

Video Article

Maintaining Biological Cultures and Measuring Gene Expression in *Aphis nerii*: A Non-model System for Plant-insect Interactions

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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 several aphid species, and with advances in the next-generation sequencing, transcriptomic studies are being extended to non-model organisms that lack 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 can 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 the 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.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58044/>

Introduction

Aphids are small, hemimetabolous insects that colonize on diverse plant families worldwide. They are distinctive for several 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 sap¹. 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 vector². The publication of the first aphid genome in 2010, the pea aphid *Acyrtosiphon pisum*³, marked an important milestone in the study of aphid biology because it provided the genomic resources for addressing questions about the insect's adaptations to the herbivorous lifestyles, including those that might lead to a better control strategies⁴. Since that time, additional genomic resources have accumulated with the publication of an annotated genome for the soybean aphid *Aphis glycines*⁵, 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)⁶. Valuable *de novo* transcriptomic resources are available as well for a number of other aphid species (e.g., *Aphis gossypii* (cotton aphid)⁷, *Sitobion avenae* (grain aphid)⁸, *Cinara pinitabulaeformis* (pine aphid)⁹, *Aphis nerii* (milkweed-oleander aphid)¹⁰).

Aphids have also made lasting contributions to our understanding of the plant-insect interactions and the ecology of the life on plants¹¹. One area where aphids have made particularly important contributions is in our understanding of the chemical ecology of the host plant interactions. Herbivorous insects express diverse adaptations for overcoming plant defenses, and some even co-opt plant defenses for their own benefit^{12,13,14}. For example, the milkweed-oleander aphid, *Aphis nerii*, is a bright yellow, invasive aphid found in temperate and tropical regions worldwide that colonizes 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^{15,16}. 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 benefits¹⁷. *A. nerii* is thought to sequester cardenolides in this way, although the mechanisms and functional benefits remain unclear^{10,18}.

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 cardenolides¹⁹, 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 insects²⁰ and the interplay between bottom-up and top-down regulation on the herbivore density²¹. *A. nerii* is thus a good candidate as an experimental model for an especially broad set of studies of the insect-plant interactions. Critical to the success of any study with *A. nerii* is the careful culture of aphid populations, which includes the culture of plants on which the aphids depend, as well as an efficient generation of high-quality -omic data. Our goal is to guide the reader through both.

Outlined below are methods for the generation and maintenance of the 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 a variety of aphid species.

Protocol

1. Plant Cultures

- 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.
- Plant seeds in a fine germinating soil (60–70% fine peat moss, perlite, vermiculite, limestone).**
 - 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.
 - Place one seed in each hole and water very well with a watering can such that the soil covers the seeds and is saturated.
 - Grow seeds in a greenhouse (see conditions below, 1.5). Water regularly, daily to every-other-day; enough to maintain the soil moisture to a moderate level.
- When the seedlings have grown their first set of full leaves, re-pot seedlings in a general potting mix (50–60% peat moss, bark, and limestone) (Figure 1A).**
 - Use 4-inch round pots that fit with a tight seal with the cup cages. Fill with general potting soil up to about 5 cm below the rim.
 - Create a hole in the soil deep enough to reach the bottom of the pot.
 - With the 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 the soil. Water very well.
 - Grow plants in the greenhouse and water regularly, daily to every other day; enough to maintain moderate soil moisture.
- Greenhouse conditions.**
 - Set the greenhouse thermostats to maintain daytime temperatures between 18–28 °C and nighttime temperatures between 16–22 °C using the manufacturer's instructions.
 - During winter months when the days are shorter, supplement the daylight with 600 W high-pressure sodium bulbs (12 h, 8 am – 8 pm).
- Control unwanted pests (e.g., thrips, aphids) with a foliar organic soap solution, however, use these products with caution.**
 - Make the soap solution according to the manufacturer's recommendation and apply using a spray bottle.
 - 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.
- 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 (Figure 1B).

