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An experimental and bioinformatics protocol for RNA-Seq analyses of photoperiodic diapause in the Asian tiger mosquito, Aedes albopictus.

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TITLE:
An experimental and bioinformatics protocol for RNA-Seq analyses of photoperiodic diapause in the Asian tiger mosquito, *Aedes albopictus*.

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SHORT ABSTRACT:
RNA-Seq analyses are becoming increasingly important for identifying the molecular underpinnings of adaptive traits in non-model organisms. Here, a protocol to identify

differentially expressed genes between diapause and non-diapause *Aedes albopictus* mosquitoes is described, from mosquito rearing, to RNA sequencing and bioinformatics analyses of RNA-Seq data.

LONG ABSTRACT:
Photoperiodic diapause is an important adaptation that allows individuals to escape harsh seasonal environments via a series of physiological changes, most notably developmental arrest and reduced metabolism. Global gene expression profiling via RNA-Seq can provide important insights into the transcriptional mechanisms of photoperiodic diapause. The Asian tiger mosquito, *Aedes albopictus*, is an outstanding organism for studying the transcriptional bases of diapause due to its ease of rearing, easily induced diapause, and the genomic resources available. This manuscript presents a general experimental workflow for identifying diapause-induced transcriptional differences in *A. albopictus.* Rearing techniques, conditions necessary to induce diapause and non-diapause development, methods to estimate percent diapause in a population, and RNA extraction and integrity assessment for mosquitoes are documented. A workflow to process RNA-Seq data from Illumina sequencers culminates in a list of differentially expressed genes. The representative results demonstrate that this protocol can be used to effectively identify genes differentially regulated at the transcriptional level in *A. albopictus* due to photoperiodic differences. With modest adjustments, this workflow can be readily adapted to study the transcriptional bases of diapause or other important life history traits in other mosquitoes.

INTRODUCTION:
Rapid advances in next-generation sequencing (NGS) technologies are providing exciting opportunities to probe the molecular underpinnings of a wide range of genetically complex ecological adaptations in a broad diversity of non-model organisms[1–3]. This approach is extremely powerful because it establishes a basis for population and functional genomics studies of organisms with an especially interesting and/or well-described ecology or evolutionary history, as well as organisms of practical concern, such as agricultural pests and disease vectors. Thus, NGS technologies are leading to rapid advances in the fields of ecology and have the potential to address problems such as understanding the mechanistic bases of biological responses to rapid contemporary climate change[4], the spread of invasive species[5], and host-pathogen interactions[6,7].

The extraordinary potential of NGS technologies for addressing basic and applied questions in ecology and evolutionary biology is in part due to the fact that these approaches can be applied to any organism at a moderate cost that is feasible for most research laboratories. Furthermore, these approaches provide genome-wide information without the requirement of *a priori* genetic resources such as a microarray chip or complete genome sequence. Nevertheless, to maximize the productivity of NGS experiments requires careful consideration of experimental design including issues such as the developmental timing and tissue-specificity of RNA sampling. Furthermore, the technical skills required to analyze the massive amounts of data produced by these experiments, often up to several hundred million DNA sequence reads, has been a particular challenge and has limited the widespread implementation of NGS

approaches.

Recent RNA-Seq studies on the transcriptional bases of diapause in the invasive and medically important mosquito *Aedes albopictus* provide a useful example of some of the experimental protocols that can be employed to successfully apply NGS technology to studying the molecular basis of a complex ecological adaptation in a non-model organism[8–10]. *A. albopictus* is a highly invasive species that is native to Asia but has recently invaded North America, South America, Europe, and Africa[11,12]. Like many temperate insects, temperate populations of *A. albopictus* survive through winter by entering a type of dormancy referred to as photoperiodic diapause. In *A. albopictus*, exposure of pupal and adult females to short (autumnal) day lengths leads to the production of diapause eggs in which embryological development is completed, but the pharate larva inside the chorion of the egg enters a developmental arrest that renders the egg refractory to hatching stimulus[15–17]. Diapause eggs are more desiccation resistant[5,18] and contain more total lipids[19] than non-diapause eggs. Photoperiodic diapause in *A. albopictus* is thus a maternally controlled, adaptive phenotypic plasticity that is essential for surviving the harsh conditions of winter in temperate environments. Despite the well-understood ecological significance of photoperiodic diapause in a wide range of insects[20,21], the molecular basis of this crucial adaptation is not well characterized in any insect[22]. In organisms such as *A. albopictus* that undergo an embryonic diapause at the pharate larval stage, it remains a particularly compelling challenge to understand how the photoperiodic signal received by the mother is passed to the offspring and persists through the course of embryonic development to cause arrest at the pharate larval stage.

This protocol describes mosquito rearing, experimental design and bioinformatics analyses for NGS experiments (transcriptome sequencing) performed to elucidate transcriptional components of photoperiodic diapause in *A. albopictus*. This protocol can be used for additional studies of diapause in *A. albopictus*, can be adapted to investigate diapause in other closely related species such as other aedine mosquitoes that undergo egg diapause[23], and is also more generally relevant to employing NGS approaches to study the transcriptional bases of any complex adaptation in any insect.

PROTOCOL:

1. Larval Rearing of two *A. albopictus* groups to adulthood.

1.1) Set two photoperiod cabinets with programmable lighting at 21 °C for optimal diapause expression[16] and approximately 80% relative humidity.

1.1.1) Program one cabinet for a 16L:8D light:dark cycle (a non-diapause inducing LD photoperiod). Set the second cabinet for an 8L:16D light:dark cycle (diapause inducing)[13].

1.1.2) Program ‘lights on’ at the same time in both cabinets to synchronize circadian time between photoperiods.

1.2) Calculate the quantity of eggs needed to perform the experiment. Aim for 300-500 eggs per cage. At least three replicate diapause cages and three replicate non-diapause cages are needed for RNA generation. This totals to ca. 1800-3000 eggs per experiment.

1.2.1) Hatch the eggs by submerging egg papers into ca. 500 ml of deionized H_2O.

1.2.2) Add ca. 1 ml food slurry consisting of ground dog food and brine shrimp as previously described[24]. Cover container with mesh, and keep the mesh in place with a rubber band.

1.2.3) Place in the LD photoperiod cabinet for ca. 24 hours.

1.3) Transfer hatched larvae to 10x10x2 cm petri dishes filled with ca. 90 ml deionized H_2O.

