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TITLE:

Maintaining Biological Cultures and Measuring Gene Expression in *Aphis nerii*: A Non-Model System for Plant-Insect Interactions.

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SHORT ABSTRACT:

The aphid *Aphis nerii* colonizes on highly-defended plants in the dogbane family (Apocyanaceae) and provides numerous opportunities to study plant-insect interactions. Here, we present a series of protocols for the maintenance of plant and aphid cultures, and the generation and analysis of molecular and -omic data for *A. nerii*.

LONG 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.

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 sap[1]. 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[2]. The publication of the first aphid genome in 2010, the pea aphid *Acyrthosiphon 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 strategies[4]. 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 plants[11]. 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[12-14]. 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 benefits[17]. *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 cardenolides[19], 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[20] and the interplay between bottom-up and top-down regulation on the herbivore density[21]. *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

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). Water regularly, daily to every-other-day; enough to maintain the soil moisture to a moderate level.

1.3. 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**).

1.3.1. 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.

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

1.3.3. 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.

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

1.4. Greenhouse conditions.

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

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

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

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

1.5.2. 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.

1.6. 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

2.1. Start the 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 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.

2.2.1. 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.

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 2,00 µL pipette tip (**Figure 2A**).

2.2.3. 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**).

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

2.3. To maintain the 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, 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 the use in experiments, place up to 5 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 5 unwinged F_1 adults on a new host plant. Remove the adults 24 h later.

2.4.3. 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.

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

3. DNA Extraction

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

3.2.2. 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.

3.2.3. Grind the aphid with the pestle to initially lyse cells.

3.2.4. For a single adult aphid, use 200 µL (split into 2 x 100 µL 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.

3.2.5. Incubate the crushed aphids in lysis buffer at 65 °C in the 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 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.

3.3.2. 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.

3.3.3. To improve DNA pellet visualization, add 2 µL glycogen (20 µg/mL) to the supernatant. If the sample size is large enough, omit this step.

3.3.4. Add 200 µL 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 hours.

3.3.5. Centrifuge at 13,000 x g for 15 min at room temperature. Remove ethanol by pipetting.

3.4. DNA wash and elution

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

3.4.2. Centrifuge at 13,000 x g for 5 min. While visualizing the pellet, carefully remove the ethanol by pipetting and add 200 µL of cold 100% molecular-grade ethanol.

3.4.3. 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.

3.4.4. Air dry the pellets for 5-10 min with the tube laying horizontally open on a tissue paper.

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

3.4.6. Quantify the resuspended DNA using a spectrophotometer.

3.4.7. Store at 4 °C.

4. Microsatellite PCR and Sequencing for Aphid Genotyping

4.1. Order the appropriate F and R primers for microsatellite sequencing (**Table 1**[20]).

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 s, 58 °C for 35 s, 72 °C for 45 s, and a final elongation step at 72 °C for 10 min.

4.3. Combine PCR samples with different fluorescent tags to reduce the number of samples sequenced and sequence the microsatellite samples at a genotyping facility.

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

5. RNA Extraction

5.1. 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.

5.2. **Tissue homogenization**

5.2.1. 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.

5.2.2. 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.

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. Add 160 µL of chloroform to sample. Shake vigorously by hand for 15 s.

5.3.2. Incubate for 2-3 min at room temperature. Centrifuge for 15 min at 12,000 x g 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 fume hood, 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 up to 24 h.

5.4.3. Centrifuge the sample 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 slow vortexing. Centrifuge for 5 min at 7,500 x g at 4 °C.

5.5.3. Repeat steps 5.5.1 – 5.5.2 to help remove phenol contaminants.

5.5.4. 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.

5.5.5. 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 °C for 10-15 min.

6. RNAseq *de novo* Transcriptome Assembly, Annotation, and Differential Expression Analysis

6.1. 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.

6.1.1. 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 100bp, paired end reads would be a recommended starting point. Total mRNA library preparation and RNA sequencing were performed by a sequencing facility.

6.2. Check the quality of reads using Fast QC[22].

6.3. Combine all sample reads and assemble the transcriptome *de novo* using Trinity[23,24] (Trimmomatic quality filtering enabled).

6.4. Refine the assembly

6.4.1. Use Transdecoder[25] to identify open reading frames (ORFs) that are a minimum of 100 amino acids in length.

6.4.2. Perform homology searches for the translated ORFs against Pfam[26] and UniProt[27] databases using BLASTP[28] and HMMER[29], respectively.

