on herbivore fitness. Protozoan parasite-infected monarchs feeding on higher cardenolide host plants have increased survival and disease tolerance (Lefèvre et al., 2010; Sternberg et al., 2012), but not without associated costs (L. Tao et al., 2016). Likewise, the negative effects of increased cardenolides on *A. nerii* observed in the laboratory may be ameliorated under varying ecological conditions. Previous studies indicate that host plants with increased cardenolides can have a negative effect on parasitoids of *A. nerii* (Colvin & Yeargan, 2013; Desneux et al., 2009), indicating that ecological context is important in *A. nerii* as in other milkweed specialists.

Effect of increased host plant toxicity on insect gene expression

Despite having been a model system for insect specialization on toxic plants and sequestration for decades, relatively few mechanisms, outside of target-site insensitivity, of cardenolide resistance and tolerance have been described in milkweed herbivores. While many milkweed-adapted species exhibit target-site insensitivity to mitigate the toxicity of cardenolides (Dobler et al., 2012; Zhen et al., 2012), these adaptations do not confer complete resistance to cardenolide toxicity (Petschenka, Fandrich, Sander, Wagschal, Boppré, et al., 2013a). Thus, both target-site insensitive and sensitive milkweed-adapted insects must possess other adaptations to tolerate host plant cardenolides. Adaptation towards cardenolides likely requires synergistic mechanisms involving the modification of cardenolides to polarize and/or degrade them, the protection of sensitive tissues from cardenolides, possibly through expression of molecules such as efflux carriers, and the efficient excretion of cardenolides and their metabolic byproducts (A. A. Agrawal et al., 2012; Petschenka & Agrawal, 2016; Petschenka, Pick, Wagschal, & Dobler, 2013b). We found a large diversity of P450s, UGTs, and ABC transporters expressed in *A. nerii* feeding on milkweed plants, and specific members of these groups were differentially expressed in *A. nerii* feeding on higher toxicity host plants. The coordinated action of P450s, UGTs, and transporters has been well documented in drug metabolism (Bock, Bock, Köhle, & Köhle, 2004), and the role of these genes in adaptation to milkweeds should be further investigated.

Given the effects of milkweed host plant species on multiple aspects of *A. nerii* fitness, it is perhaps surprising that milkweed species had such a small effect on *A. nerii* gene expression, particularly on primary metabolism genes. However, importantly, this host plant differential expression analysis does not analyze differences between tissue types and encompasses only genes that were commonly differentially expressed between all three genotypes and, thus, represents the most conservative consensus of differential gene expression. Only 0.21% of transcripts were highly differentially expressed (padj < 0.05, $\log_2$FC $\geq$ 1), 1.1% differentially expressed regardless of fold change (padj < 0.05), when *A. nerii* were reared on low compared to high toxicity host plants. Other studies examining specialist insect transcriptomic responses to novel host plants or variable plant chemistry have demonstrated a range of magnitude of response with some studies finding upwards of 20% of genes differentially expressed between treatments (De Panis et al., 2016; Ragland et al., 2015; Rispe et al., 2016). Nevertheless, the small effect of variable host plants on *A. nerii* gene expression is in accordance with other studies that find lower differential expression in specialist compared to generalist lepidopterans when feeding on host plants varying in defenses (Govind et al., 2010; Schweizer et al., 2017). One assumption is that plasticity in gene expression is a strategy for managing the cost of production of detoxification enzymes and fine-tuning response to environmental signals, such as plant allelochemicals (Brattsten, 1979). Constitutive expression may be favorable in conditions where mechanisms of sensing and

gene regulation are costly (Savageau, 1977; Terriere, 1984) or where fast responses to predictably adverse environments are necessary (Geisel, 2011). Strategies for constitutive and inducible gene expression are predicted to be different between generalist and specialist insect herbivores (Ali & Agrawal, 2012; Schweizer et al., 2017). At the moment, few studies have evaluated the patterns and performance of specialist and generalist insects in the context of genome-wide responses to plant defenses (Govind et al., 2010; Schweizer et al., 2017).

A majority of differentially expressed genes have putative functions in milkweed adaptations including several canonical detoxification pathways (P450s, esterase FE4, GST, UGTs, multidrug resistance-associated proteins, and ABC transporters), as well as several transcripts with proposed hydrolysis functions (legumains, glucosidases, lipases, maltases). Multidrug transporters were recently identified to protect *Drosophila melanogaster* from cardenolide toxicity (Groen et al., 2017) and are likely important in *A. nerii*'s adaptation to milkweed hosts. GSTs and UGTs both act to conjugate groups to substrates to facilitate excretion of toxic compounds (King et al. 2000; Sheehan et al. 2001). In contrast, P450s and esterases facilitate degradation of toxins through oxidation and hydrolysis, respectively (Scott 1999; Jackson *et al.* 2013). It is notable that two active transport protein groups previously associated with cardenolide tolerance, organic anion transporters and p-glycoprotein-like transporters in lepidopterans, did not have highly similar proteins differentially expressed in *A. nerii* (Groen et al., 2017; Petschenka, Pick, Wagschal, & Dobler, 2013b; Torrie et al., 2004).

A primary barrier mitigating the toxicity of ingested cardenolides may involve the restriction of movement of cardenolides from the gut to the hemocoel, and this may entail restructuring of gut membranes including cuticular structures (Dobler et al., 2011; Linser & Dinglasan, 2014). Indeed, genes involved in the structure and development of the cuticle were significantly differentially expressed when reared on different milkweed host plants and were significantly enriched in the differentially expressed gene set as compared to the full transcriptome. Metabolism of cardenolides through oxidation or hydrolysis has also been proposed as a potential primary mechanism of resistance for cardenolide adapted insects (A. A. Agrawal et al., 2012), and here, we observe a high number of hydrolysis promoting genes (legumains, glucosidases, lipases, maltases) differentially expressed as well as enrichment of GO terms involved in catalysis of hydrolysis in *A. nerii* feeding on higher cardenolide host plants. These genes could function to break bonds in the core steroid structure of cardenolides, thus disrupting the very specific binding of cardenolides to sensitive Na^+/K^+ ATPases (Keenan, DeLisle, Welsh, Paula, & Ball, 2005; Paula, Tabet, & Ball, 2005).

Furthermore, the expression of multiple P450s and differential expression of specific CYP genes in *A. nerii* feeding on more toxic host plants parallels the role of P450s in xenobiotic resistance and host plant adaptation in other insects (Berenbaum, Favret, & Schuler, 1996; Puinean et al., 2010; X.-Y. Tao et al., 2012; Wybouw et al., 2015; F. Zhu, Moural, Nelson, & Palli, 2016). P450s were classically described in the adaptive radiation of Papidionidae lepidopteran species (Berenbaum et al., 1996; Schuler & Berenbaum, 2013), and since, specific CYP6 genes have been identified in phytotoxin resistance in the white fly, *Bemisia tabaci*, and in aphid species (Halon et al., 2015; Peng et al., 2016; Ramsey et al., 2014). Two different CYP6 genes were differentially regulated in opposite directions in *A. nerii* feeding on *G. physocarpus*, suggesting a role for specific P450s in mediating milkweed toxicity.

UGTs catalyze the conjugation of glucuronic acid to a substrate and produce more polar compounds. Their enrichment in *A. nerii* feeding on host plants with increased and more non-polar cardenolides suggests a role for their involvement in *A. nerii*'s adaptation towards cardenolides; non-polar cardenolides are considered to be more toxic to insects than polar cardenolides, as these compounds can passively cross membranes. Zust and Agrawal (2015) identified a polar cardenolide in *A. nerii* feeding on *A. syriaca*, a low cardenolide milkweed species, that was not represented in its dietary cardenolide profile, indicating that *A. nerii* possess the ability to modify cardenolides post-ingestion. This finding, in combination with our finding that UGTs increase in expression when aphids feed on more non-polar cardenolides, suggests a possible role for UGTs in converting cardenolides to less toxic, more polar forms. Moreover, it has been observed that during sequestration in lepidopterans, cardenolides are converted into more polar forms and thus UGTs may play an important role in the sequestration of these compounds in other insects (Brower et al., 1984; Seiber et al., 1980). UGTs were also differentially expressed in *Heliconius melpomene* larvae when feeding on more chemically defended *Passiflora*

host plants (Yu et al., 2016), suggesting a broader, overlooked role for UGTs in host plant specialization and secondary metabolite detoxification.

Novel genes putatively involved in cardenolide adaptation and sequestration

Remarkably, relatively little is known about the genes and proteins involved in insect sequestration of plant toxins outside of a few model systems (Erb et al., 2016; Petschenka & Agrawal, 2016). One efflux carrier has been described to protect sensitive tissues from cardenolides in a cardenolide-adapted lepidopteran species, however, no specific carriers have been identified in cardenolide sequestration (Petschenka & Agrawal, 2016). Previous analyses of cardenolides in *A. nerii* whole bodies compared to their honeydew revealed that aphids excrete more polar compounds while retaining non-polar cardenolides, suggestive of passive sequestration (Malcolm, 1990; Züst & Agrawal, 2016). Efficient excretion of polar cardenolides observed previously with the large number of detoxification genes differentially expressed here suggest that *A. nerii*'s tolerance of cardenolides may arise from efficient conversion of non-polar cardenolides to less toxic forms by UGTs and a large number of enzymes to metabolize cardenolides.

In active sequestration, transport proteins are proposed to translocate polar cardenolides through gut membranes in some species (Frick & Wink, 1995; Petschenka, Pick, Wagschal, & Dobler, 2013b), including monarchs (Frick & Wink, 1995), oleander moths (*Syntomeida epilais*) (Nickisch-Rosenegk et al., 1990), and large milkweed bugs (*Oncopeltus fasciatus*) (Scudder, Moore, & Isman, 1986). In the leaf beetle, *Chrysomela populi*, a multidrug resistance-associated protein, identified as an ABC transporter subfamily C, is involved in the transport of plant metabolites to defensive glands (Strauss et al., 2014). We identified the differential expression of a transcript homologous to ABC transporter G family member 20 and two multidrug resistance-associated proteins representing a probable function in cardenolide sequestration. Enzymatic assays and tissue specific expression of transporters are necessary to confirm the role of ABC transporters in cardenolide adaptation and/ or sequestration. Esterases and GSTs have also been shown to be involved in insect sequestration of insecticides (Hemingway, 2000; Kostaropoulos, Papadopoulos, Metaxakis, Boukouvala, & Papadopoulou-Mourkidou, 2001), and our identification here of transcripts in each of these classes prompts future investigations into their involvement in cardenolide sequestration. Moreover, several transcripts with homologies to various receptors and transporters provide interesting candidates for novel genes involved in sequestration.

Conclusions

In summary, we present the first whole transcriptome analysis for cardenolide degradation and metabolism in a milkweed specialist and show that *A. nerii* differentially express a targeted set of conserved pathways involved in enzymatic and transport/excretory detoxification to order to tolerate increased secondary metabolites, but not without a cost to their development and reproduction. Hydrolysis, polarization, and transport of cardenolides by a number of proteins are likely processes mediating *A. nerii*'s tolerance of cardenolides in milkweed host plants, however, future experiments are necessary to empirically test candidate gene interactions with cardenolides. These may include experiments using artificial diets with commercially available polar and non-polar cardenolides or experiments knocking down specific candidate genes. Moreover, given the critical role the gut of aphids plays in the general detoxification of compounds, and the barrier it provides against cardenolide damage to sensitive, internal tissues, specific tissue experiments are required to understand the genes immediately involved in *A. nerii*'s tolerance to cardenolides and their coevolution with milkweed host plants.