1.3.1) Maintain ca. 30 larvae per dish. Transfer larvae to clean dishes every 48 – 72 hours, for example every Monday, Wednesday, and Friday (M-W-F)[24].

1.3.2) Feed ca. 1 ml food slurry consisting of ground dog food and brine shrimp in deionized water every M-W-F as previously described[24].

1.4) Set up three to four adult cages for each photoperiod treatment, where each cage comprises a biological replicate.

1.4.1) From opposite sides of 9.5 L buckets, cut out one 10- by-14 cm hole, and another hole with 15 cm diameter. Cover the first with mesh. Cut approximately one foot length of an orthopedic stocking, and glue one end around the inside of the other hole.

1.4.2) Cut the foot end of the stocking off and knot shut – open only when access to the interior of the cage is needed. For the cage lid, cut out all of the interior, leaving only the rim, and replace the interior plastic with mesh[24].

1.4.3) Note the photoperiod, replicate number, cage start date, and other information relevant to the experiment with permanent marker on the side of the cage.

1.5) Line the bottom of the adult cages with wet filter paper. Dampen the filter paper with enough deionized H_2O to increase local humidity in the cage, but avoid standing water, which can stimulate oviposition on the filter paper[24]. Check the filter paper daily for drying, re-wet when necessary.

1.6) To produce sufficient eggs for an RNA library, include at least 100 females/9.5 L cage, with no more than 500 mosquitoes per cage.

1.7) Collect pupae M-W-F and place in a small cup of clean H_2O at a density of no more than 50 pupae per 25 ml H_2O. Transfer the pupae cup to an adult cage. Place cages in the respective photoperiod cabinet - *A. albopictus* pupae are photosensitive[15].

1.8) Ensure daily that H_2O in cups is clean and clear, and remove dead pupae, because build-up of dead pupae can cause mass mortality. Remove H_2O cups after all pupae emerge.

1.9) Place organic raisins on the top mesh of the cage to provide sugar for emerged adults. Monitor raisins, and change them every 3-5 days to prevent mold accumulation.

2. Maintenance of adults to allow mating and egg production.

2.1) Maintain cages at high humidity (approx. 80%) by lining the cage bottom with a moist filter paper, and provide access to non-moldy raisins, as described above (Sections 1.5 and 1.9).

3. Blood-feeding.

3.1) Prepare to blood-feed females between two to six days after eclosion to ensure that females have been exposed to at least eight unambiguous short days before oviposition commences for nearly 100% diapause eggs[14].

3.2) Prepare the Hemotek Membrane Feeding system. Plug the feeding units into the power supply. Adjust the temperature of each unit to 37 °C using the adjustment screw. Use an electronic thermometer and probe to measure the temperature of the feeding unit during calibration.

3.3) Prepare the meal reservoir. Stretch a square of collagen feeding membrane over the aperture of the meal reservoir and secure it with an O-ring. Carefully pull the corners to remove wrinkles; trim the excess membrane with scissors.

Note: Collagen membrane may not work well for all mosquito species, and it may be necessary to try several types to find the optimal membrane if working with a species other than *A. albopictus*. Parafilm works well with *Culex pipiens*.

3.3.1) If blood is stored frozen, thaw at room temperature for at least 1 hour before using.

3.3.2) Hold the reservoir so the membrane is facing down, unsupported, and the filling ports are facing up. Use a transfer pipette or syringe to fill the reservoir with approximately 3 ml of whole blood from chickens that has sodium citrate as an anti-coagulant. Seal filling ports with plastic plugs.

3.4) Attach the prepared reservoir to the feeder by screwing it onto the stud on the heat transfer plate on the bottom of the feeder. Invert the feeder and place it on top of the cage, membrane side down, so that mosquitoes can feed through the mesh of the cage. Keep the feeder on the cage for approximately 45 min to maximize feeding.

4. Stimulate Oviposition.

4.1) Four to five days post blood meal, equip each cage with a dark colored 50 ml cup lined with unbleached seed germination paper (egg paper) or textured non-bleached paper towel and fill halfway with deionized water[9]. If more than 250 mosquitoes are in a cage, use two cups.

Note: For small cages or single-female vials, "hay infusion" in oviposition containers may increase oviposition due to the odor of the microbial flora[25].

5. Collect and Store Eggs.

5.1) Commence egg collection within 4-5 days of blood feeding because egg production typically peaks approximately five days after blood feeding, and then subsides over the next week.

5.2) Vary egg collection frequency on necessities of the experiment. For general purposes, collect egg papers on an M-W-F schedule. Remove egg papers from each cage and replace with fresh paper. Place recently removed papers in petri dishes and store in SD photoperiod cabinet to avoid confounding effects of egg storage.

5.3) Allow egg papers to remain wet for 2 days post-oviposition to allow serosal cuticle formation, which increases egg desiccation resistance[26].

5.4) Approximately 48 hours post collection, dry eggs in open air. Dry the paper such that it is limp and slightly damp to the touch, but not so wet that the paper is dark from H_2O or stimulates hatching of eggs.

Note: A 6.5" x 4" paper may take approximately 3.5 hrs to dry. Be cautious not to over-dry egg papers, as this will result in egg desiccation[27].

5.5) Reserve additional eggs from both LD and SD photoperiods to assess diapause incidence and interpret the photoperiodic effect (see Measuring Diapause, Section 6).

5.6) For long-term storage, keep egg papers at 21 °C and approximately 80% humidity in petri dishes. Keep petri dishes in a Tupperware storage container with a flask of water to maintain local humidity as embryonic development takes four to five days at 21 ^{o}C.

6. Measure Diapause Incidence.

6.1) Use additional reserved embryos (see Stimulate Oviposition, Section 4) that are 7 – 20 days old to quantify the diapause response.

6.2) Record the number of eggs present on each egg paper.

6.3) Stimulate eggs to hatch by completely submerging individual egg papers in a 90 ml petri

dish with approximately 80 ml deionized H_20. Add approximately 0.25 ml food slurry.

6.4) After 24 hours tally the number of hatched first instar larvae. Place the petri dish on a black surface to visualize larvae and place a light source on one side of dish. Larvae will move away from the light source, allowing for a clear tally of individual larvae. Remove individual larvae with a pipette while counting to prevent recounting individual larvae.

6.5) Place egg papers in a new petri dish and re-dry. Re-hatch eggs after ~1 week and again tally eggs hatched using the above method.