6.4.3. 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%).

6.4.4. Collapse any complete, translated ORFs that are at least 99% identical at the amino acid level using CD-HIT[30].

6.4.5. Collapse the remaining, incomplete ORFs that are at least 95% identical at the nucleotide level using CD-HIT[30].

6.4.6. 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[31].

6.6. Transcriptome annotation

6.6.1. First, annotate the refined transcriptome using HMMER against the Pfam database[26,29].

6.6.2. Second, annotate the transcriptome using BLASTP against the UniProt database[27,28].

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 the original read files[32].

Note: If performing this step after a Trinity assembly, one may instead use the Trimmomatic output from that step.

6.7.2. Perform local alignments for each sample using Bowtie2[33].

6.7.3. Extract the read counts from each sample individually using SAMtools[34].

6.7.4. Calculate the differential expression between samples of interest using DESeq2 with the default parameters and a parametric fit[35].

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.

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

7.2. Quantitate RNA extractions using a spectrophotometer to check for the quality and obtain the concentration.

7.3. Synthesize cDNA samples using a commercially available kit as per the manufacturer's recommendation.

7.4. Determine the 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^(-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 the differential expression for genes of interest[36].

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 aphid[10].

DNA and RNA extractions: Single, adult *A. nerii* will yield approximately 100 – 200 ng/µL DNA (80 µL elution; **Figure 4A**) and 150 – 300 ng/µL RNA (30 µL 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 AND TABLE LEGENDS:

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.

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 µL pipette tip, and a 200 µL 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.

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.

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.

Figure 5: Representative microsatellite peaks. 6-FAM-tagged peaks are visualized in blue. LIZ-500 ladder is shown in orange.

Figure 6: qPCR verification of a differentially expressed gene. Representative mRNA relative quantity (RQ) expression (calculated using the ΔΔCt 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**).

Table 1: Microsatellite primer sequences used to genotype *Aphis ner*ii[20].

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 interactions[40] 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.

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DISCLOSURES:

The authors have nothing to disclose.

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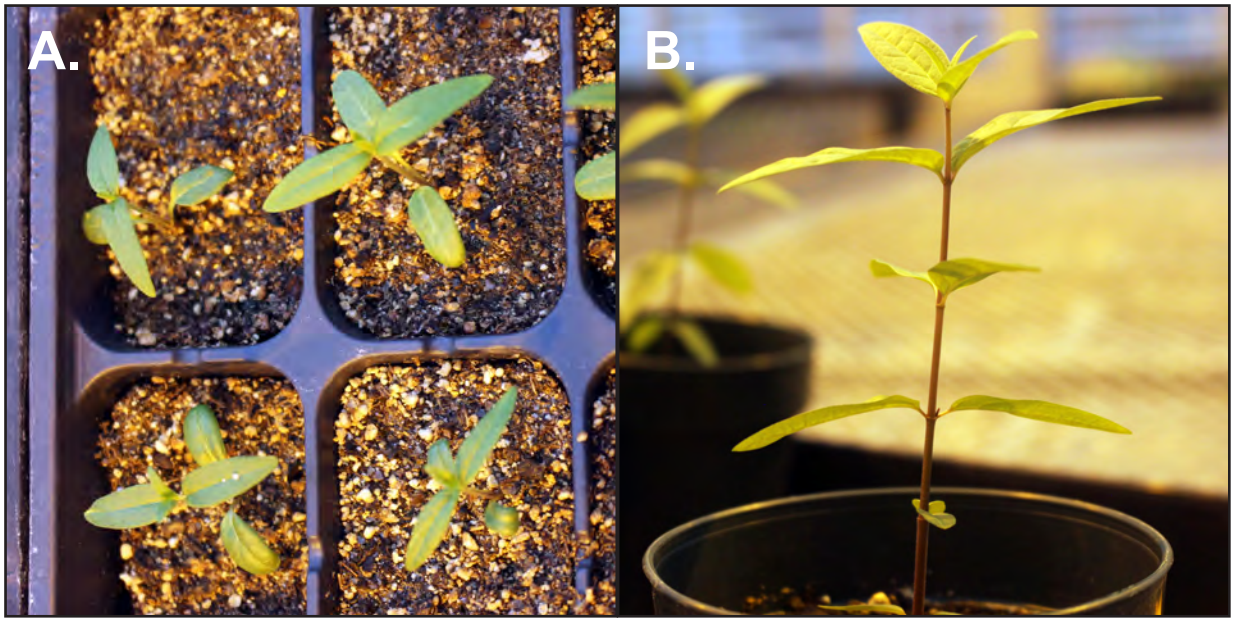