The biochemical adaptations plants and insects exhibit and the signatures found in the proteins and enzymes that confer resistance and counter-resistance have provided the foundation for our understanding of the coevolution process (Ehrlich & Raven, 1964; Xianchun Li, Schuler, & Berenbaum, 2007). In recent years, the focus has turned increasingly on the gene regulatory mechanisms that underpin plant defenses and insect

adaptations to plant defense chemistry. It remains unclear how specialist and generalist insects might differ in the patterns and extent of expression plasticity (Govind et al., 2010; Schweizer et al., 2017) or in the architecture of the signaling networks that translate environmental variation into genomic responses (Ragland et al., 2015; Wybouw et al., 2015). Disentangling how these differences shape responses to new host plants or other stressors is a pivotal step in understanding the evolution of specialization. More broadly, there are open questions about the nature of the evolutionary factors that lead to specialization and whether the classic Ehrlich & Raven coevolutionary scenario is well-supported by the current evidence (D. A. Peterson, Hardy, & Normark, 2016). To address these questions and to provide synthesis, there is a need for broad comparative phylogenetic studies of joint patterns of differential expression in specialist and generalist insects (Ali & Agrawal, 2012).

TRANS-GENERATIONAL TRANSCRIPTOMIC RESPONSE TO NATURAL VARIATION IN HOST PLANT TOXICITY AND INSECTICIDES IN A SPECIALIST INSECT

Author Contributions: Stephanie S.L. Birnbaum designed, executed, and analyzed all experiments in this Chapter. S.S.L.B. wrote this Chapter and created all Figures and Tables. Patrick Abbot edited the manuscript.

Abstract

Insects have been challenged by plant secondary metabolites throughout their evolutionary history. An important mechanism thought to promote insecticide resistance is the ability of insects to use preexisting detoxification systems originally evolved for tolerance of plant defenses. Yet, it remains unclear what level of convergence exists in metabolic mechanisms employed against various natural and xenobiotic chemicals. How do transcriptomic responses to these stressors change in response to novel and long-term exposure? We employed an experimental evolution approach in a milkweed-specialist aphid (*Aphis nerii*) model to test the effects of a more toxic host plant species and two insecticides (a neonicotinoid, imidacloprid, and a general homopteran blocker, pymetrozine) on aphid gene expression and fitness over multiple generations of selection. Aphids were transferred from low toxic plants and selected on three stress treatments (a more toxic host plant species, imidacloprid, pymetrozine) for five generations. Whole transcriptome gene expression changes and changes in development time and fecundity were compared at generations one and five. While there were no consistent fitness costs or benefits to long-term exposure, exposure to stress is associated with increased transcriptional plasticity and changes in genes associated with the metabolism of secondary metabolites as well as genes important in transcription, translation, and post-translation processes.

Introduction

Adaptations that insects have acquired over evolutionary time to overcome plant defenses are hypothesized to provide a basis for the evolution of insecticide resistance. Various insecticides and plant secondary metabolites have common targets in insects, and it is thought that adaptations to plant toxins may prime, or "pre-adapt" insects to tolerate insecticides (Alyokhin & Chen, 2017; Dermauw, Pym, Bass, Van Leeuwen, & Feyereisen, 2018; Hardy et al., 2017; Hawkins, Bass, Dixon, & Neve, 2018). However, this "pre-adaptation" hypothesis has rarely been directly tested (Hardy et al. 2017). Are the genes important in host plant adaptation also employed in the resistance or tolerance of insecticides?

Resistance to pesticides has been well documented and threatens food security worldwide, and an understanding of the likely molecular mechanisms is necessary for predictions of when resistance should be expected and efficient agricultural practices (ffrench-Constant, 2013; Gould, Brown, & Kuzma, 2018). Resistance can arise from *de novo* mutations or from selection on existing genetic variation, and experimental evolution approaches provide opportunities to examine whole genome or transcriptome changes over the course of adaptation to xenobiotic or stress treatments (Burke, 2012; Burke, Liti, & Long, 2014; Hawkins et al., 2018; Sørensen, Schou, & Loeschcke, 2017; Vogwill, Lagator, Colegrave, & Neve, 2012). While sexual recombination is an important source for genetic variation (Burke et al., 2014; Jacomb, Marsh, & Holman, 2016), experiments with asexual organisms allow for the examination of variation based on random mutations, genetic drift, or selection on standing variation alone.

The pre-adaptation hypothesis is premised on the idea that resistance to synthetic pesticides and plant chemical defenses share common underlying mechanisms, conferring a degree of cross-resistance that accelerates adaptation upon novel exposure (Hardy et al. 2017). Target-site modifications, changes in metabolism or detoxification, and sequestration can play important roles in both adaptation to toxic plants and insecticide resistance. Target-site modifications have been described in insect adaptations to toxic plants (Dobler et al., 2012; Zhen et al., 2012) and in *Tribolium* resistance to cyclodienes (Andreev, Kreitman, Phillips, Beeman, & ffrench-Constant, 1999). Differential expression and diversification of detoxification genes, including esterases, glutathione S-trasnferases, UDP-glycosyltransferases, ABC transporters (Birnbaum et al., 2017; Crava et al., 2016; Halon et al., 2015; Jackson et al., 2013; Krempl et al., 2016; Fen Li et al., 2017a; Rix, Ayyanath, & Cutler, 2015; Sun, Pu, Chen, Wang, & Han, 2017), and, most notably, cytochrome P450s, has been characterized in plant adaptations in diverse herbivorous insects (Bansal & Michel, 2018; Calla et al., 2017; Peyser, Lanno, Shimshak, & Coolon, 2017; Puinean et al., 2010) and in resistance to diverse insecticides (Bajda et al., 2015; Clements, Schoville, Peterson, Lan, & Groves, 2016; Denecke et al., 2017; Ilias et al., 2015; X.-Y. Tao et al., 2012; F. Zhu et al., 2016).

Changes in the expression of genes involved in degradation or sequestration of insecticides is a major mode of evolution of pesticide resistance (Dermauw et al., 2018; Délye, Jasieniuk, & Le Corre, 2013). Initial genomic responses to new stressors, such as those that herbivorous insects experience when encountering novel host plants or insecticides, are expected to be mostly mediated by changes in gene regulation, as opposed to novel mutations that confer heritable adaptive advantages. Such transcriptional plasticity is known to play an important role in adaptation to novel environments (Mäkinen et al., 2017; Scheiner & Holt, 2012), however, changes in whole transcriptome expression in response to a novel plant over time are largely ignored in studies of plant-insect transcriptional interactions. Gene expression differences in response to a novel environment may also be maladaptive or a general response to stress. Over time, adaptive plasticity can be selected upon through the survival and reproduction of adapted individuals. Genes initially differentially expressed in a novel condition that are responsible for an adaptive response may become constitutively expressed over time through a process known as genetic assimilation; or plasticity may be maintained or increase in a process known as the Baldwin effect (Crispo, 2007).

Clearly, the target of insecticide and mode and tempo of exposure play important roles in the mechanism of resistance evolution (N. Liu, 2015). Moreover, the genes and patterns of gene expression involved in both insecticide resistance and adaptations to plant allelochemicals have not been investigated outside key studies in the model generalist pest spider mite, *Tetranychus urticae* (Dermauw et al. 2013). Experimental evolutionary approaches provide unique opportunities to track the how gene expression changes over the course of exposure to novel stressors. At the moment, we know of few studies that have taken experimental evolutionary approaches to test the pre-adaptation hypothesis with respect to cross-resistance, gene regulation, and the initial responses to novel host plants and insecticides. The pre-adaptation hypothesis would predict a degree of convergence in the patterns of gene expression changes (either adaptive or maladaptive) when insect herbivores initially encounter either. Moreover, few studies have coupled such experimental evolutionary work to empirical measures of fitness.

Here, we investigate transcriptomic plasticity and fitness changes a specialist insect, *Aphis nerii*, in response to multiple generations of selection on toxic host plants and to two insecticides with different targets, pymetrozine and imidacloprid. *A. nerii* specialize on milkweed host plant species that vary in their toxicity, primarily based on cardenolides which specifically impair sodium-potassium pump activity (A. A. Agrawal et al., 2012). Unlike most other milkweed-herbivores, *A. nerii* do not possess adaptive target-site modifications (Dobler et al. 2012; Zhen et al. 2012). Instead, *A. nerii* employ a narrow set of differentially expressed genes when reared on more toxic milkweed plant species for multiple generations (Birnbaum et al. 2017). Even specialist insects that feed on different but closely related host plants face additional nutritional and metabolic challenges whilst feeding on different plant species, whereas insecticides, e.g. neurotoxins (imidacloprid) or growth regulators (pymetrozine), typically have a more targeted, narrow molecular effect in insects.

What level of convergence exists in metabolic mechanisms employed against various natural and xenobiotic chemicals? How do transcriptomic responses to these stressors change in response to novel and long-term exposure? We compared transcriptional responses of control *A. nerii* populations reared on *Asclepias incarnata* to the three stressors (more toxic host plants [*Gomphocarpus physocarpus*], pymetrozine, imidacloprid) after one and five generations of exposure. *A. nerii* differentially express a greater number of genes after long-term exposure to all three treatments compared to expression after one generation, and after five generations, expression differences of aphids reared on more toxic host plants largely overlap with gene expression changes in response to either of the two insecticides. This work provides novel insights into how specialist insect transcriptomes respond to different natural and xenobiotic chemicals and how these responses change over time during exposure.

Methods

Insect and plant cultures

Milkweed species that dramatically vary in their cardenolide composition (Gowler et al., 2015) and that have been previously used in milkweed herbivore experiments (Birnbaum et al., 2017; Sternberg et al., 2012) were selected. Seeds of *Asclepias incarnata* (low toxicity) and *Gomphocarpus physocarpus* (high toxicity) were obtained commercially (Joyful Butterfly, Blackstock, SC, USA) and grown as previously described. Briefly, seeds were first planted in a fine germination mix (Sungro Propagation Mix), and after 2–3 weeks, they were repotted in 4-inch pots filled with a general-use potting soil (Sungro 3B Mix). Plants were grown in the Vanderbilt University glasshouse.

Three *Aphis nerii* clones, previously genotyped (Birnbaum et al., 2017), were used in experiments. Two clones of *Aphis nerii* were collected in the spring of 2014 from Atlanta, GA (GH14), and Miami, FL (MIA14). A third *A. nerii* clone was collected in the summer of 2015 from Nashville, TN (Sc15). All experimental aphids were housed in a controlled environmental chamber at 22°C and 16-hr light:8-hr dark cycle.