6.6) Place egg papers with the remaining un-hatched eggs in new 90 ml petri dishes with approximately 80 ml bleaching solution[28]. Ensure that the egg papers are completely submerged in the bleaching solution and leave under a fume hood overnight to avoid the odor of bleach.

Note: Bleaching solution can be stored for ~1 week at 4 °C, but should otherwise be made fresh.

6.7) Inspect eggs using a light microscope as the bleaching will clear the chorion and allow visualization of embryonated, un-hatched eggs. If the egg is embryonated, the egg will have an off-white color with eyes appearing as two small black dots opposite each other on the dorsal side. Tally the number of un-hatched, embryonated eggs[13].

6.8) Determine diapause incidence with the following formula:% diapause = no. embryonated un-hatched eggs/(no. hatched eggs + no. embryonated un-hatched eggs) * 100 [13].

7. RNA extraction from eggs/pharate larvae.

CAUTION: Use Trizol in a laminar flow hood.

7.1) Brush mosquito eggs containing developing embryos or pharate larvae at distinct development time points from egg papers to glass grinders using a camel-hair brush. Grind the eggs in Trizol (1 ml per 50–100 mg of tissue) until completely pulverized. Use at least 400 eggs per library to yield sufficient RNA.

7.1.1) Alternatively, snap freeze eggs in liquid nitrogen and stored at -80 °C in microcentrifuge tubes for up to a month before grinding in Trizol.

7.2) Perform RNA extraction in Trizol followed by isopropanol precipitation according to manufacturer's instructions.

7.3) Treat the bench with RNase decontamination solution or other agents to remove any residual nucleases to avoid RNA degradation.

7.3.1) Treat the extracted RNA with DNase. According to manufacturer's instructions, incubate the RNA samples with DNase for 30 minutes at 37 °C. Use 1 µl DNase for up to 10 µg of RNA in a 50 µl reaction. Increase the amount of DNase if there are more than 10 µg of RNA in one reaction.

7.3.2) Inactivate DNase by adding 5 µl suspended DNase inactivation reagent. Incubate 5 minutes at room temperature, mixing three times during incubation period (gentle vortexing).

7.3.3) Centrifuge at 10,000 x g for 1.5 minutes. Transfer the supernatant containing the treated RNA samples to fresh tubes for subsequent steps.

7.4) Assess the quality of the total RNA samples by fluorometry. Send the samples to a specialty facility with proper instrument for this task. The facility will perform on-chip gel electrophoresis to determine the sizes of RNA species in the sample, visualized by fluorescent dye instilled in the chip. The results will be returned as an electropherogram.

7.4.1) Determine the integrity of total RNA samples by the presence or absence of degradation products, as evidenced by the presence of peaks between the 18S and 5S ribosomal RNA peaks on the resulting electropherogram (Figure 1B).

8. RNA sequencing.

8.1) Send total RNA samples with sufficiently high quality (Figure 1A) and quantity (usually >3 µg per library) to a commercial sequencing center for construction of enriched paired-end mRNA libraries and mRNA sequencing, following standard protocols.

8.2) If more than one lane is being used for sequencing a single experiment, split individual libraries into two lanes for sequencing to account for technical variation among lanes during sequencing.

9. Illumina read cleaning.

Note: Figure 2 summarizes the bioinformatics portion of this protocol. For a full list of all programs and resources used in the bioinformatics section of this protocol, refer to Table 1. In addition, Supplemental File 1 contains command line examples for each of the following bioinformatics protocol steps.

9.1) Use ssaha2[29] (Table 1) to identify matches of 95% identity or higher to the NCBI UniVec Core database (Table 1), *A. albopictus* rRNA sequence (GenBank #L22060.1), and sequencing adapters (detailed command-line examples provided in Supplemental File 1). Remove read pairs with matches using Perl or a similar scripting tool, for example by adapting the provided Perl script (Supplemental File 2).

9.2) Clean remaining reads with the SolexaQA package[30] (Table 1; Supplemental File 1): trim

regions with a phred score equivalent of less than 20 using the default settings of DynamicTrim.pl.

9.2.1) Remove reads shorter than 25 bp with LengthSort.pl on both forward and reverse reads simultaneously. Evaluate the quality of the cleaned fastq files with FastQC (Table 1) – in particular, verify that the per-base sequence quality and the per-sequence quality scores are above 20.

10. Digital normalization.

10.1) Perform one round of digital normalization on the cleaned reads using the khmer tool[31] (Table 1; Supplemental File 1), specifically normalize-by-median.py (using k-mer size 20, a coverage cut-off of 20, and x = 1e10).

10.2) Alternatively, if a machine with high RAM is available (hundreds of GBs), use Trinity's normalize_by_kmer_coverage.pl script (Table 1).

11. *de novo* Transcriptome Assembly.

11.1) Obtain access to a computer or computer cluster with up to 256 Gb of RAM and 24 cpus, depending on the size of the assembly.

11.2) Use Trinity[32] (Table 1; Supplemental File 1) to assemble the digitally normalized read set into contigs. To reduce memory usage, use --min_kmer_cov 2.

12. Assembly evaluation.

12.1) Run assemblathon_stats.pl from the Assemblathon2 project[33] on the Trinity contig output. This script performs basic calculations relevant to evaluating assembly quality, such as number of scaffolds, N50, assembly composition, and more (Table 1; Supplemental File 1).

13. Annotation of the assembled transcriptome.

13.1) Perform Blastx (Table 1) of the assembly against a reference protein set; for mosquitoes, *Drosophila melanogaster, Anopheles gambiae, Culex pipiens*, and *Aedes aegypti* are suitable references. Specifically, format the reference protein fasta file for blast, followed by blastx (Supplemental File 1).

14. Map reads to the assembly using RSEM[34] (Table 1)**.**

14.1) Create a 'transcript-to-gene-map' file, in which the first column contains the reference gene IDs, and the second column the contig IDs. In a spreadsheet editor, swap the first and second columns from the Blastx output, and write these columns to a .txt file. Use the LineBreak program to convert line breaks in the resulting .txt file to Unix format.

14.2) Create a reference dataset from the transcriptome fasta file using the rsem-prepare-reference script, provided in the RSEM package (Supplemental File 1).

14.3) Calculate the expression values separately for each library using the rsem-calculate-expression command, provided in the RSEM package (Supplemental File 1). As reads, use the paired fastq files resulting from the read-cleaning step (step 9.2).