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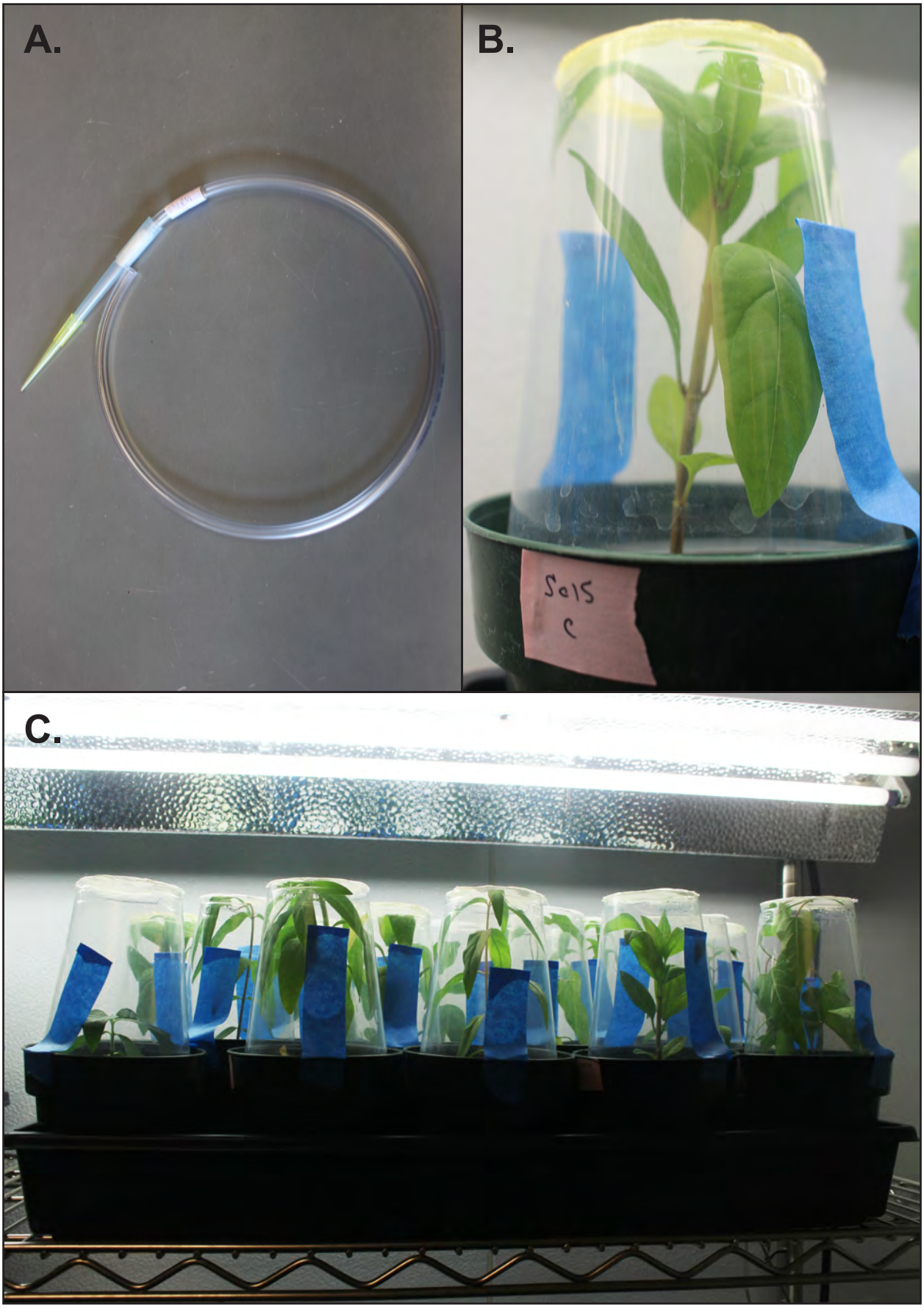