Pymetrozine (Endeavor, 50%) and Imidacloprid (Prime Source Imidacloprid 2F T/I, 21.4%) were commercially obtained and sublethal concentrations were used (pymetrozine = 0.1873 g/L; imidacloprid = 0.25 ug/L). Pymetrozine disrupts homopteran feeding and development through unknown actions, and imidacloprid is a well-known neonicotinoid, acetylcholine agonist. *A. incarnata* plants were sprayed with either pymetrozine or imidacloprid in a fume-hood; plants were completely coated and allowed to air-dry.

Experimental design

A. nerii stock clones have been maintained on *A. incarnata* long-term (> 20 generations), and these aphids were used as the control treatment (Fig. 18). To investigate trans-generational responses, *e.g.* responses over time, to increased toxicity host plants and insecticides, *A. nerii* from each of the three genotypes reared on *A. incarnata* were transferred to three stress or "exposure" treatments: more toxic host plants, *G. physocarpus*, *A. incarnata* plants treated with pymetrozine, and *A. incarnata* treated with imidacloprid. Three to five adult aphids from each treatment were transferred each generation for five generations to new plants of the same treatment, with the first generation designated as Generation 0 and the last Generation 5 (an initial generation, followed by five transferred generations). *A. nerii* were also passaged on *A. incarnata* as controls. Treatments for each genotype were maintained in triplicate. Adult aphids were flash frozen for RNAseq at Generation 0 (*A. incarnata*) and Gens. 1 and 5 (*A. incarnata*, *G. physocarpus*, *A. incarnata* + pymetrozine, *A. incarnata* + imidacloprid) resulting in a total of 27 samples.

Adults from Gen. 5 were then challenged with new plant treatments for fitness measurements ("challenge" treatments). To assess fitness after novel exposure to challenge treatments, adults from *A. incarnata* were transferred to *A. incarnata*, *G. physocarpus*, *A. incarnata* + pymetrozine, and *A. incarnata* + imidacloprid treatments. To investigate how exposure to toxic plants for five generations influences fitness and

tolerance of insecticides, adults from *G. physocarpus*-exposed lines were transferred to *G. physocarpus*, *A. incarnata* + pymetrozine, and *A. incarnata* + imidacloprid treatments. Last, to assess fitness after five generations of exposure to insecticides adults from *A. incarnata* + pymetrozine-exposed and *A. incarnata* + imidacloprid-exposed lines were transferred to *A. incarnata*, and *A. incarnata* + pymetrozine and *A. incarnata* + imidacloprid treatments, respectively. The average number of offspring produced after 48 hours and the time for those offspring to develop to adults were recorded. Stress-exposed aphids transferred to novel treatments, *e.g. G. physocarpus*-exposed on *A. incarnata* + pymetrozine, were also flash frozen for RNAseq resulting in another 12 samples (Gen. 6).

RNAseq methods

RNA was extracted from five pooled adults from each treatment at four generations (Gen. 0, 1 5, 6, n = 15/genotype) using a standard Trizol extraction protocol. Sample quality and concentration was assessed using a 2100 Bioanalyzer. RNA libraries were constructed from 1ug total RNA per sample using the NEBNext Ultra II Library Prep and poly(A) mRNA magnetic isolation kit. RNA-sequencing libraries were quantified using a 2100 Bioanalyzer. The 39 samples were sequenced on an Illumina NovaSeq across 20% of an S2 flow cell with paired-end 101bp reads.

The previously published and annotated transcriptome was used as a reference for the alignment and quantitation for each of the 39 individual samples. Read files were quality-filtered and trimmed (Trimmomatic; Bolger, Lohse, & Usadel, 2014) to remove Illumina adapters and poor-quality reads. Reads were then aligned with BOWTIE2 (v 2.2.5) using the local alignment option in paired-end mode. BOWTIE2 alignment rate ranged between 69-99% for all libraries.

Differential expression analyses

Multidimensional scaling plots were created to visualize which factors are associated with the greatest variation in the data. Genes with counts ≤ 10 in 90% of the samples were removed, and data were transformed using the variance-stabilizing transformation function in the DESEQ2 package in R (Love, Huber, & Anders, 2014), which converts data normalized for library size to a log2-scale (Anders & Huber, 2010). The plotMDS function in R was used to visualize a principle component analysis for the first four axes.

Uniquely aligned read counts were extracted from each of the individual alignments (SAMtools; Ruan, 2009), and differential expression was calculated between the initial control samples and the three exposure treatments over time with DESeq2 using default parameters with a parametric fit (Love, Huber, & Anders, 2014). For the differential expression design matrices, the three aphid genotypes were used as biological replicates. Differential expression was calculated between control, Gen. 0 *A. incarnata*, aphids and Gen. 1 and 5 aphids in each of the three treatments: *G. physocarpus*, pymetrozine, imidacloprid. Heatmaps were generated for the top 500 most variably differentially expressed genes using the heatmap.2 function in R. For each data set comparison, the 500 most variably expressed transcripts across samples were extracted, and counts data was used in the heatmap.2 function; data were scaled by row, *i.e.* transcript count values.
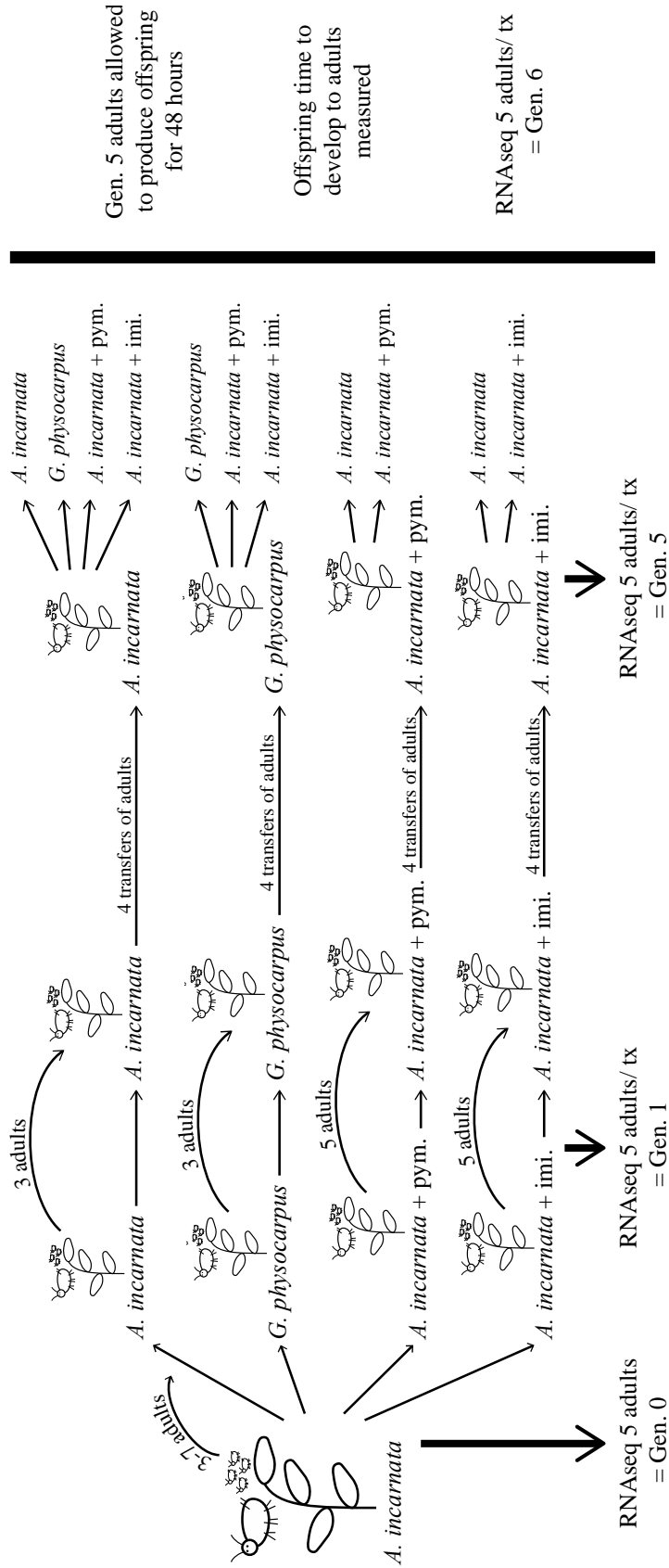
Figure 18. Experimental design schematic (see Methods text for more details).

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To test whether differentially expressed genes were enriched for particular functional categories, functional enrichment tests were performed using the go-basic.obo and the R function dhyper (R Core Team 2014). The GO terms of significantly differentially expressed ($padj < 0.05$) *A. nerii* genes in each of the sets of differentially expressed genes were compared against those of the entire *A. nerii* transcriptome (12,162 genes). GO enrichment was also assessed in each of the set of overlapping differentially expressed genes, *e.g.* genes differentially expressed in all three treatments. Statistical significance was adjusted for multiple comparisons using the Benjamini & Hochberg (BH) method in the R function p.adjust (Benjamini & Hoch- berg, 1995).

Sets of differentially expressed genes were also mapped to KEGG metabolic pathways to functionally characterize gene expression changes. KEGG orthology (KO) IDs were extracted from the annotated differentially expressed *A. nerii* genes and mapped to metabolic pathways using the online KEGG Mapper tool (v3.1) against the KO database.

A co-expression network was constructed using the WGCNA package in R (Langfelder & Horvath, 2008). Before constructing the networks, genes with counts ≤ 10 in 90% of the samples were removed, and data were transformed using the variance-stabilizing transformation function in the DESEQ2 package in R (Love, Huber, & Anders, 2014), which transforms data normalized for library size to a log2-scale (Anders & Huber, 2010). The soft-threshold power was set to four. Once modules were detected, we analyzed significant module correlation with each of the three factors in our experiment: exposure regime, challenge treatment, and generation. GO enrichment and Kegg analyses (described above) were performed on significantly correlated modules.

Fitness data statistical analyses

Adults from Generation 5 were challenged with new plant treatments and the average number of offspring per adult was recorded; the time for offspring to develop to adults was also recorded. The effects of exposure regime, challenge treatment, and genotype on average fecundity and time to develop to adults were analyzed using three-way ANOVA tests; data distribution and the distribution of residuals were evaluated using Shapiro–Wilk tests.