14.3.1) If RNA from a biological replicate was split into two lanes for sequencing, include both fastq files in the expression calculation to generate a single file.

14.4) Convert the expression results from each library to a matrix easily processed by other programs using the provided script rsem-generate-data-matrix, provided in the RSEM package (Supplemental File 1).

15. Differential expression analysis.

15.1) Install R and EdgeR (Table 1).

15.2) Use read.delim to load the RSEM results from step 14.3 (Supplemental File 1). If necessary, round the counts to the nearest integer.

Note: The EdgeR guide recommends limiting the dataset to genes with high enough expression to detect significance.

15.3) To format the data for EdgeR, generate a DGEList object from the loaded data file (Supplemental File 1). Then, normalize the data using TMM normalization (Supplemental File 1). Estimate the common and tagwise dispersions of the data (Supplemental File 1).

15.4) Identify differentially expressed genes with a Benjamini-Hochberg corrected p-value < 0.05 (Supplemental File 1). Plot the distribution of log-fold-change vs. abundance (Supplemental File 1).

REPRESENTATIVE RESULTS:

Fluorometry of two representative RNA samples showed two bands at approximately 2,000 nt (Figure 1A, B). The insect 28S ribosomal RNA is comprised of two polynucleotide chains held together by hydrogen bonds, which are easily disrupted by brief heating or agents that disrupt hydrogen bonds[35]. The resulting two components are approximately the same size as the 18S ribosomal RNA. The second RNA sample showed high levels of degradation (Figure 1B).

Photoperiodic treatment of a representative group of *A. albopictus* mosquitoes resulted in high diapause incidence in short-day-reared mosquitoes, and low diapause incidence in long-day-reared mosquitoes, although there was some variation among replicates (Table 2). For example, replicate SD2 shows lower (80%) diapause incidence than the remaining replicates

(87.18% - 97.67%). This replicate also had the smallest sample size, so it is recommended to set aside a sufficient number of eggs (>150) for the diapause measurement in order to obtain an accurate result.

Post-sequencing read cleaning on one representative library from adult *A. albopictus* females removed a substantial number of reads (from 83,853,322 to 52,736,065 total reads for one representative library). Digital normalization further reduced the number of total reads to 41,435,934. A Trinity assembly of these reads generated 76,377 contigs, with an N50 of 1,879, mean contig length of 1,023.1, and a maximum contig length of 20,892 (Figure 3). Differential expression analyses from a similar workflow of embryos reared under diapause-inducing conditions at 11 and 21 days post-oviposition revealed 3,128 differentially expressed genes between these two time periods (Figure 4).

Figure 1: Fluorometry profiles of example high-quality (A) and low-quality (B) RNA extractions from *A. albopictus.*
The x-axis represents the sizes of the nucleotide fragments, and the y-axis represents the fluorescent readings. Note the difference in the y-axis scale between panels (A) and (B). Arrows mark the positions of the different ribosomal RNAs. The apparent bands close to the green marker band indicate degradation.

Figure 2: Summary of the bioinformatics workflow from read preparation to differential expression. Each box represents a step in the bioinformatics section of this protocol, accompanied by the corresponding number of each protocol step.

Figure 3: Histogram of contig lengths from a Trinity *de novo* transcriptome assembly. The average contig length is 1,023.1. Note that the distribution of contig lengths is heavily skewed towards shorter contigs; this is typical of *de novo* transcriptome assembly.

Figure 4: Log_2-fold-change vs. abundance of TMM-normalized gene expression of diapause pharate larvae at 11 days vs. 21 days post-oviposition. Each point designates a unigene; differentially expressed unigenes are in red. Unigenes with higher expression at 11 days post-oviposition have positive fold-change values, whereas unigenes with higher expression 21 days post-oviposition have negative fold-change values.

Table 1: Programs and resources used for the bioinformatics procedures in this protocol. URLs are listed to easily access each of the resources needed in this protocol.

Table 2: Diapause incidence calculations. Results from five replicates per photoperiod of diapause incidence calculations. Numbers of hatched larvae from two separate hatchings are included, as are the number of un-hatched, embryonated eggs, all of which are necessary to calculate diapause incidence.

DISCUSSION:
This protocol presents methods to discover differentially expressed genes due to

photoperiodically induced diapause in *A. albopictus*. The protocol is significant in that it uniquely combines mosquito rearing and bioinformatics techniques to make all experimental aspects of a molecular physiology program accessible to novice users – in particular for those focusing on the photoperiodic diapause response. Existing methods, to our knowledge, do not provide as much detail in the rearing protocol – which is often necessary to identify rearing mistakes - nor do they provide insight on experimental design during the rearing stage that will enable successful bioinformatic analysis downstream. The methods presented here have been optimized for *A. albopictus*, especially the rearing methods, which generally take six weeks from one laboratory generation to the next. However, in future applications this method could be adapted with modest adjustments to other mosquito species that exhibit photoperiodic diapause[23]. Furthermore, the general experimental design and bioinformatics workflow are applicable to the study of other polyphenisms.

Several points not detailed in the protocol should be considered when rearing *A. albopictus* larvae. First, *A. albopictus* can be found in a wide variety of natural and artificial container habitats as described in previous papers[36, 37]. Used tire lots are a common source of larvae for establishing laboratory colonies. Populations collected above 32°N latitude in North America can be expected to exhibit a strong diapause response[13]. The *A. albopictus* strain used in this protocol was collected from Manassas, VA, and was reared in a laboratory setting for more than eight generations prior to experimental manipulation. Second, lighting in the photoperiod cabinets should be chosen with care. Bulbs in cabinets with built-in lighting functions can cause temperature spikes within the cabinet when the lighting turns on or off. Anecdotal observation suggests these temperature spikes can disrupt the diapause response. To prevent this, built-in lighting functions should be disabled and cabinets should be equipped with a 4-watt cool-fluorescent bulb. Third, larvae are sensitive to H_20 quality and food abundance. Therefore, over-feeding may lead to bacterial accumulation and larval mortality. Fourth, there are alternative methods to blood-feed adult female mosquitoes. Glass membranes are an alternative artificial membrane system[38, 39], although the HemoTek system performs better in the authors' experience. Live animals (usually chicken or rodent) can also be used[38]– in this case, it is essential to first obtain appropriate certification from your Institutional Animal Care and Use Committee (IACUC). Fifth, although there is no clear published evidence that eggs are photosensitive[15], anecdotal observations suggest that eggs from an SD photoperiod treatment exhibit slightly reduced diapause incidence when exposed to an LD photoperiod within 10 days of oviposition. Thus, store both SD and LD eggs under SD conditions to produce a maximal diapause response in the SD eggs and avoid any confounding effect of photoperiod (SD vs. LD) during egg storage.