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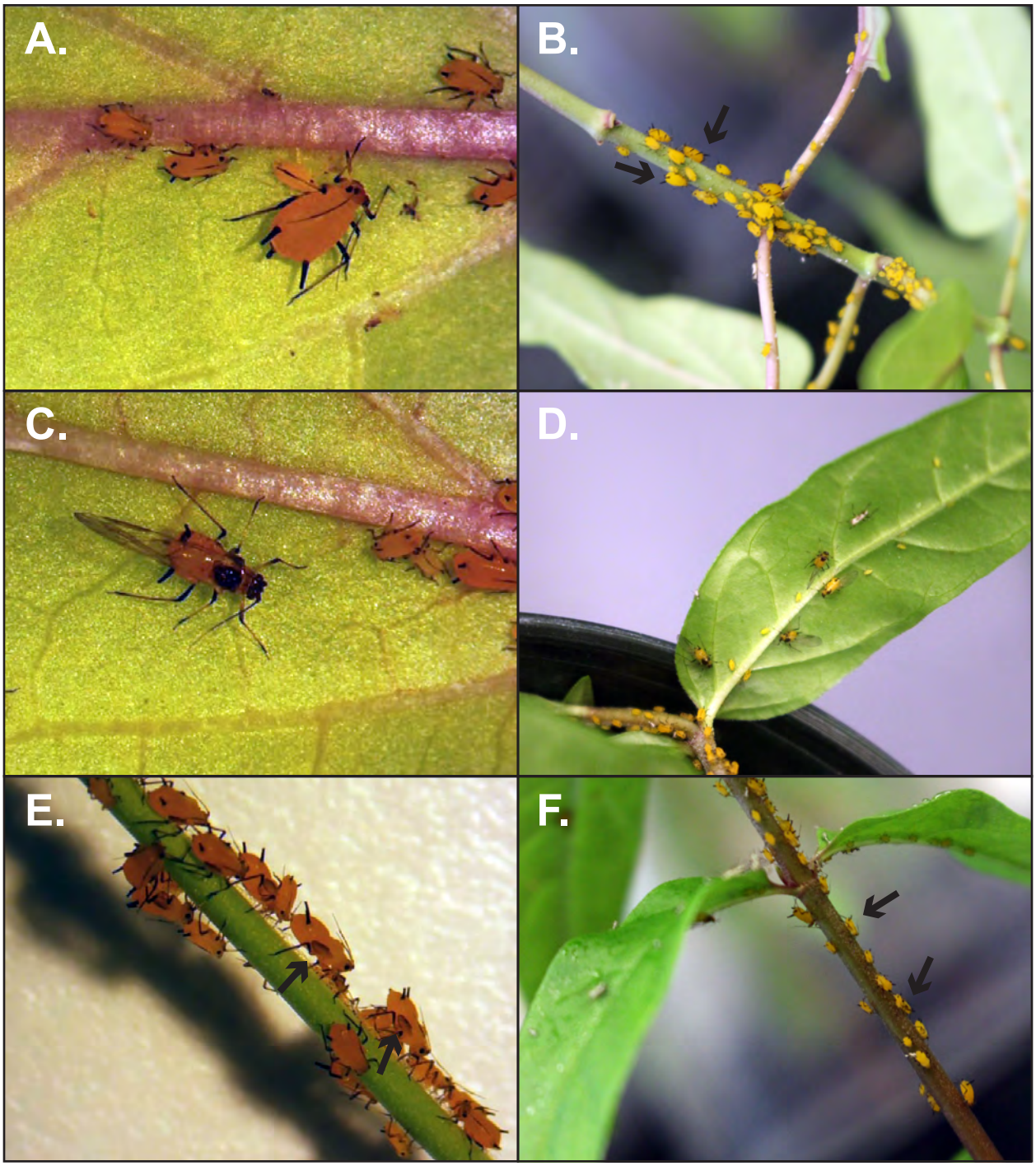