2. Aphid Cultures

- Start the laboratory aphid populations from an existing lab isoclonal population or start from the field-collected aphids following the directions below.**
 - When starting a laboratory population from an existing lab isoclonal population, transfer aphids as described in 2.3.1–2.3.3.
- When starting the new isoclonal, field-collected aphid populations and place a single, reproducing, adult aphid on a suitable host plant as mentioned in Step 1.6.**
NOTE: Populations may be started from winged (alate) or unwinged (apterous) adults.
 - Manually inspect plants from the greenhouse for unwanted pests prior to the use with laboratory aphids. Freeze any plants with unwanted aphids. If desired, use an ethanol vacuum flask to remove thrips or other pests.
NOTE: Be sure to rinse plants that have been treated with soap prior to use as described in Step 1.5.2.
 - 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 2,00 µL pipette tip (Figure 2A).
 - Securely cover plants with aphids with a cup cage created with a plastic cup with the top cut off, covered with a fine mesh and secured with tape (Figure 2B).
 - Place aphid-infested plants in a tray and keep in a controlled environmental chamber (16L:8D, 22 °C, 70% humidity).
- To maintain the stock populations, transfer aphids to fresh, new plants weekly (2.2.1–2.2.3).**
 - 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.
 - Securely cover aphid-infested plants with a cup cage created with a plastic cup with the top cut off, covered with a fine mesh and secured with tape.
 - Place plants in a tray and keep aphids in an environmental chamber (16L:8D, 22 °C, 70% humidity).
 - 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.
- To create same age populations for the use in experiments, place up to 5 adults (preferably unwinged) from the stock population onto a new host plant.**
 - Remove the adults 24 h later.

2. About 5–7 days later, once the F_1 offspring have matured to adulthood, place up to 5 unwinged F_1 adults on a new host plant. Remove the adults 24 h later.
3. Once the F_2 population has matured to adulthood, this population is ready to be used in experiments.
NOTE: This process ensures that the experimental population is roughly the same age and are born of roughly same age mothers.

5. Confirm the genotypic differences between field-caught isoclonal lines using microsatellite genotyping (described below, Sections 3 & 4).

3. DNA Extraction

1. Preparation

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

2. Tissue homogenization and lysis

1. Place the aphid near the bottom of a sterile, 1.5 mL microcentrifuge tube.
2. Place the sterile pestle in the tube with the 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. Grind the aphid with the pestle to initially lyse cells.
4. For a single adult aphid, use 200 μ L (split into 2 x 100 μ L aliquots) of the lysis buffer. Add the first aliquot to grind and resuspend the crushed aphid until the sample is visibly disintegrated, then use the second aliquot to wash off the pestle.
5. Incubate the crushed aphids in lysis buffer at 65 °C in the water bath or heat block for 30 min.

3. DNA precipitation

1. While the tube is warm, add 14 μ L of 8 M KOAc. Invert the tube to mix. Store the sample on ice for 30 min.
Note: The protocol can be paused here, and samples can be stored at -20 °C up to 24 h.
2. Centrifuge at 13,000 x g for 15 min at room temperature. Transfer the supernatant to new 1.5 mL tube with a pipette. Be careful not to remove any of the pelleted debris.
3. To improve DNA pellet visualization, add 2 μ L glycogen (20 μ g/mL) to the supernatant. If the sample size is large enough, omit this step.
4. Add 200 μ L of cold 100% molecular-grade ethanol to the supernatant. Invert tubes to mix and incubate at room temperature for at least 15 min.
NOTE: The protocol can be paused here, and samples can be stored at -20 °C up to 24 h.
5. Centrifuge at 13,000 x g for 15 min at room temperature. Remove ethanol by pipetting.