High RNA quality is essential for generating high quality RNA-Seq data. Abundant care should be taken during the RNA extraction to avoid any nuclease contamination. Low quality RNA samples, such as that shown in Figure 1B, are not appropriate for sequencing. Assessing the RNA quality before sending the samples for sequencing is imperative. Characteristic bands of RNA molecules might be visible for different types of insect tissue used for RNA extraction, such as the four bands smaller than 18S shown in the high quality RNA electropherogram in Figure 1A. Consistent patterns of RNA bands other than the two bands at 18S across samples under

distinct biological treatments can strongly indicate that these bands do not result from degradation, but represent biological composition of the RNA molecules in the specific tissue types chosen in the experimental design.

The bioinformatics workflow outlined here allows a user with some command-line and scripting skills to obtain a list of differentially expressed genes from Illumina sequencing data generated from replicated RNA libraries from two contrasting experimental conditions. While this example concerns genes differentially expressed due to photoperiod, this workflow can be applied to any experimental design with two or more treatments, in any organism. There are many other ways to arrive at a list of differentially expressed genes; however, this protocol is likely to be the most straightforward approach for the novice user. More experienced bioinformaticians may want to take extra measures to improve the contiguity and redundancy of their assembly. Biologists with little to no bioinformatics experience may also complete at least part of this pipeline within the iPlant[40] Discovery Environment, which is a free graphical-user-interface driven analysis environment. It is likely that iPlant's functionality will grow larger in the future in order to accommodate full RNA-Seq pipelines from de novo transcriptome assemblies. Finally, note that the excellent User's Guide thoroughly discusses the many ways to use EdgeR[41] (Table 1) for differential expression analysis.

In some cases, mis-assemblies can generate chimeric contigs. There are several methods that can help to identify these mis-assemblies, for example, Uchime[42]. However, from past experience, the number of detected chimeras is exceedingly low (< 0.1%); therefore, employing a chimera detection program may not be worth the extra effort.

Processing high-throughput, next-generation sequencing data requires the ability to 1) store large amounts of data (for a single project, >500 Gb); 2) manipulate large data files that cannot be opened in traditional word processors or spreadsheet programs; 3) perform analyses that require large amounts of RAM, e.g., for *de novo* assembly; and 4) analyze large datasets, either through programs driven by a command-line interface (which requires the ability to install these programs, which is often non-trivial), or through analysis suites with graphical user interfaces (e.g. Galaxy[43] or iPlant[40]). Researchers with some proficiency in Unix command line and a scripting language will gain the most benefit from access to a local computing cluster - either University-owned, via a collaborator, or purchased for their own laboratory. For example, the above workflow was accomplished using a laboratory-owned Macintosh (12 cores, 64 GB RAM, 1 Tb hard drive), and a University-owned computer cluster for the Trinity assembly. If similar resources are not available, researchers can still turn to iPlant to perform large-scale analyses at no cost, and with relatively lower investment in training due to the graphical interface environment. However, those performing and interpreting the analyses still need to understand the assumptions of each program used.

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The authors have nothing to disclose.

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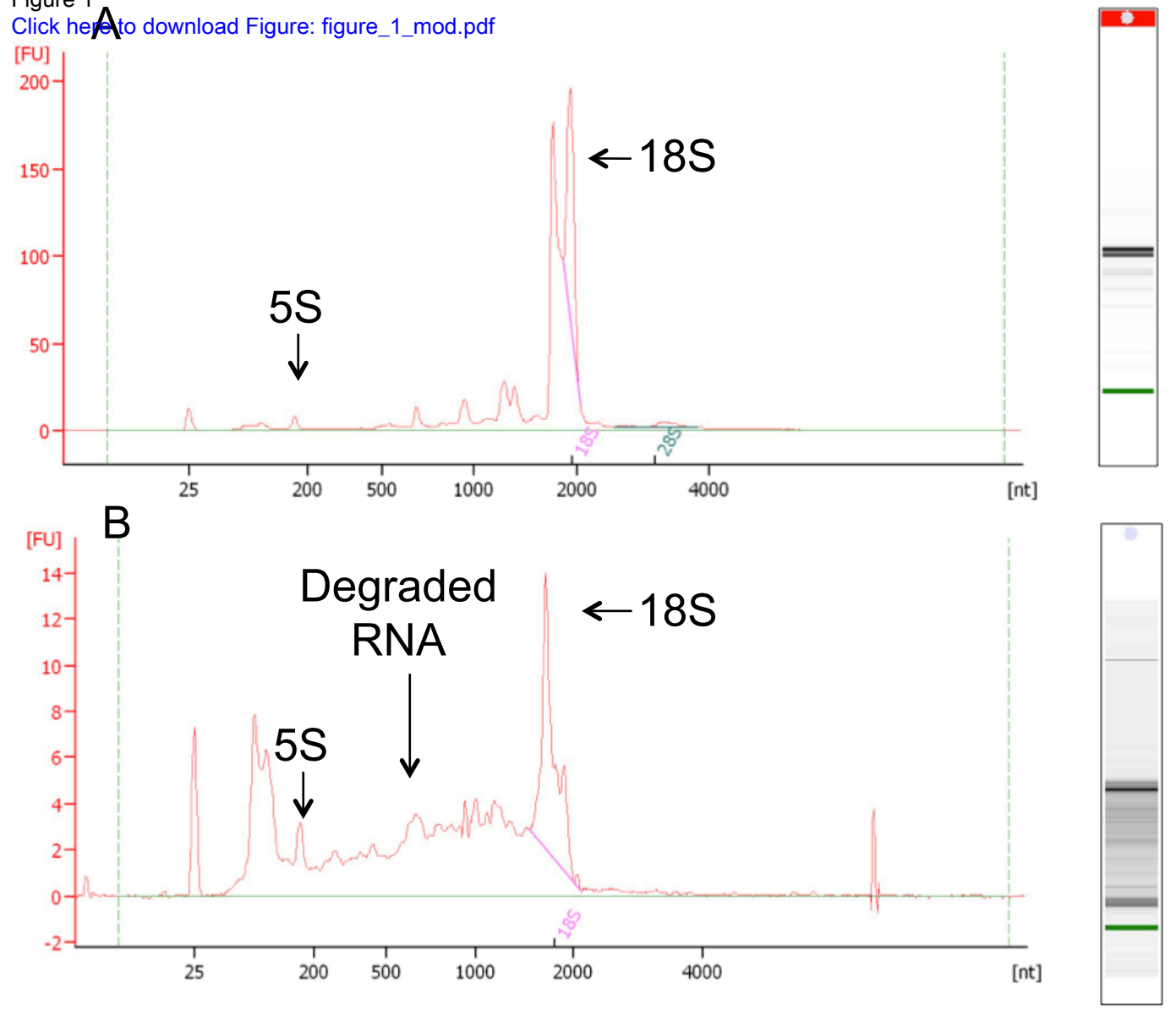