Results

Effects of selection on A. nerii *gene expression*

When visualizing differential gene expression at Gens. 1 and 5, distinct patterns appear (Fig. 19). After one generation of exposure, genes differentially expressed in response to imidacloprid contrast with those differentially expressed in response to *G. physocarpus* and pymetrozine and are primarily upregulated (Fig. 19A). In contrast, differential gene expression after five generations of exposure is more varied, and samples from the three stress treatments do not group together in consistent patterns (Fig. 19B). Overall, *A. nerii* gene expression clusters primarily based on generation (Fig. 20). A heatmap of the 500 most variably differentially expressed genes across the entire RNAseq sample set reveals dynamic changes in gene expression whereby samples from Generations 0 and 1 group together and separately from samples from Generations 5 and 6 (Fig. 21). These results indicate highly variable gene expression over the course of five generations of *A. nerii's* exposure to different stress treatments.
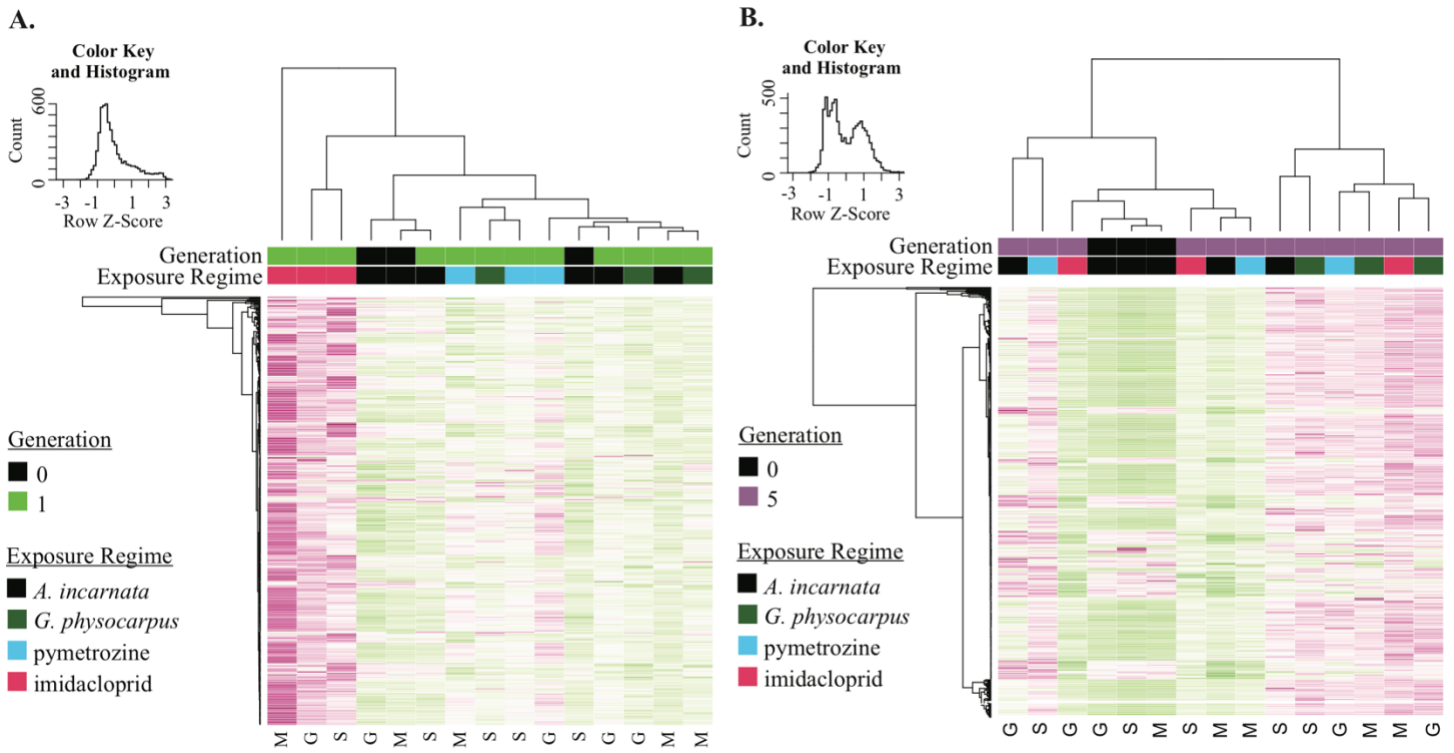
Figure 19. Heatmaps of **(A)** all differentially expressed genes at Gen. 1 and of **(B)** the 500 most variably differentially expressed genes at Gen. 5 (G = GH14, M = MIA14, S = Sc15). **A.** After one generation of exposure, imidacloprid-exposed *A. nerii* show distinct patterns of upregulation, while expression patterns of *G. physocarpus*- and pymetrozine-exposed aphids are more similar and show patterns of downregulation. **B.** Patterns of gene expression after five generations of exposure are more varied across all exposure regimes.
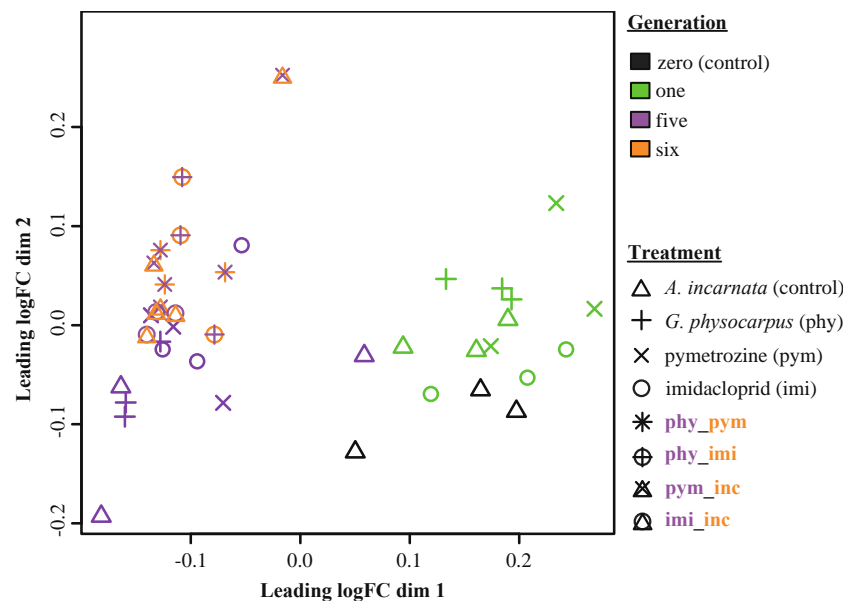
Figure 20. MDS plot of all samples in experiment reveals that samples group primarily based on generation with Gens. 0 and 1 forming a group separate from Gens. 5 and 6. Generations are shown by different colors and exposure regimes are shown by different symbol shapes.

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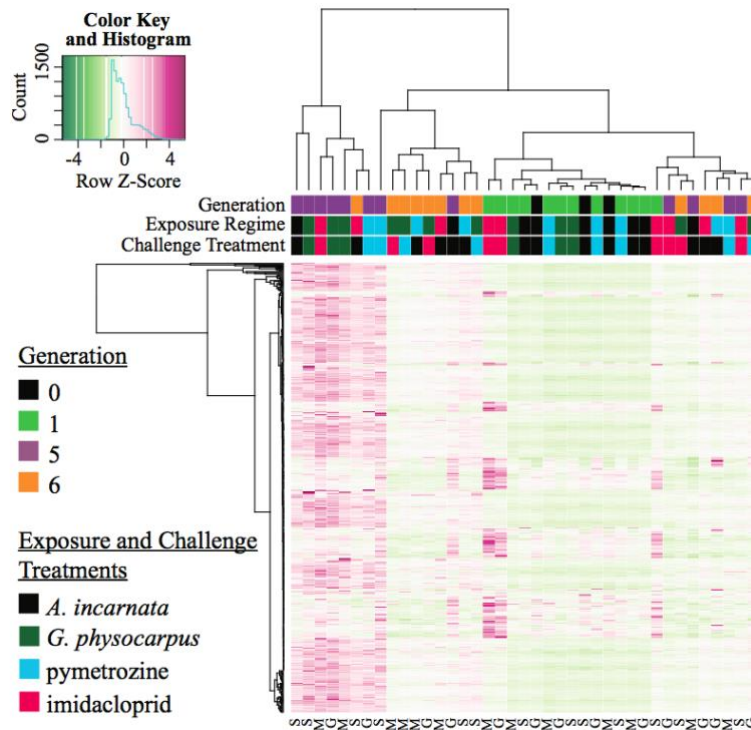
Figure 21. A heatmap of the 500 most variably expressed genes across the entire dataset reveal dynamic changes in gene expression (G = GH14, M = MIA14, S = Sc15). Samples from Gens. 0 and 1 form one group while Gens. 5 and 6 form three clusters.