4. DNA wash and elution

1. Add 200 μ L of cold 70% molecular-grade ethanol. Then flick the tube to resuspend and wash the pellet.
2. Centrifuge at 13,000 x g for 5 min. While visualizing the pellet, carefully remove the ethanol by pipetting and add 200 μ L of cold 100% molecular-grade ethanol.
3. Centrifuge at 13,000 x g for 5 min. While visualizing the pellet, carefully remove ethanol by pipetting.
NOTE: Repeat the 100-70-100 ethanol wash if necessary.
4. Air dry the pellets for 5–10 min with the tube laying horizontally open on a tissue paper.
5. Resuspend the DNA pellet in 80 μ L of low TE (10 mM Tris-HCl, 0.1 mM EDTA).
6. Quantify the resuspended DNA using a spectrophotometer.
7. Store at 4 °C.

4. Microsatellite PCR and Sequencing for Aphid Genotyping

1. Order the appropriate F and R primers for microsatellite sequencing (Table 1²⁰).
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.
2. Perform PCR with single aphid DNA samples (described in Section 3) and fluorescently labeled microsatellite primers.
 1. Mix PCR reactions according to the manufacturer's protocol (0.2 μ M each F/R primer, 2.5 mM $MgCl_2$, 50–200 ng DNA template).
 2. Use the following thermocycler settings: initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 58 °C for 35 s, 72 °C for 45 s, and a final elongation step at 72 °C for 10 min.
3. Combine PCR samples with different fluorescent tags to reduce the number of samples sequenced and sequence the microsatellite samples at a genotyping facility.
4. Analyze the .fsa raw sample files using microsatellite analysis software.

5. RNA Extraction

1. Collect aphids' 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.
2. Tissue homogenization
 1. With the sterile pestle in the tube with aphid, freeze in liquid nitrogen for 10–15 seconds, until the sample stop sizzling. Crush the aphid well with the pestle as described in step 3.2.
NOTE: Optimal tissue disintegration is achieved when the aphid is positioned between the pestle and side of the tube.

2. In the fume hood, add 800 μL of guanidinium thiocyanate-phenol-chloroform extraction reagent to the sample (1–5 adult aphids). Homogenize samples with the pestle and dispose of the pestle.
3. **Phase separation**
NOTE: All steps should be performed in a fume hood.
 1. Incubate the homogenized samples for 5 min at room temperature. Add 160 μL of chloroform to sample. Shake vigorously by hand for 15 s.
 2. Incubate for 2–3 min at room temperature. Centrifuge for 15 min at 12,000 $\times g$ at 4 $^{\circ}\text{C}$.
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 $\sim 480 \mu\text{L}$.
4. **RNA precipitation**
 1. In a fume hood, transfer the aqueous phase to a fresh, RNase-free tube. Do not disturb the intermediate phase.
 2. Precipitate the RNA by adding 400 μL of isopropanol and incubate the sample at -20 $^{\circ}\text{C}$ for 10 min.
NOTE: The protocol can be paused here, and samples can be stored at -20 $^{\circ}\text{C}$ up to 24 h.
 3. Centrifuge the sample for 10 min at 12,000 $\times g$ at 4 $^{\circ}\text{C}$.
5. **RNA wash and elution**
 1. Remove the supernatant; watch for the RNA pellet.
 2. Wash the RNA pellet with 1 mL of 75% ethanol in DEPC-treated water. Mix by slow vortexing. Centrifuge for 5 min at 7,500 $\times g$ at 4 $^{\circ}\text{C}$.
 3. Repeat steps 5.5.1–5.5.2 to help remove phenol contaminants.
 4. Remove the supernatant and air dry the pellet for 5–10 min with tube laying horizontally open on a sterile bench. Do not let the RNA pellet dry completely.
 5. 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 $^{\circ}\text{C}$ for 10–15 min.