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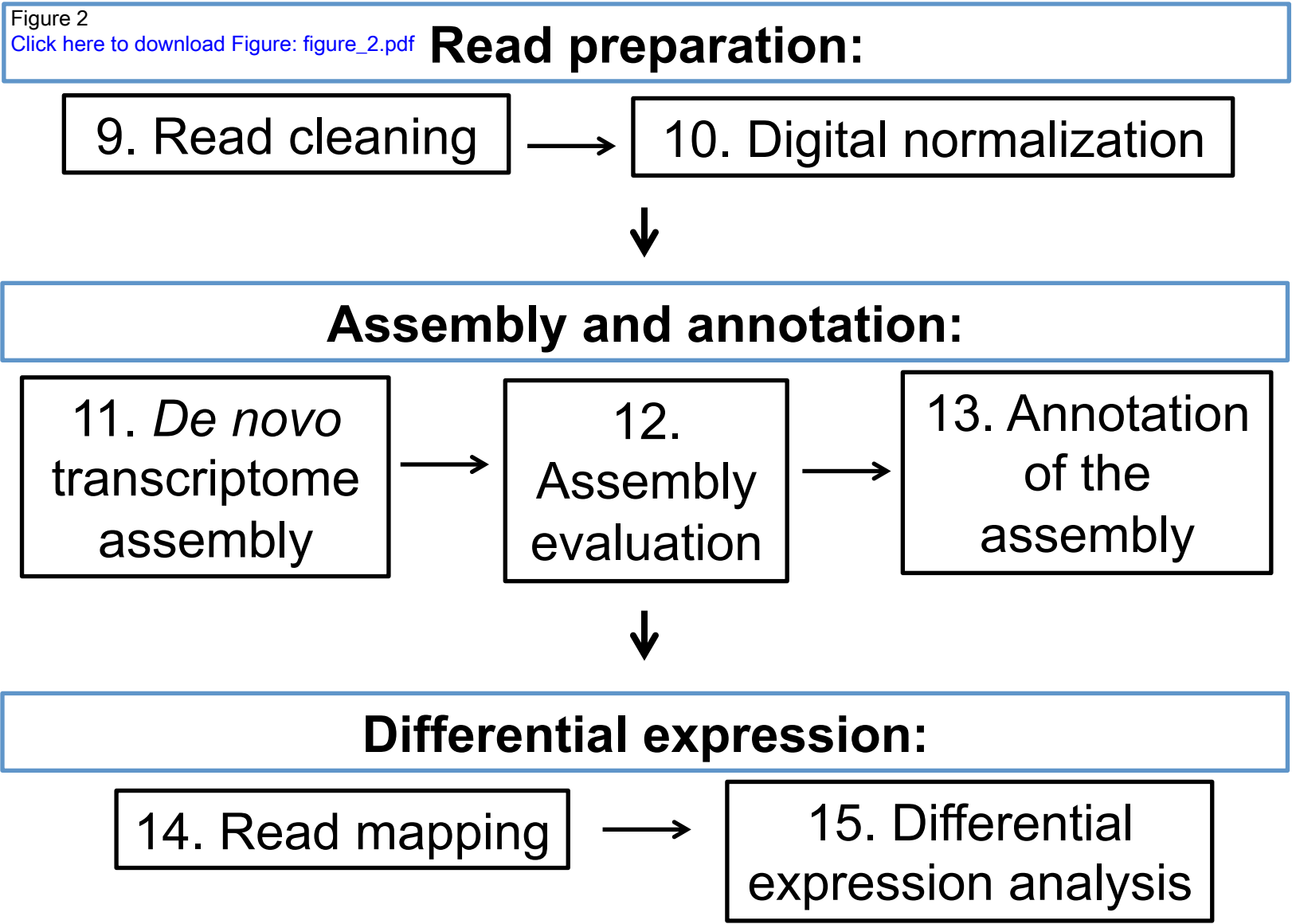