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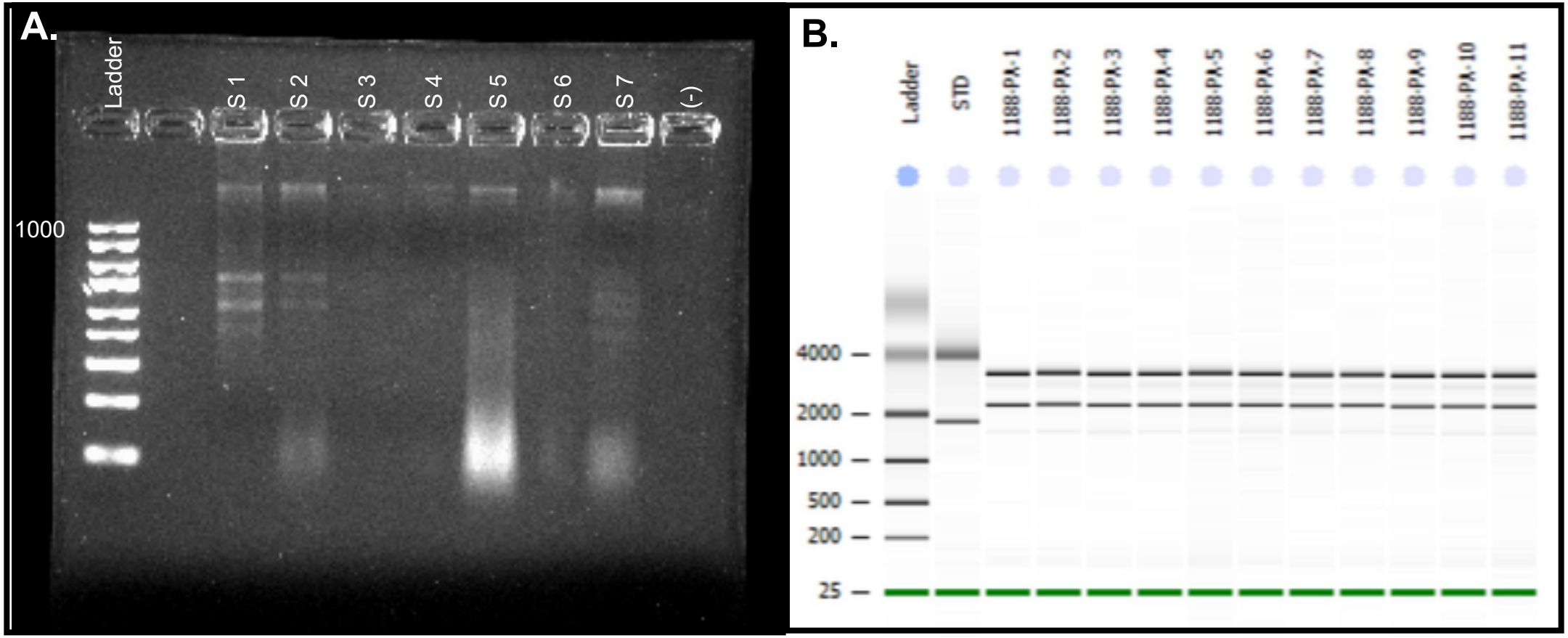