6. RNAseq *De Novo* Transcriptome Assembly, Annotation, and Differential Expression Analysis

1. **Analyze RNA sample concentration and quality using a chip-based capillary electrophoretic system.**
NOTE: A chip-based capillary electrophoresis system is the preferable method of choice than analyzing with a spectrophotometer because it provides a more accurate and sensitive measure of RNA concentration and quality.
 1. If samples are of suitable quality (≥ 250 ng total, RIN (RNA Integrity Number) ≥ 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 100 bp, paired end reads would be a recommended starting point. Total mRNA library preparation and RNA sequencing were performed by a sequencing facility.
2. Check the quality of reads using Fast QC²².
3. Combine all sample reads and assemble the transcriptome *de novo* using Trinity^{23,24} (Trimmomatic quality filtering enabled).
4. **Refine the assembly**
 1. Use Transdecoder²⁵ to identify open reading frames (ORFs) that are a minimum of 100 amino acids in length.
 2. Perform homology searches for the translated ORFs against Pfam²⁶ and UniProt²⁷ databases using BLASTP²⁸ and HMMER²⁹, respectively.
 3. 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%).
 4. Collapse any complete, translated ORFs that are at least 99% identical at the amino acid level using CD-HIT³⁰.
 5. Collapse the remaining, incomplete ORFs that are at least 95% identical at the nucleotide level using CD-HIT³⁰.
 6. Assign the remaining nucleotide sequences with unique, species-specific identifiers (e.g., APHNE 0001).
5. Assess the completeness of the refined assembly, using BUSCO (<http://busco.ezlab.org/>) and the Arthropoda gene dataset³¹.
6. **Transcriptome annotation**
 1. First, annotate the refined transcriptome using HMMER against the Pfam database^{26,29}.
 2. Second, annotate the transcriptome using BLASTP against the UniProt database^{27,28}.
 3. Third, annotate the transcriptome using BLASTP against the coding sequences of selected insects with published, annotated genomes.
 4. Last, annotate the transcriptome using BLASTP against the pea aphid protein database only.
 5. Use Trinotate to generate GO annotations from UniProt accessions.
 6. Use Trinotate to organize all the annotation results into a SQLite database and generate an annotation report.
7. **Differential expression analysis**
NOTE: Using the refined transcriptome as a reference, align and quantify each library separately.
 1. Use Trimmomatic to quality-filter and trim the original read files³².
Note: If performing this step after a Trinity assembly, one may instead use the Trimmomatic output from that step.
 2. Perform local alignments for each sample using Bowtie2³³.
 3. Extract the read counts from each sample individually using SAMtools³⁴.
 4. Calculate the differential expression between samples of interest using DESeq2 with the default parameters and a parametric fit³⁵.

7. qPCR Verification of Differentially Expressed Genes

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.

1. Generate RNA samples as described above (step 5).
2. Quantitate RNA extractions using a spectrophotometer to check for the quality and obtain the concentration.
3. Synthesize cDNA samples using a commercially available kit as per the manufacturer's recommendation.
4. **Determine the primer efficiencies for genes of interest to ensure accurate two-fold PCR amplification.**
 1. Based on original RNA concentrations, perform serial dilutions (10^1) to obtain 3 cDNA concentrations.
 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/ μ L, 10 ng/ μ L, 100 ng/ μ L).
 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).
 4. Use the following equation to calculate the primer efficiency (E) where m is the slope calculated in 7.4.3:

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

NOTE: Primer efficiencies between 90-110% are suitable for analyses. This process ensures equal amplification of all genes included in the calculations.
5. Use the $\Delta\Delta C_t$ method with a housekeeping gene to quantify the differential expression for genes of interest³⁶.

Representative Results

Plant cultures: Seeds will take approximately two to four weeks, depending on the season, to grow large enough to be re-potted (**Figure 1A**). Re-potted seedlings will take another two to four weeks to grow to an optimal size for aphid cultures (**Figure 1B**).

Aphid cultures: Adult *A. nerii* are distinguished by some darkened cauda and may be unwinged (apterous, **Figure 3A, B**) or winged (alate, **Figure 3C, D**). Developing wing pads become visible when nymphs reach the third instar (**Figure 3E, 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 aphid¹⁰.