A. nerii differentially expressed a small set of genes in each of the three stress treatments (Fig. 22; *G. physocarpus*- 223, pymetrozine- 336, imidacloprid- 89) after one generation of exposure compared to Gen. 0 control aphids on *A. incarnata*. Seven genes were differentially expressed in control Gen. 1 *A. incarnata* aphids compared to control, Gen. 0 *A. incarnata* aphids. *G. physocarpus* and pymetrozine treatments shared the greatest set of commonly differentially expressed genes, and only a small set of shared genes are differentially expressed in all three treatments (Fig. 23A). GO enrichment tests of the annotated genes in these differentially expressed genes sets (Table 9) revealed significant GO terms in the gene set expressed by pymetrozine only. A total of 45 GO terms are enriched in this gene set, and they primarily encompass terms related to transporter activity, ion channel activity, and regulation of action potentials. When considering only genes differentially expressed at the 2-fold or higher level, several significant GO terms are enriched in the *G. physocarpus* complete gene set (Table 9). These terms describe processes related to cuticle and growth development and oxidoreductase activity, among others. These results suggest that after one generation of exposure to more toxic host plants and insecticides, *A. nerii* transcriptionally regulate a narrow set of genes which appear targeted towards adaptations to stress and exposure to xenobiotics.
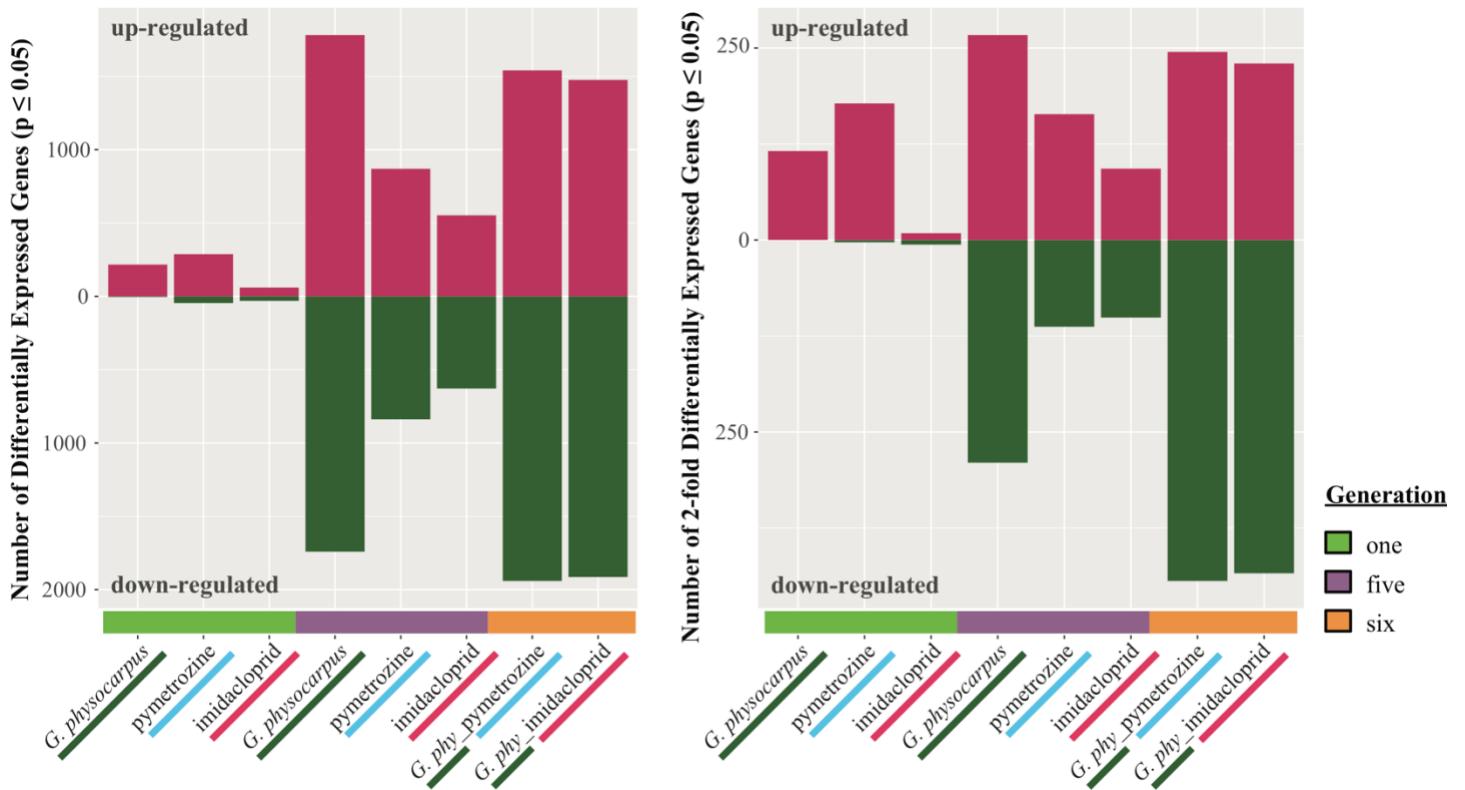
Figure 22. Number of differentially expressed genes in each treatment at Generations 1, 5, and 6 compared to control aphids at Generation 0 (*G. phy*_pymetrozine = *G. physocarpus* exposed aphids challenged with pymetrozine, *G. phy*_imidacloprid = *G. physocarpus* exposed aphids challenged with imidacloprid). Left) all differentially expressed genes (adj. p < 0.05). Right) 2-fold differentially expressed genes (log(fold change) ≥ 1, adj. p < 0.05).
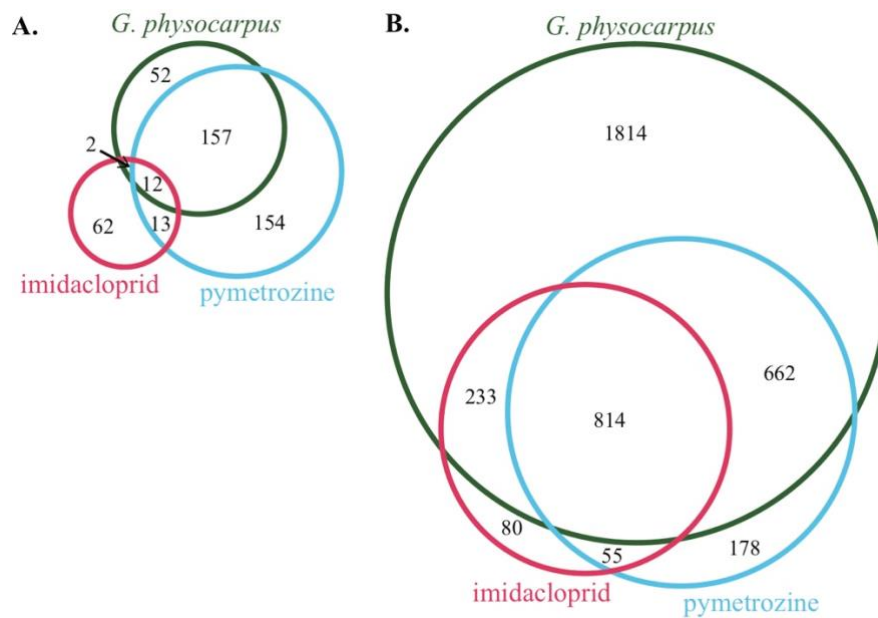
Figure 23. Overlap of differentially expressed genes between all three stress treatments, *G. physocarpus* (P), pymetrozine (Pym), and Imidacloprid (Imi) at **(A)** Generation 1 and **(B)** Generation 5.

GO.term	GO.space	GO.description	Number of Genes	adj. p value	Gen. 1 phy. 2FC	Gen. 1 pym. only	Gen. 5 phy. 2FC	Gen. 5 pym. & imi.
GO:0000412	B	histone peptidyl-prolyl isomerization	3	0.006			*	
GO:0034764	B	positive regulation of transmembrane transport	4	0.018		*		
GO:0034767	B	positive regulation of ion transmembrane transport	4	0.018		*		
GO:0045760	B	positive regulation of action potential	3	0.018		*		
GO:1904064	B	positive regulation of cation transmembrane transport	4	0.018		*		
GO:0008365	B	adult chitin-based cuticle development	2	0.019	*			
GO:0032485	B	regulation of Ral protein signal transduction	2	0.019	*			
GO:0050665	B	hydrogen peroxide biosynthetic process	2	0.019	*			
GO:0022409	B	positive regulation of cell-cell adhesion	4	0.021		*		
GO:0004368	M	glycerol-3-phosphate dehydrogenase activity	2	0.024	*			
GO:0016901	M	oxidoreductase activity, acting on the CH-OH group of donors, quinone or similar compound as acceptor	2	0.024	*			
GO:0052590	M	sn-glycerol-3-phosphate:ubiquinone oxidoreductase activity	2	0.024	*			
GO:0052591	M	sn-glycerol-3-phosphate:ubiquinone-8 oxidoreductase activity	2	0.024	*			
GO:0040007	B	growth	8	0.024	*			
GO:0030513	B	positive regulation of BMP signaling pathway	3	0.025	*			
GO:0032044	C	DSIF complex	2	0.026				*
GO:0000413	B	protein peptidyl-prolyl isomerization	3	0.027			*	
GO:0022407	B	regulation of cell-cell adhesion	5	0.033		*		
GO:0051050	B	positive regulation of transport	9	0.033		*		
GO:0098900	B	regulation of action potential	3	0.033		*		

Table 9. The 20 most significantly enriched GO terms across the entire experiment (phy. = *G. physocarpus*, pym. = pymetrozine, imi. = imidacloprid).
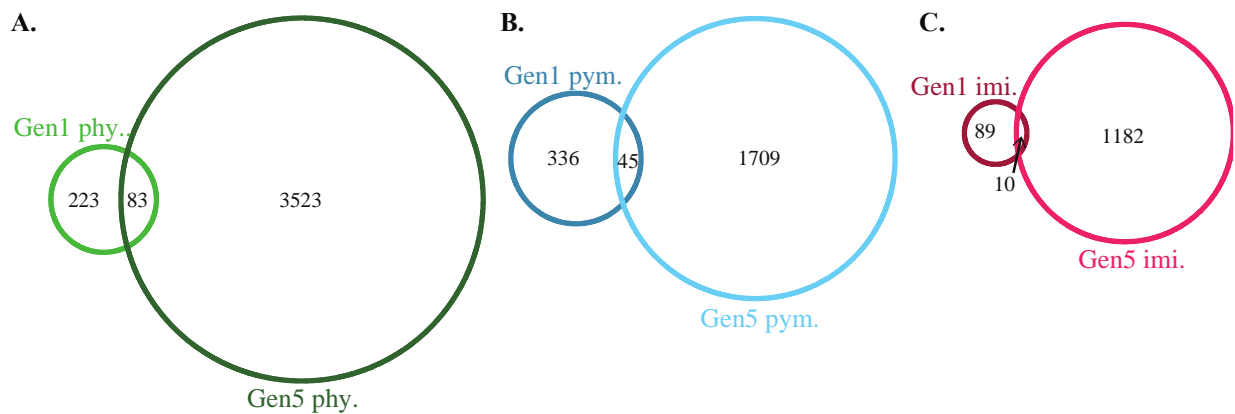

Figure 24. Overlap of differentially expressed genes between Generation 1 and 5 for all three stress treatments, (**A**) *G. physocarpus*, (**B**) pymetrozine, and (**C**) imidacloprid.
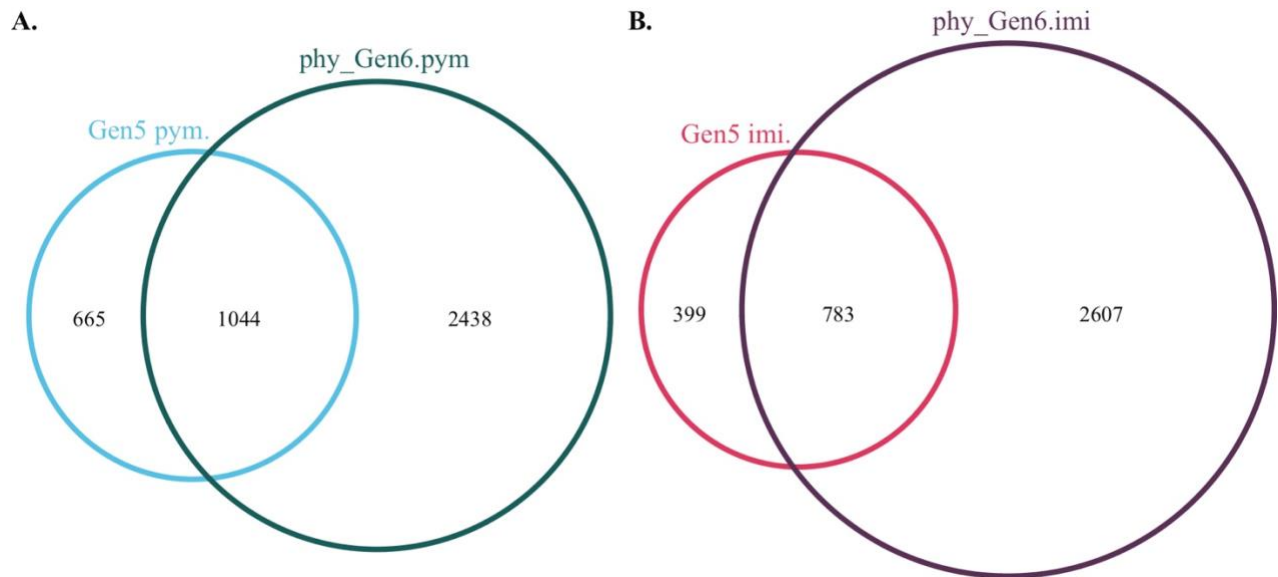
Figure 25. A. Overlap of genes differentially expressed between those DE after five generations of pymetrozine exposure and those differentially expressed (DE) in *G. physocarpus* exposed aphids challenged with pymetrozine. **B.** Overlap of genes differentially expressed between those DE after five generations of imidacloprid exposure and those DE in *G. physocarpus* exposed aphids challenged with imidacloprid.