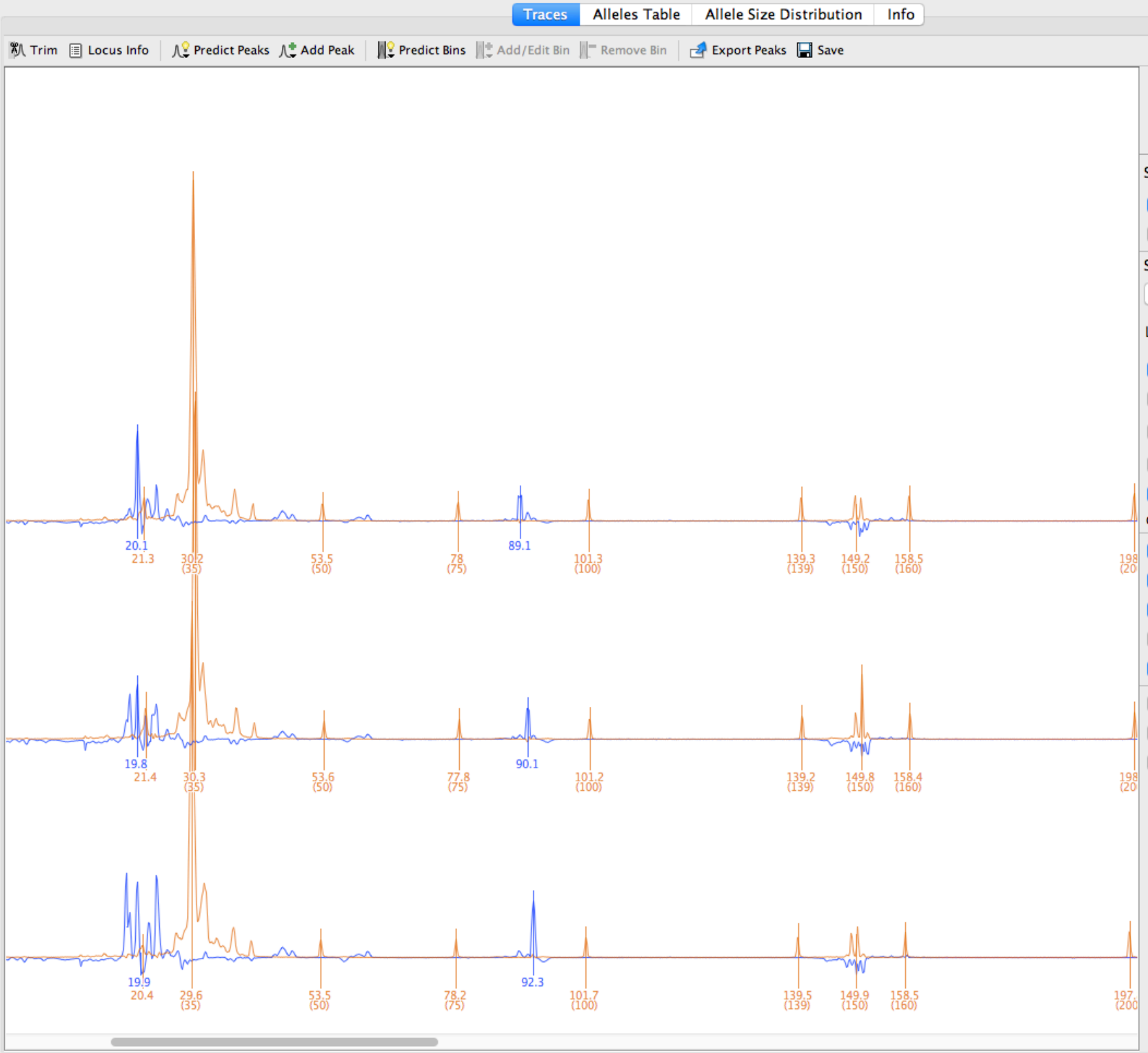

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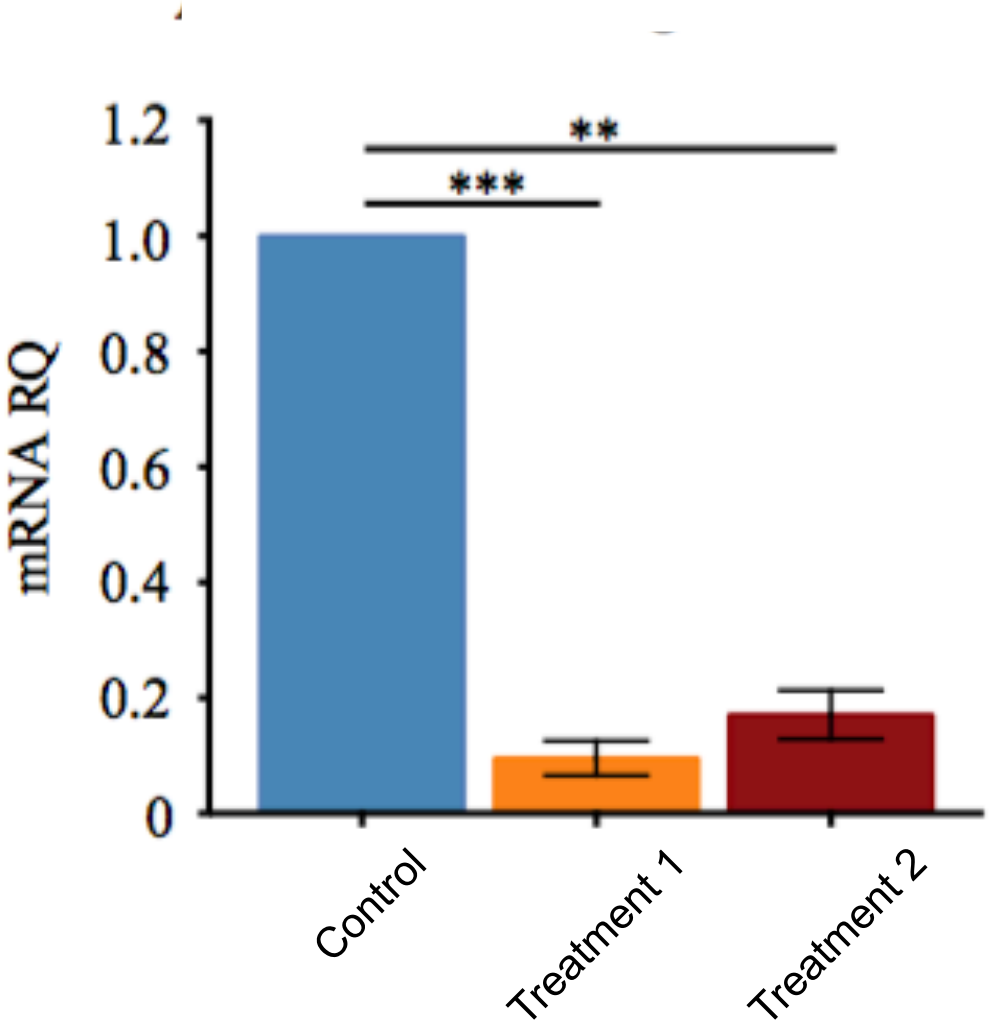