DNA and RNA extractions: Single, adult *A. nerii* will yield approximately 100–200 ng/ μ L DNA (80 μ L elution; **Figure 4A**) and 150–300 ng/ μ L RNA (30 μ L elution; **Figure 4B**). Representative microsatellite peaks are shown in **Figure 5**. Representative relative expression of a candidate gene under three conditions (control, Treatment 1, Treatment 2) are calculated in **Table 2** and shown in **Figure 6**.

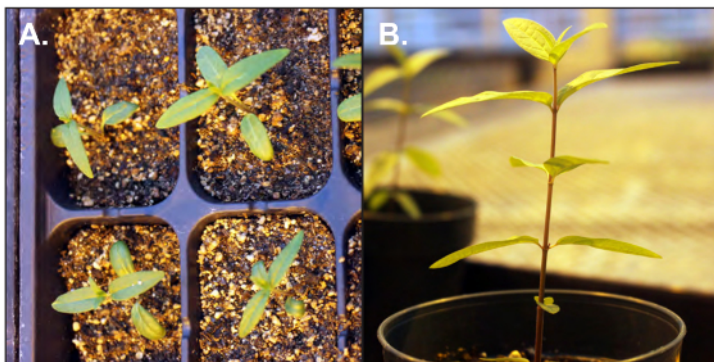


Figure 1: Representative plants for aphid cultures. (A) Seedlings can be re-potted after they have developed their first full set of true leaves. (B) Plants can be used for aphid cultures when they have developed 3-4 sets of true leaves. [Please click here to view a larger version of this figure.](#)

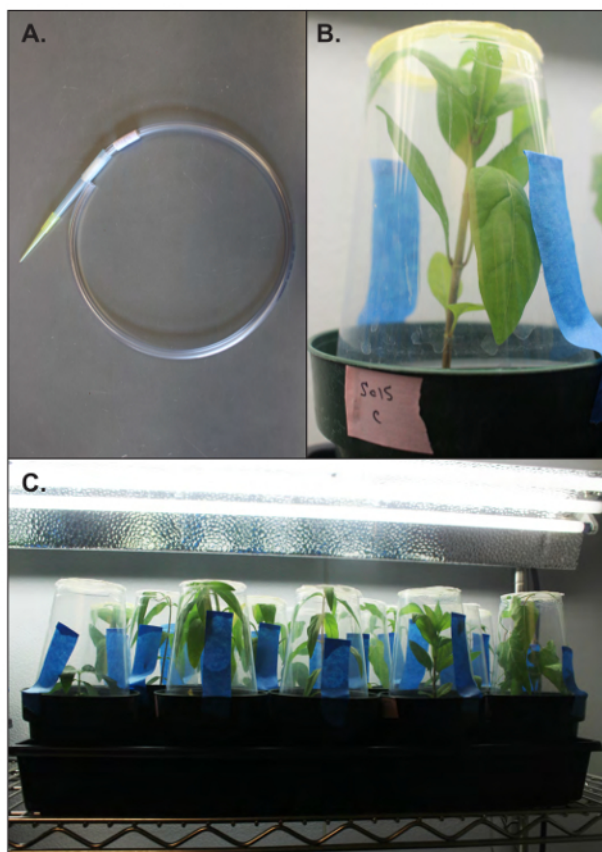


Figure 2: Examples of tools used for culturing aphids. (A) Mouth pipettes can be created using 3/16" ID x 1/4" OD plastic tubing, a 1,000 μ L pipette tip, and a 200 μ L pipette tip. (B, C) Use cup cages (clear plastic cups with the top cut off and secured with fine mesh) to securely fit over the top 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. [Please click here to view a larger version of this figure.](#)