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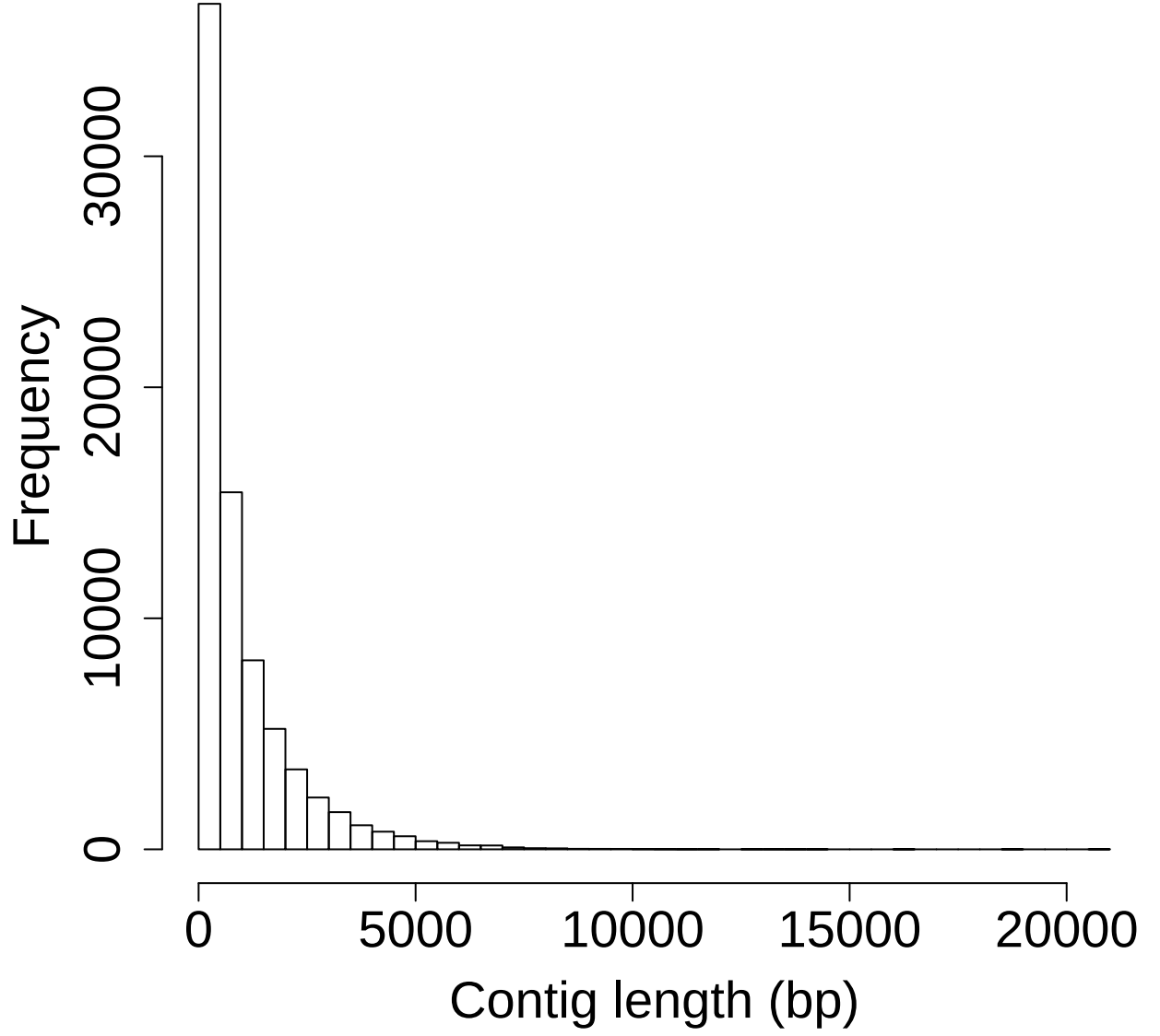

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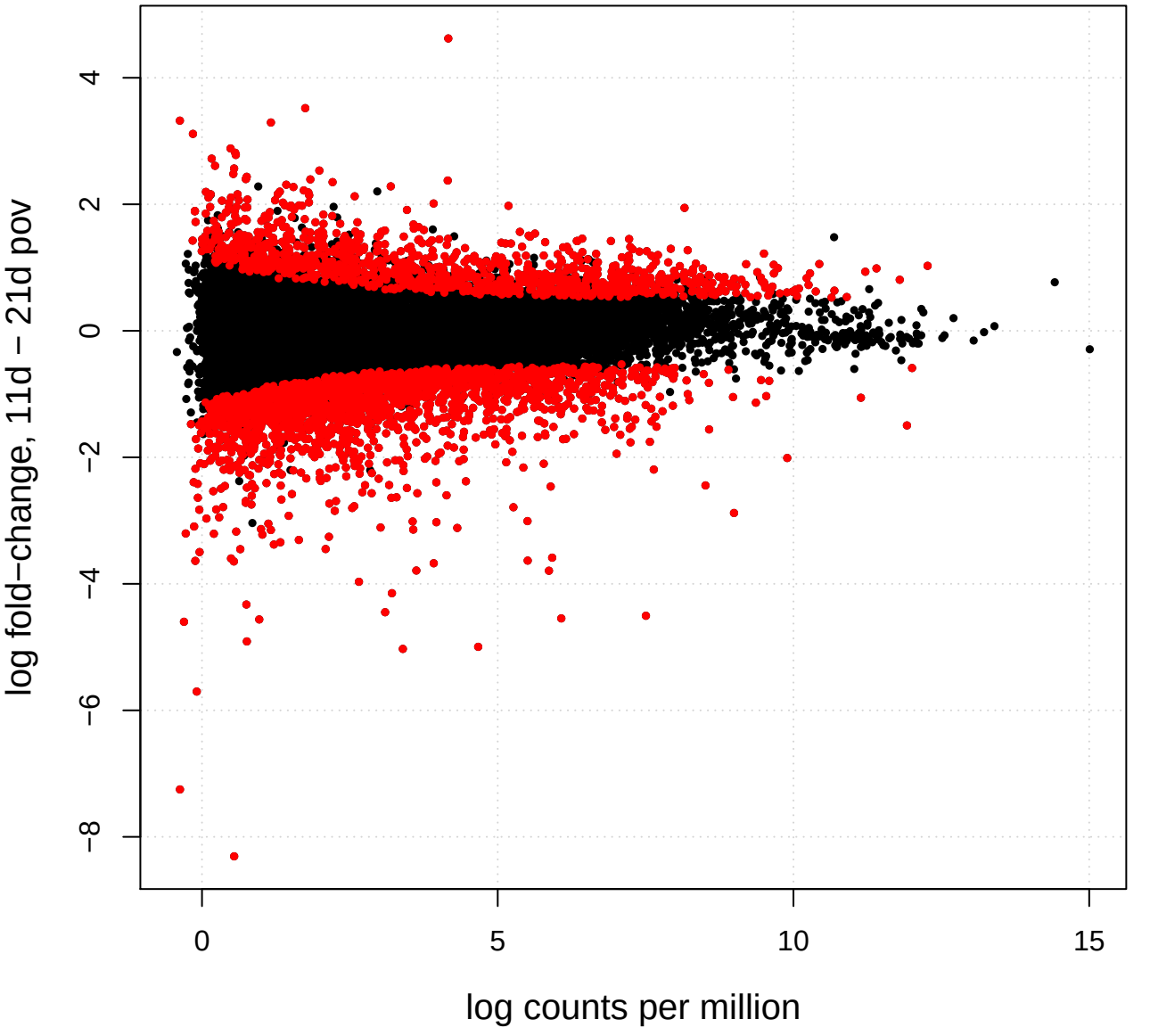