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Primer Name	Direction	Sequence (5'-3')
Ago24_F	forward	TTTTCCCGGCACACCGAGT
Ago24_R	reverse	GCCAAACTTTACACCCCGC
Ago 53_F	forward	TGACGAACGTGGTTAGTCGT
Ago 53_R	reverse	GGCATAACGTCCTAGTCACA
Ago 59_F	forward	GCGAGTGGTATTCGCTTAGT
Ago 59_R	reverse	GTTACCCTCGACGATTGCGT
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

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Target	Sample	Ct Mean	Ct Std. Dev	ΔCt	avg. ΔCt	ΔΔCt	RQ=2^(-ΔΔCt)
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
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
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
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
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
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
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
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
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

RQ SEM

0

0

0

0.031024057

0.013927153

0.043930413

0.041877788

0.069203951

0.008204101

Table of Materials

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Name of Material / Equipment	Company	Catalog Number
Sun Gro Fafard Germination Mix	Hummert International	10-0952-2
Sun Gro Fafard 3B/ Metro Mix	Hummert International	10-0951-2
2x 4" Round Standard Pot	Anderson Pots	1503
DreamTaq DNA Polymerase	ThermoFisher Scientific	EP0701
Trizol	ThermoFisher Scientific	15596026
SuperScrip	ThermoFisher Scientific	18080051
Power SYBR Green PCR Master Mix	ThermoFisher Scientific	4367659

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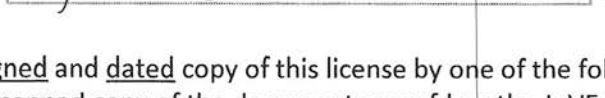
Signature: 

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KEYWORDS:

Aphid, greenhouse, milkweed, microsatellite, RNAseq, qPCR

SHORT ABSTRACT:

The aphid *Aphis nerii* specializes on highly-defended plants in the dogbane family (Apocyanaceae) and provides numerous opportunities to study plant-insect interactions. Here, we present a series of protocols for the maintenance of plant and aphid cultures, and the generation and analysis of molecular and -omic data for *A. nerii*.

LONG 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 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[1]. 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[2]. The publication of the first aphid genome in 2010, the pea aphid *Acyrthosiphon pisum*[3], 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[4]. 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 plant-insect interactions and the ecology of life on plants[11]. 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[12-14]. 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[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 benefits[17]. *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 cardenolides[19], 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[20] and the interplay between bottom-up and top-down regulation on herbivore density[21]. *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 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:

1. Plant Cultures

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) (Figure 1A).

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 (Figure 1B).

2. Aphid Cultures

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 (Figures 3A, 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 2,00 µL pipette tip (Figure 2A).

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 (Figure 2B).

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).

3. DNA extraction

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 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 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.

4. Microsatellite PCR and sequencing for aphid genotyping

4.1. Order the appropriate F and R primers for microsatellite sequencing (Table 1[20]).

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.

5. RNA extraction for RNAseq

5.1. 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.

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 stop 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 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.

6. RNAseq *de novo* transcriptome assembly, annotation, and differential expression analysis

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 (≥ 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 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[22].