After five generations of exposure, *A. nerii* differentially expressed a greater number of genes (Fig. 22) compared to Gen. 0 control aphids on *A. incarnata*. A large number of these genes are differentially expressed in all three treatments, and the majority of genes differentially expressed in either of the two insecticide treatments are also differentially expressed in aphids selected on more toxic host plants (Fig. 23B). GO enrichment tests of the annotated genes in each of the differentially expressed gene sets revealed only one significant GO term, DSIF complex (cellular component- GO:0032044), in the shared gene set of pymetrozine and imidacloprid (Table 9). When considering only genes differentially expressed at the 2-fold or higher level, two significant GO terms are enriched in the *G. physocarpus* complete gene set (Table 9). These terms describe histone and protein peptidyl-prolyl isomerization (biological process- GO:0000412, GO:0000413). These results contrast with the more targeted differential expression after one generation of exposure, and suggest that after five generations of exposure, *A. nerii* transcriptionally regulate a broad set of genes. Of note, all three exposure treatments are associated with expression changes in genes related to transcription modification, and *A. nerii* exposed to more toxic host plants differentially express genes associated with alterations in protein folding.

A. nerii genes differentially expressed after one generation of exposure are not differentially expressed after five generations (Fig. 24). Transcriptional responses between generations share the most overlap in the more toxic plant treatment, *G. physocarpus* (83 genes). GO enrichment tests of the genes differentially expressed in each of the three stress treatments at both Gen. 1 and 5 did not reveal any significant GO terms. In a previous study, we characterized differential expression of *A. nerii* adapted long-term (> 10 generations) on *G. physocarpus* relative to expression on *A. incarnata* (Birnbaum et al., 2017). There is little overlap between genes initially identified as differentially expressed between host plants varying in toxicity and those differentially expressed after one and five generations of exposure to all three stressors. However, not surprisingly, *G. physocarpus* exposed aphids share the greatest overlap with genes differentially expressed in long-term adapted *G. physocarpus* aphids, followed by pymetrozine exposed aphids. Very few genes differentially expressed in only imidacloprid aphids are also differentially expressed in long-term adapted *G. physocarpus* aphids. These results suggest that dynamic, plastic changes in gene expression are associated with *A. nerii's* interactions with more toxic host plants and insecticides.

A. *nerii* adapted to *G. physocarpus* were also exposed to pymetrozine and imidacloprid to examine how adaptation to more toxic plants may alter transcriptional responses to insecticides. *A. nerii* differentially express the greatest number of genes after *G. physocarpus*-adapted aphids are exposed to insecticides (Fig. 22). A large number of genes were commonly differentially expressed between both pymetrozine and imidacloprid exposed aphids after five generations and *G. physocarpus* adapted aphids exposed to pymetrozine and imidacloprid, respectively (Fig. 25). GO enrichment tests of the annotated genes in the overlap between genes differentially expressed in both insecticide-selected aphids and *G. physocarpus*-selected aphids exposed to insecticides did not reveal any significantly enriched terms. Visualization of the most variably expressed differentially expressed genes after one and five generations of exposure to each stressor and those of *G. physocarpus* exposed aphids challenged with insecticides reveals that gene expression clusters into four groups (Fig. 26). Expression of control and Gen. 1 *A. nerii* form one cluster; Gen. 5 samples primarily fall into a second cluster; and a second two clusters are formed by samples from Gen. 5 and 6. These results provide compelling evidence for the pre-adaptation hypothesis, because *A. nerii* maintained on more toxic host plants and then exposed to insecticides appear primed to regulate a large number of those same genes that are differentially expressed in *A. nerii* when exposed to insecticides for five generations.

Modules inferred by weighted gene co-expression network analysis (WGCNA)

To look at broad patterns of gene expression, a co-expression network was built to examine the correlation between gene expression and exposure regime, challenge treatment, and generation. Eighteen modules were identified, ranging from 33 to 5332 genes each and 17 modules were significantly correlated with at least one factor (Table 10). Six modules were significantly correlated with all three factors, exposure regime, challenge treatment, and generation; two additional modules were significantly correlated with challenge treatment and generation; one additional module was significantly correlated with exposure regime and challenge treatment; one additional module was significantly correlated with only exposure regime and seven additional modules were significantly correlated with only generation. All but two gene modules did not contain significant GO terms. One module significantly correlated with both exposure regime and challenge treatment was significantly enriched for two GO terms involved in single-organism transport and localization (GO:0044765, GO:1902578). The second module was significantly correlated with both challenge treatment and generation and was enriched for two GO terms involved in leukocyte and lymphocyte homeostasis (GO:0001776, GO:0002260).

Kegg pathway IDs were extracted for all genes in each of the significantly associated modules and were mapped to Kegg orthology pathways. The top five pathways for each significant module are listed in Table 10. Of note, several modules were associated with pathways involved in the biosynthesis of secondary metabolites and the production of proteins (*e.g.* ribosome, RNA transport, and splicesome).
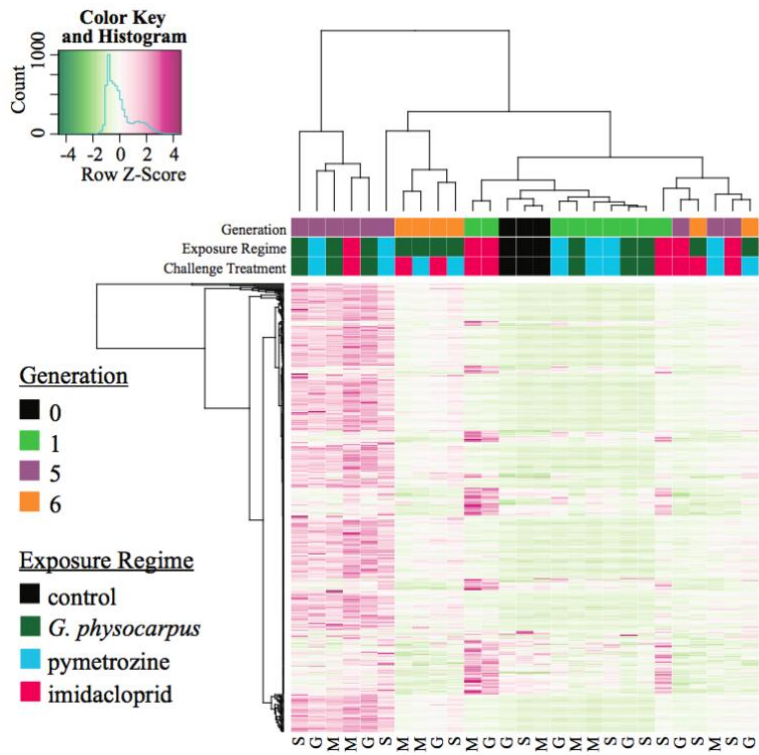
Figure 26. Heatmap of the 500 most variably differentially expressed genes between control, Gen. 1 *G. physocarpus*, Gen. 5 *G. physocarpus* and insecticide, and Gen. 6 samples (G = GH14, M = MIA14, S = Sc15).

Module Number	# Genes in Module	ko ID	ko Pathway Description	# ko Annotated Genes	G	E	C
1	5332	KO01100	metabolic pathways	379	*	*	*
		KO01110	biosynthesis of secondary metabolites	135	*	*	*
		KO01130	biosynthesis of antibiotics	96	*	*	*
		KO01120	microbial metabolism in diverse environments	69	*	*	*
		KO04714	thermogenesis	64	*	*	*
2	1993	KO01100	metabolic pathways	58	*	*	*
		KO05165	human T-cell leukemia virus 1 infection	17	*	*	*
		KO01110	biosynthesis of secondary metabolites	16	*	*	*
		KO04144	endocytosis	16	*	*	*
		KO05200	pathways in cancer	16	*	*	*
3	1179	KO01100	metabolic pathways	43	*	*	*
		KO03013	RNA transport	15	*	*	*
		KO03040	splicesome	14	*	*	*
		KO03008	ribosome biosynthesis in eukaryotes	14	*	*	*
		KO04110	cell cycle	13	*	*	*
5	276	KO01100	metabolic pathways	12		*	
		KO05200	pathways in cancer	8		*	
		KO00230	purine metabolism	8		*	
		KO05165	human papillomavirus infection	6		*	
		KO04020	calcium signaling pathway	6		*	
6	262	KO01100	metabolic pathways	22	*	*	*
		KO04144	endocytosis	6	*	*	*
		KO05231	choline metabolism in cancer	5	*	*	*
		KO05203	viral carcinogenesis	5	*	*	*
		KO01110	biosynthesis of secondary metabolites	5	*	*	*
7	190	KO03010	ribosome	15	*	*	*
		KO01100	metabolic pathways	11	*	*	*
		KO03013	RNA transport	4	*	*	*
		KO05165	human T-cell leukemia virus 1 infection	4	*	*	*
		KO04141	protein processing in endoplasmic reticulum	3	*	*	*
8	180	KO01100	metabolic pathways	11	*	*	*
		KO04146	peroxisome	4	*	*	*
		KO04934	Cushing syndrome	3	*	*	*
		KO05165	human T-cell leukemia virus 1 infection	3	*	*	*
		KO04921	oxytocin signaling pathway	3	*	*	*
9	130	KO03040	splicesome	6	*		
		KO05168	Herpes simplex infection	3	*		
		KO05200	pathways in cancer	3	*		
		KO03010	ribosome	2	*		
		KO03013	RNA transport	2	*		

Module Number	# Genes in Module	ko ID	ko Description	# ko Annotated Genes	G	E	C
10	95	KO01100	metabolic pathways	9	*		
		KO01110	biosynthesis of secondary metabolites	5	*		
		KO04934	Cushing syndrome	4	*		
		KO04977	vitamin digestion & absorption	3	*		
		KO00230	purine metaboism	3	*		
11	78	KO01100	metabolic pathways	5		*	*
		KO00562	inositol phosphate metabolism	2		*	*
		KO04151	PI3K-Akt signaling pathway	1		*	*
		KO01110	biosynthesis of secondary metabolites	1		*	*
		KO04971	gastric acid secretion	1		*	*
12	57	KO01100	metabolic pathways	4	*		
		KO04120	ubiquitin mediated proteolysis	3	*		
		KO05200	pathways in cancer	3	*		
		KO04520	adherens junction	2	*		
		KO01110	biosynthesis of secondary metabolites	2	*		
13	56	KO03010	ribosome	6	*		
		KO03040	spliceosome	2	*		
		KO05203	viral carcinogenesis	2	*		
		KO03022	basal transcription factors	2	*		
		KO04934	Cushing syndrome	1	*		
14	56	KO01100	metabolic pathways	4	*		
		KO04540	gap junction	2	*		
		KO05206	microRNAs in cancer	2	*		
		KO05231	choline metabolism in cancer	2	*		
		KO00561	glycerolipid metabolism	1	*		
15	48	KO01100	metabolic pathways	2	*		
		KO04212	longevity regulating pathway-worm	1	*		
		KO01110	biosynthesis of antibiotics	1	*		
		KO00260	glycine, serine, & threonine metaboism	1	*		
		KO00520	amino acid sugar & nucleotide sugar metaboism	1	*		
16	43	KO01110	biosynthesis of secondary metabolites	1	*		*
		KO01100	metabolic pathways	1	*		*
		KO04145	phagosome	1	*		*
		KO00062	fatty acid elongation	1	*		*
		KO03320	PPAR signaling pathway	1	*		*
17	34	KO01522	endocrine resistance	1	*		*
		KO01100	metabolic pathways	1	*		*
		KO04115	p53 signaling pathway	1	*		*
		KO05206	microRNAs in cancer	1	*		*
		KO04658	Th1 & Th2 cell differentiation	1	*		*

Module Number	# Genes in Module	ko ID	ko Description	# ko Annotated Genes	G	E	C
18	33	KO01100	metabolic pathways	5	*		
		KO00604	glycosphingolipid biosynthesis- ganglio series	2	*		
		KO04142	lysosome	2	*		
		KO00260	glycine, serine, & threonine metabolism	1	*		
		KO00531	glycosaminoglycan degradation	1	*		

Table 10. Kegg pathways associated with significant modules (G = generation, E = exposure regime, C = challenge treatment).