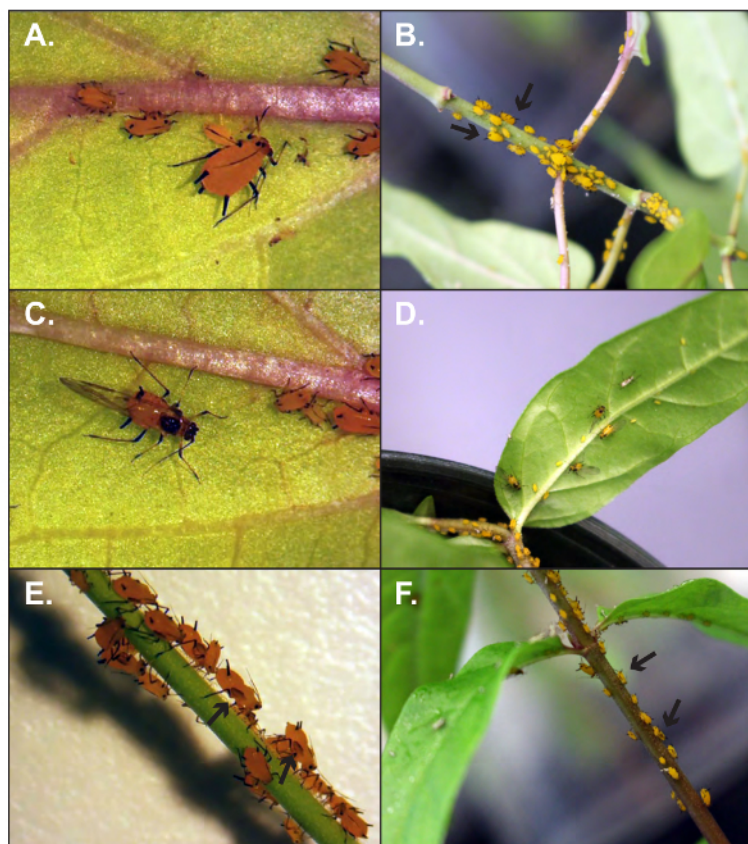


Figure 3: Representative adult and nymph *Aphis nerii*. (A, B) Apterous (unwinged) adult *A. nerii* are identified by darkened cauda at their posterior end. (C, D) Alate (winged) adults are identified by fully developed wings and darkened cauda at their posterior. (E, F) Developing *A. nerii* nymphs go through four instar stages and developing wing pads become apparent during the third instar stage. [Please click here to view a larger version of this figure.](#)

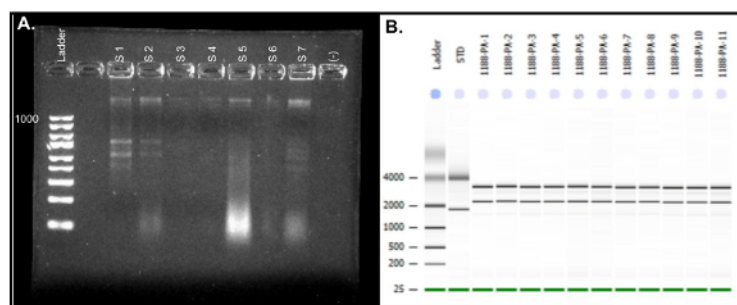


Figure 4: Representative gels. (A) DNA extractions (1kb ladder). Seven *A. nerii* DNA extractions are visualized in lanes 3-9. Negative control is in lane 10. (B) RNA extractions. Eleven *A. nerii* RNA extractions are visualized in lanes 3-13. [Please click here to view a larger version of this figure.](#)