6.3. Combine all sample reads and assemble the transcriptome *de novo* using Trinity[23,24] (Trimmomatic quality filtering enabled).

6.4. *Refine the assembly*.

6.4.2. Use Transdecoder[25] to identify open reading frames (ORFs) that are a minimum of 100 amino acids in length.

6.4.3. Perform homology searches of the translated ORFs against Pfam[26] and UniProt[27] databases using BLASTP[28] and HMMER[29], 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[30].

6.4.6. Collapse the remaining, incomplete ORFs that are at least 95% identical at the nucleotide level using CD-HIT[30].

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[31].

6.6. Transcriptome annotation

6.6.1. First, annotate the refined transcriptome using HMMER against the Pfam database[26,29].

6.6.2. Second, annotate the transcriptome using BLASTP against the UniProt database[27,28].

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[32].

Note: If preforming 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[33].

6.7.3. Extract read counts from each sample individually using SAMtools[34].

6.7.4. Calculate differential expression between samples of interest using DESeq2 with default parameters and a parametric fit[35].

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.

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 manufacturers 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

485 independent variables (three points total).
486
487 7.4.4. Use the following equation to calculate the primer efficiency (E) where m is the slope
488 calculated in 7.4.3:
489 E = 10^(-1/m)
490
491 **Note:** Primer efficiencies between 90-110% are suitable for analyses. This process ensures
492 equal amplification of all genes included in the calculations.
493
494 7.5. Use the $\Delta\Delta C_t$ method with a housekeeping gene to quantify differential expression for
495 genes of interest[36].
496
497 **REPRESENTATIVE RESULTS:**
498 *Plant cultures.* Seeds will take approximately two to four weeks, depending on the season, to
499 grow large enough to repot (Figure 1A). Repotted seedlings will take another two to four weeks
500 to grow to an optimal size for aphid cultures (Figure 1B).
501
502 *Aphid cultures.* Adult *A. nerii* are distinguished by some darkened cauda and may be unwinged
503 (apterous, Figure 3A, B) or winged (alate, Figure 3C, D). Developing wing pads become visible
504 when nymphs reach the third instar (Figure 3E, F). Stock cultures are best maintained by
505 transferring one to three mid-instar and one adult-aged unwinged aphids; this ensures a
506 healthy, mixed age population. Populations to be used for experiments should be cultured
507 using unwinged aphids as described above (2.4). One *A. nerii* adult can produce 3-10 offspring
508 per day, dependent on the host plant and age of the aphid[10].
509
510 *DNA and RNA extractions.* Single, adult *A. nerii* will yield approximately 100 – 200 ng/µl DNA (80
511 µl elution; Figure 4A) and 150 – 300 ng/µl RNA (30 µl elution; Figure 4B). Representative
512 microsatellite peaks are shown in Figure 5. Representative relative expression of a candidate
513 gene under three conditions (control, Treatment 1, Treatment 2) are calucated in Table 2 and
514 shown in Figure 6.
515
516 **FIGURE AND TABLE LEGENDS:**
517
518 **Figure 1: Representative plants for aphid cultures. (A)** Seedlings can be repotted after they
519 have developed their first full set of true leaves. **(B)** Plants can be used for aphid cultures when
520 they have developed 3-4 sets of true leaves.
521
522 **Figure 2: Examples of tools used for culturing aphids. (A)** Mouth pipettes can be created using
523 3/16" ID x 1/4" OD plastic tubing, a 1000 ul pipette tip, and a 200 ul pipette tip. **(B, C)** Use cup
524 cages (clear Solo cup with top cut off and secured with fine mesh) to securely fit over the top of
525 4 in. pots used for aphid cultures. This allows for ample light and ventilation to create a suitable
526 environment for the aphids and plant, and keeps the aphids contained.
527
528 **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.

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.

Figure 5: Representative microsatellite peaks. 6-FAM-tagged peaks are visualized in blue. LIZ-500 ladder is shown in orange.

Figure 6: qPCR verification of a differentially expressed gene. Representative mRNA relative quantity (RQ) expression (calculated using the ΔΔCt 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).

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

Table 2: Calculations for qPCR ΔΔC_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.

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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[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 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 in order to fully capitalize on the experimental opportunities these offer. Insect herbivores live in complex communities on their host plants, and both intraspecific interactions[38,39] as well as interspecific interactions[40] 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.

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The authors have nothing to disclose.

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