Effects of exposure on A. nerii *fitness*

Comparisons of short- and long-term exposure to more toxic host plants and insecticides had complex effects on aphid fitness. Exposure regime and challenge treatment, and the interaction between the two factors, had significant effects on *A. nerii* time to develop to adults (exposure regime- $F_{3,499} = 5.62$, p = $8.59*10^{-4}$; Challenge treatment: $F_{3,499} = 69.22$; p < $2*10^{-16}$; Exposure*Challenge: $F_{4,499} = 8.07$; p = $2.6*10^{-6}$); exposure regime, challenge treatment, and genotype had significant effects on 48-hour fecundity (exposure regime- $F_{3,69} = 101.1$, p < 2^{-16}; challenge treatment- $F_{3,69} = 29.1$, p < 2.8^{-12}; genotype- $F_{2,69} = 12.3$, p < 2.6^{-5}).

Overall, aphids newly exposed to insecticides (*i.e.*, *A. nerii* reared on *A. incarnata* and exposed to pymetrozine or imidacloprid) develop more slowly and have fewer offspring after 48 hours compared to control aphids reared on *A. incarnata* (Fig. 27, 28A). Sc15 aphids differed in this trend and had greater offspring when newly exposed to pymetrozine as compared to control aphids reared on *A. incarnata* (Fig. 28D).

Interestingly, when *A. nerii* are newly exposed to more toxic host plants, *G. physocarpus,* they develop more quickly (Fig. 27), but have lower fecundity compared to control aphids (Fig. 28A). After long-term exposure on *G. physocarpus* for five generations, time to development increased (Fig. 27A), however, there was no effect of selection on fecundity (Fig. 28A). *A. nerii* exposed to *G. physocarpus* for five generations and challenged with insecticides, overall, have equivalent (pymetrozine) or intermediate (imidacloprid) development times compared to *A. nerii* newly exposed and exposed for five generations to insecticides (Fig. 27). A similar trend is seen in average fecundity after 48 hours; *G. physocarpus*-exposed *A. nerii* challenged with pymetrozine have intermediate fecundity compared to aphids newly and those long-term exposed to pymetrozine, and *G. physocarpus* exposed *A. nerii* challenged with imidacloprid have equivalent fecundity to those newly exposed to imidacloprid (Fig. 28A).

Pymetrozine exposure had overall negative effects on *A. nerii* development time, regardless of exposure regime (Fig. 27). on development time except in Sc15 aphids, which had faster development times compared to control aphids. Pymetrozine exposure had either no effect (GH14) or negative effects (MIA14, Sc15) on fecundity (Fig. 28). When pymetrozine-exposed aphids were challenged with control *A. incarnata* plants, they had lower fecundity compared to *A. incarnata*-exposed control aphids.

Exposure on imidacloprid decreased time to development as compared to newly exposed imidacloprid *A. nerii* (Fig. 27). When *G. physocarpus*-exposed aphids were challenged with imidacloprid, they developed more quickly than when challenged with imidacloprid from control, *A. incarnata* plants. Imidacloprid-exposed aphids challenged with control *A. incarnata* developed more quickly than control *A. incarnata*-exposed aphids. Long-term exposure to imidacloprid had negative effects on fecundity when aphids were challenged with imidacloprid (compared to newly exposed imidacloprid aphids) and control plants (compared to *A. incarnata*-selected control aphids) (Fig. 28A).
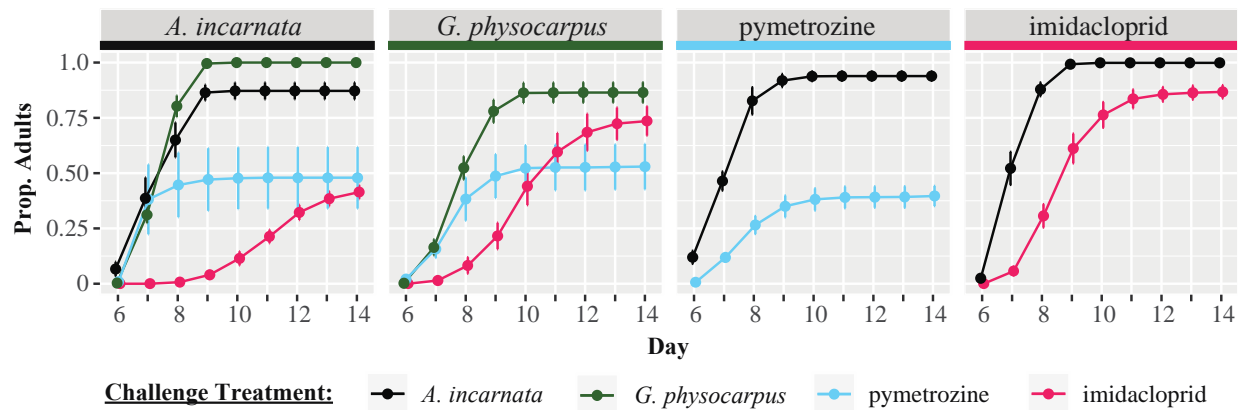
Figure 27. Effects of selection on time to development. Overall effect of selection on *A. nerii* time to develop to adults. Group 1) Control *A. incarnata*-selected aphids were exposed to *A. incarnata*, *G. physocarpus*, pymetrozine, and imidacloprid. Group 2) *G. physocarpus*-selected aphids were exposed to *G. physocarpus*, pymetrozine, and imidacloprid. Group 3) Pymetrozine-selected aphids were exposed to pymetrozine and control *A. incarnata* plants. Group 4) Imidacloprid-selected aphids were exposed to imidacloprid and control *A. incarnata* plants.
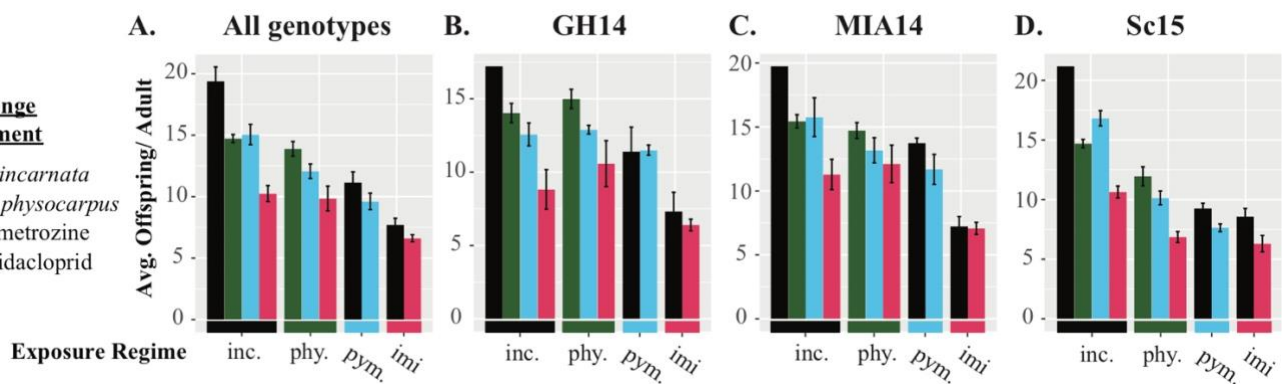
Figure 28. Effects of selection on fecundity. **A.** Overall effect of selection on *A. nerii* fecundity. **B.** Effect of selection on GH14 aphid fecundity. **C.** Effect of selection on MIA14 aphid fecundity. **D.** Effect of selection on Sc15 aphid fecundity. Group 1) Control *A. incarnata*-selected aphids were exposed to *A. incarnata*, *G. physocarpus*, pymetrozine, and imidacloprid. Group 2) *G. physocarpus*-selected aphids were exposed to *G. physocarpus*, pymetrozine, and imidacloprid. Group 3) Pymetrozine-selected aphids were exposed to pymetrozine and control *A. incarnata* plants. Group 4) Imidacloprid-selected aphids were exposed to imidacloprid and control *A. incarnata* plants.

Discussion

Adaptations insects possess to overcome plant defenses are hypothesized to be employed in the evolution of resistance to insecticides (Alyokhin & Chen, 2017), but the degree of convergence of changes in gene expression over short- and long-term exposure is unknown. Gene expression plasticity and the evolutionary history of herbivorous insects play important roles in the evolution of diet breadth and resistance to xenobiotics in insects (Dermauw et al., 2018; Hawkins et al., 2018). In response to a novel environment, initially plastic organisms may either increase or decrease plasticity over the course of adaptation. The degree of gene expression plasticity over the course of adaptation and the level of convergence of adaptive mechanisms

are important factors to consider in mitigating the risks of insecticide evolution. Here, we performed transcriptomic gene expression analyses in a specialist insect to investigate the role of plasticity and level of convergence in adaptive metabolic mechanisms in long- and short-term adaptation to toxic host plants and two insecticides with varying targets.

A number of studies in diverse systems have shown the importance of transcriptional plasticity in the evolution of organisms and their ability to persist through stressful conditions (Y. Huang & Agrawal, 2016; Xiaotong Li et al., 2018; Mathers et al., 2017; Mäkinen et al., 2017; Silva-Brandão et al., 2017; Yampolsky et al., 2014). Here, *A. nerii* demonstrate patterns of increased plasticity associated with evolution to novel conditions in that aphids differentially expressed a greater number of genes after five generations of exposure to all three stressors compared to differential expression after one generation. This finding is in accordance with a trans-generational transcriptomic study in spider mites, which also found little overlap between genes differentially expressed after initial exposure and five generations of adaptation on a challenging host plant (Dermauw et al., 2013). The relatively small overlap between genes differentially expressed after one and five generations of exposure to *G. physocarpus* with those previously identified as differentially expressed in aphids long-term adapted (more than 10 generations) to more toxic host plants (Birnbaum et al., 2017) indicates that *A. nerii* gene expression may remain plastic for many generations in response to novel environments. Alternatively, the variability in gene expression across this and previous experiments may reflect variations in host plant quality and nutrition. Clearly, expression plasticity plays an important role in the ability to tolerate and adapt to stress treatments.

The pre-adaptation hypothesis suggests that insects will exhibit similar patterns of differential expression to toxic or challenging host plants as to xenobiotic pesticide chemicals (Alyokhin & Chen, 2017). To attempt to prevent or delay the evolution of pesticide resistance, mixtures of multiple compounds or rotation of pesticides are recommended (Délye et al., 2013), however, depending on the target or mode of action of the compounds, convergence in resistance mechanisms may be expected.