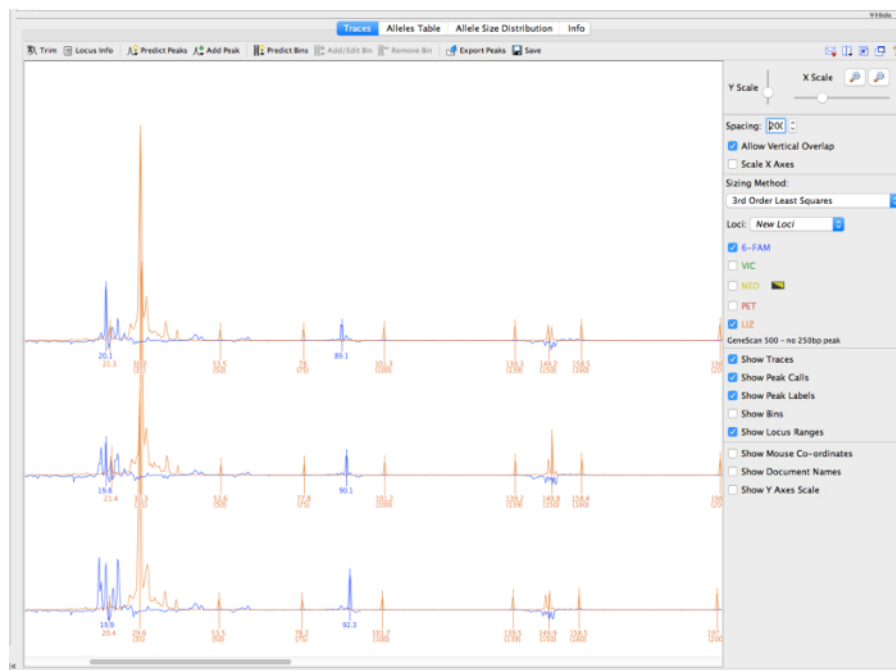


Figure 5: Representative microsatellite peaks. 6-FAM-tagged peaks are visualized in blue. LIZ-500 ladder is shown in orange. [Please click here to view a larger version of this figure.](#)

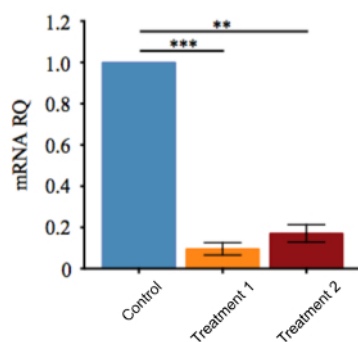


Figure 6: qPCR verification of a differentially expressed gene. Representative mRNA relative quantity (RQ) expression (calculated using the $\Delta\Delta C_t$ method, **Table 2**) shown for a candidate gene of interest under three conditions: control, treatment 1, treatment 2. Graph shows decreased expression of candidate gene under treatments 1 and 2 compared to the control (**Table 2**). [Please click here to view a larger version of this figure.](#)

Primer Name	Direction	Sequence (5'-3')
Ago24_F	forward	TTTCCCGGCACACCGAGT
Ago24_R	reverse	GCCAACTTTACACCCCGC
Ago 53_F	forward	TGACGAACGTGGTTAGTCGT
Ago 53_R	reverse	GGCATAACGTCCTAGTCACA
Ago 59_F	forward	GCGAGTGGTATTGCTTAGT
Ago 59_R	reverse	GTTACCCTCGACGATTGCGT
Ago 66_F	forward	TCGGTTTGCCAACGTCGGGC
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	GGTACATTCGTGTTCGATTT
Ago 126_R	reverse	TAAACGAAAAAACCACGTAC

Table 1: Microsatellite primer sequences used to genotype *Aphis nerii*²⁰.

Target	Sample	Ct Mean	Ct Std. Dev	ΔCt	avg. ΔCt	$\Delta\Delta Ct$	$RQ=2^{(-\Delta\Delta Ct)}$	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 2: Calculations for qPCR $\Delta\Delta C_t$ verification of candidate gene. Candidate gene expression is calculated relative to ef1a (Figure 6). 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

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^{18,37}. A number of genomic resources have recently emerged for *A. nerii*¹⁰, 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 the reproduction and dispersal. These traits, which directly relate to why they are serious crop pests, means that aphid cultures require almost daily attention, as well as extreme care if isogenic lines are required for experiments. The 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 they offer. Insect herbivores live in complex communities on their host plants, and both intraspecific interactions^{38,39} as well as interspecific interactions⁴⁰ shape the ultimate response of *A. nerii* to their host plants. The host plants, *A. nerii* specialize on, represent a diverse set of plants that express divergent life history strategies^{15,21}, 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.

Disclosures

The authors have nothing to disclose.

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