Overall, the high degree of overlap between aphids adapted over five generations to more toxic host plants and to insecticides lends support to the pre-adaptation hypothesis and points to a degree of convergence in adaptations to novel host plants and insecticides. A large proportion of genes differentially expressed after five generations of insecticide exposure are also differentially expressed in *G. physocarpus* exposed aphids newly exposed to insecticides, suggesting that adaptation to more toxic host plants may predispose *A. nerii* to more plastic, adaptive gene expression changes in response to xenobiotics. There was little evidence that *G. physocarpus*-exposed *A. nerii* have increased fitness when newly challenged with insecticides, except for possibly for development time in imidacloprid exposed aphids. Overall, *G. physocarpus* exposed aphids challenged with imidacloprid have intermediate development times between newly challenged imidacloprid *A. nerii* and *A. nerii* exposed to imidacloprid for five generations.

Transcriptional changes should reflect the degree of differentiation between treatments, as seen by Govind *et al.* (2010), whereby lepidopteran larvae fed on progressively defenseless *Nicotiana attenuata* plants exhibited progressively greater differential expression (Govind et al., 2010). Here, the degree of differential expression across the three stressors in this experiment reflects the target specificity of each stressor and the spectrum of defenses employed in adaptation to each treatment. Imidacloprid-exposed aphids differentially express the smallest number of genes, possibly reflective of the insecticide's specific agonist activity on the nicotinic acetylcholine receptor (nAChR). Pymetrozine-exposed aphids differentially express an intermediate number of genes. Given pymetrozine's unspecific and unknown mode of action, this is perhaps not surprising. Previous studies have found that pymetrozine impairs aphid feeding (Fuog et al. 1988; Kayser et al. 1994; Tjallingii 1988) and reduces aphid fecundity and lifespan (Gerami, Jahromi, & Ashouri, 2005). Interestingly, a previous study in whiteflies found that pymetrozine and imidacloprid resistance mechanisms are uncorrelated (Gorman et al., 2010), and this is in accordance with our findings of few genes commonly differentially expressed between pymetrozine and imidacloprid exposed aphids. *G. physocarpus* exposed aphids differentially express the greatest number of genes, reflective of the differences between *A. incarnata* and *G. physocarpus* host plants which undoubtedly provide the greatest variation to *A. nerii*. Pymetrozine and *G. physocarpus*

exposed aphids share the greatest overlap of differentially expressed genes, and this may reflect similar challenges in feeding in response to each stressor.

Despite changes in gene expression, *A. nerii* do not appear to demonstrate consistent adaptive phenotypes associated with exposure to stressors (ffrench-Constant & Bass, 2017). Overall, *A. nerii* exposed to *G. physocarpus* and pymetrozine for five generations develop more slowly compared to *A. nerii* newly exposed to challenge treatments. However, *A. nerii* exposed to imidacloprid for five generations develop more quickly than newly exposed imidacloprid *A. nerii*. Furthermore, on average, *A. nerii* have reduced fecundity after exposure to insecticides for five generations, but no change in fecundity between one and five generations of exposure on *G. physocarpus*. Overall, these results suggest complex trade-offs between metabolic adaptation or tolerance of stress, development, and fecundity. Numerous other studies have found trade-offs associated with insecticide resistance (Kliot et al., 2014; Kliot & Ghanim, 2012; Xiaoyu Li et al., 2017b; Lin Jie Zhang et al., 2015b), and costs of resistance can even be dependent on the specific mutations involved in adaptation (Bajda et al., 2018). It would be of interest to investigate life history fitness parameters after more than five generations of exposure.

In response to more toxic host plants and to insecticides, genes initially differentially expressed may be adaptive or maladaptive, but over time, it is hypothesized that gene expression plasticity should reflect organismal responses against stress or specific treatments (Burke, 2012; Sørensen et al., 2017). Weighted gene coexpression network analysis (WGCNA) and MDS plots reveal that *A. nerii* gene expression was most strongly influenced by generation. Most modules were significantly correlated with generation (15/17), followed by challenge treatment (9) and exposure regime (8). Of note, several modules significantly correlated with all three factors were associated with Kegg pathways annotated to the biosynthesis of secondary metabolites. Many other modules were associated with metabolic pathways involved in protein production and modification, and amino acid metabolism. GO enrichment tests and manual analysis of differentially expressed genes indicate that canonical detoxification genes were not predominantly employed in *A. nerii*'s survival to the three stressors after one and five generations. This is in contrast with previous analyses of *A. nerii* gene expression on more toxic host plants (Birnbaum et al., 2017) as well as trans-generational transcriptomic experiments in spider mites (Dermauw et al., 2013; Wybouw et al., 2015). The results from this experiment suggest that pathways associated with secondary metabolites and translational processes are important in the evolution of tolerance to toxic host plants and insecticides.

Experimental evolution approaches combined with next-generation sequencing techniques allow for explorations of the genetic mechanisms required for organismal adaptation over time. Here, exposure to stress is associated with increased transcriptional plasticity and changes in genes associated with the metabolism of secondary metabolites as well as genes important in transcription, translation, and post-translation processes. This work provides experimental evidence for the effects of natural and xenobiotic chemicals on insect gene expression plasticity over the course of adaptation.

CHAPTER VII

CONCLUSION AND FUTURE DIRECTIONS

Herbivorous insects are among the most numerous and diverse group of eukaryotes described, and their intimate relationships with host plants provide unique and powerful models for understanding the molecular mechanisms underlying species interactions. The vast majority of herbivorous insects are specialized towards one or a few plant families, genera, or species, usually constrained by particular plant phytochemical profiles. Scientists have sought to understand the patterns restricting or releasing host plant diet breadth for decades, and recently, studies using a variety of genetic methods have begun to elucidate the molecular mechanisms underlying the evolution of plant-insect interactions (Chapter II). Moreover, as crop agriculture has intensified, it has become increasingly clear that pest evolution can rapidly occur, and it is critically important to understand mechanisms of pesticide resistance, how these mechanisms relate to host plant adaptations, and when the evolution of resistance should be expected.

Here, utilizing the long-studied milkweed-herbivore system (Chapter III), I have applied transcriptomics in the non-model milkweed aphid (*Aphis nerii*) system (Chapter IV) to identify alternative mechanisms contributing to the adaptation of insects towards toxic plants (Chapter V) and to understand the relationship of adaptations towards toxic plants and those involved in the evolution of insecticide resistance (Chapter VI).

I found that after long-term selection on more toxic host plants, *A. nerii* differentially expressed a narrow set of genes (225; 1.1%) compared to gene expression on control, lowly toxic host plants. The majority of these genes belong to canonical detoxification families, and GO enrichment test revealed the importance of hydrolase, glucosidase, and oxioreductase activity, as well as the importance of cuticular components. However, these gene expression differences were associated with costs to development and fecundity, proxies for fitness. These results provide interesting candidate genes potentially involved in cardenolide adaptations, however, more detailed genetic analyses in amenable systems (*e.g.* cell transformation assays or CRISPR-CAS9 in *Drosophila* to express candidate *A. nerii* genes or use of transgenic plant lines with varying cardenolide production) are required to fully understand insect protein-cardenolide interactions.

In a separate set of experiments, I used transcriptomics in an experimental evolution framework to experimentally explore the shape of transcriptional plasticity as insects adapt to new stressors. I exposed *A. nerii* to more toxic host plants and to two insecticides, quantified gene expression changes after one and five generations of exposure, and evaluated the effects of novel exposure and exposure for five generations on aphid fitness. I found that *A. nerii* differentially express a greater set of genes after five compared to one generation of exposure, and the stress treatments had complex, overall negative effects on aphid fitness. Gene co-expression network analysis and Kegg metabolic pathway mapping revealed the importance of pathways associated with secondary metabolism and processes in nucleotide and amino acid metabolism, transcription, translation, and post-translation processes. Very few of the detoxification related transcripts that were initially identified as associated with long-term exposure of *A. nerii* to more toxic plants were not differentially expressed after either one or five generations of exposure in any of the stress treatments. These results indicate that gene expression patterns can be highly variable dependent on the time frame of insect exposure to novel host plants or to other stressors. Future gene expression studies expanding the experimental evolution approach to investigate time points further out during adaptation to stressors to are needed comprehensively understand how transcription changes during the course of organismal tolerance in stressful environments.

The studies presented here illuminate a number of novel genes (*e.g.* P450s, UGTs, ABC transporters) putatively involved in *A. nerii*'s adaptation to cardenolides and milkweed pants. Comparisons of gene

expression in gut tissues compared to whole body or of expression when feeding on diets enriched with commercially available polar (ouabain) or apolar (digitoxin, digoxin) cardenolides will help to further characterize the role of candidate genes in *A. nerii*'s milkweed adaptations. For example, we might expect greater differential expression of cuticular genes in gut tissues compared to whole body or greater differential expression of UGTs in response to polar, compared to apolar, cardenolides.

Moreover, the mechanisms underlying insect adaptations to toxic host plants are still largely not comprehensively understood in a diversity of insect or plant systems. For example, do insects of different feeding guilds (*e.g.* leaf-chewers or phloem-feeders), with varying life history strategies (*e.g.* hemimetabolous or holometabolous, or with different patterns of host plant association (different life stages either partially or entirely feed on same host plant) employ similar detoxification mechanisms against host plants? More work needs to be done to understand the differences or similarities in adaptations across diverse insect species.

Furthermore, theories of insect associations with toxic plants have been largely focused on interactions with other trophic levels and it is assumed that this behavior provides protection against pathogens or parasitoids. However, in the *Aphis nerii* system, I have found that *A. nerii* variable effects of feeding on more toxic host plants and infected with fungal pathogens in the lab (Birnbaum and Gerardo, in prep), but they exhibit higher rates of parasitism by parasitoid wasps (*e.g.*, by *Lysiphlebus testaceipes* (identified from field collected mummies by USDA, personal communication) when feeding on more toxic plants in the field (Birnbaum, unpublished data). These differences indicate differential effects of host plant toxicity on different top-down pathogens. Thus, *A. nerii* provide a unique, ecologically relevant system to explore exceptions to the common, long theorized patterns thought to underlie toxic plant-insect associations. It will be interesting to more fully investigate *A. nerii* interactions with parasitoids with experiments in the lab investigating host plant effects on parasitoid success, interactions between detoxification and immune pathways, and the molecular associations associated with additive or synergistic effects of exposure to multiple stressors.

Furthermore, the milkweed-aphid system provides a unique opportunity to investigate plant-insect interactions in a comparative framework utilizing natural differences in host plant species toxicity and diet breadth of aphid species. For example, more studies can be done investigating aphid interactions with milkweed plants that vary in cardenolide toxicity within and between species to examine the strength of plant phylogenetic signals in aphid evolution towards milkweed toxins, as measured through variables such as aphid performance or detoxification mechanisms. Other experiments may utilize aphid species that span the spectrum from generalist to specialist (*e.g.*, *Myzus persicae*, *Aphis nerii*, *A. asclepiadis*, *Myzocallis asclepiadis*) to investigate commonalities and differences in detoxification mechanisms. Paired with studies investigating interactions with parasitoids, this system could also provide key data to illuminate the differential effects of the evolution of molecular mechanisms of tolerance/ resistance and top-down effects that potentially underlie the evolution of insect associations with toxic plants.

Thus, because of the ability to combine organismal-based experiments in the field and laboratory with transcriptome/ gene expression based studies, the milkweed aphid system provides an exciting model to explore the evolutionary ecology of insect-toxic plant interactions in a molecular framework.

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