Table 1
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Program/Resource	Website URL (accessed 1/13/2014)
perl	http://www.perl.org/get.html
python	http://www.python.org/download/
ssaha2	http://www.sanger.ac.uk/resources/software/ssaha2/
NCBI UniVec Core	ftp://ftp.ncbi.nih.gov/pub/UniVec/UniVec_Core
SolexaQA	http://solexaqa.sourceforge.net/
FastQC	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
khmer	https://github.com/ged-lab/khmer
Trinity	http://trinityrnaseq.sourceforge.net/
Trinity normalization script	http://trinityrnaseq.sourceforge.net/trinity_insilico_normalization.html
Assemblathon 2 evaluation scripts	https://github.com/ucdavis-bioinformatics/assemblathon2-analysis
BLAST+(which includes makeblastdb and blastx)	ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/
linebreak	https://code.google.com/p/linebreak/
RSEM	http://deweylab.biostat.wisc.edu/rsem/
EdgeR User's Guide	http://www.bioconductor.org/packages/2.13/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf
R	http://www.r-project.org/
EdgeR	http://www.bioconductor.org/packages/2.13/bioc/html/edgeR.html

Table 2
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Treatment	Replicate	No. of eggs from 1st hatch	No. of eggs from 2nd hatch	No. embryonated, unhatched eggs	% Diapause
SD	1	10	0	68	87.18
SD	2	3	0	12	80.00
SD	3	1	0	42	97.67
SD	4	5	0	46	90.20
SD	5	6	0	79	92.94
LD	1	79	4	9	9.78
LD	2	28	0	4	12.50
LD	3	92	1	6	6.06
LD	4	30	0	5	14.29
LD	5	43	0	3	6.52

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Incubator - Model 818	Thermo-Scientific	3751	120V
Controlled environment room	Thermax Scientific	N/A	Walk-in controlled environment room built to custom specifications by Thermax Scientific Products. A larger alternative to an incubator. http://thermmax.com/
Cool Fluorescent bulb	Philips	392183	4 Watt
Petri Dish 100mm x 20mm	Fisher	08-772-E	
Filter Paper 20.5cm	Fisher	09-803-6J	
9.5L Bucket	Plastican	Bway Products	http://www.bwayproducts.com/sites/portal/plastic-products/plastic-open-head-pails/117
Utility Fabric-Mosquito Netting White	Joann	10173292	http://www.joann.com/utility-fabric-mosquito-netting-white/10173292.html
Orthopedic stockings	Albahealth	23650-040	product no. 081420
Organic Raisins	Newman's Own	UPC: 884284040255	
Oviposition cups (brown)	Fisher Scientific	03-007-52	The product is actually an amber 125 mL bottle that we saw the top off of.
Recycled Paper Towels	Seventh Generation	30BPT120	
Modular Mates Square Tupperware Set	Tupperware		http://order.tupperware.com/pls/htprod_www/coe$www.add_items
Glass Grinder	Corning Incorporated	7727-2	These Tenbroeck tissue grinders break the eggs and release RNA into the TRI Reagent.
TRI Reagent	Sigma Aldrich	T9424	Apply 1ml TRI Reagent per 50-100mg of tissue. Caution - this reagent is toxic.
TURBO DNA-free	Ambion/Life Technologies	AM1907	This kit generates greater yield than traditional DNase treatment followed by phenol/chloroform cleanup, and it is simpler to use.

RNaseZap	Ambion/Life Technologies	AM9782	Apply liberally on the bench surfaces and any equipment that might be in contact with the RNA samples. The solution is slightly alkaline/corrosive, can cause irritation and is harmful when swallowed.
2100 Bioanalyzer	Agilent Technologies	G2939AA	Place up to 12 RNA samples on one chip.
Hemotek Membrane Feeder	Hemotek	5W1	This system provides 5 feeding stations that can be used simultaneously. Includes PS5 Power Unit and Power cord; 5 FUI Feeders + Meal Reservoirs and O-rings; Plastic Plugs, Hemotek collagen feeding membrane; Temperature setting tool; and Plug extracting tool. The company's mailing address is: Hemotek Ltd; Unit 5 Union Court; Alan Ramsbottom Way; Great Harwood; Lancashire, UK; BB6 7FD; tel: +44 1254 889 307.
Digital Thermometer and Probe	Hemotek	MT3KFU	MicroT3 thermometer and KFU probe. This is used to set the temperature of each FUI feeding unit.
Chicken Whole Blood, non-sterile with Sodium Citrate	Pel-Freez Biologicals	33130-1	The 500 ml of blood were frozen and stored in 20 ml aliquots at -80 degrees C for up to 1 year. Thaw blood at room temperature for at least 1 h before using.

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Author response: We have proofread the manuscript. We have also performed the following modifications:

- We have modified step 1.2 and sub-steps to clarify how to calculate the number of eggs to hatch (l. 132-141).
- We have slightly changed the text in step 7.2 (l. 299) to clarify that the RNA extraction is performed in Trizol.
- We have switched steps 7.3.1 and 7.3.2 to improve the logical flow of the methods (l. 299-303).
- We have exchanged the representative electopherogram that was depicted in Figure 1a with another electropherogram that more clearly demonstrates the tissue-specific banding patterns between the 18S and 5S bands. Also, we have removed the arrows indicating the 28S RNA band – these were misleading, as the 28S RNA is degraded in *A. albopictus* and other Diptera.
- In the Discussion, l. 539, we have removed the reference to the organisms that iPlant accepts in order to avoid confusion.

2. Please provide additional details to step 7.4.1 (the electrophoresis step) and step 7.4.2.

Author response: We have clarified the methods in step 7.4 (l. 316-323). We do not think it is appropriate to add more details to the electrophoresis step, as this step is performed at a specialized facility – the only task that the reader would perform is to send it to the facility. The previous sub-structuring of this step made this unclear. We have merged step 7.4.1 into step 7.4.
What was previously step 7.4.2 is now 7.4.1. We have added more details to this step, and also now refer to Figure 1B:

"7.4) Assess the quality of the total RNA samples by fluorometry. Send the samples to a specialty facility with proper instrument for this task. The facility will perform

on-chip gel electrophoresis to determine the sizes of RNA species in the sample, visualized by fluorescent dye instilled in the chip. The results will be returned as an electropherogram.

7.4.1) Determine the integrity of total RNA samples by the presence or absence of degradation products, as evidenced by the presence of peaks between the 18S and 5S ribosomal RNA peaks on the resulting electropherogram (Figure 1B)."

3. Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length (2.75 pages or less). Please see JoVE's instructions for authors for more information.

Author response: We have updated the highlighting on l. 121, 174, 218, 227, 253, 280, and 287.

4. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. You are able to highlight up to 2.75 pages and you only highlighted 2 pages. Is there anything else you would like to put on video. Also in step 9, only one sentence is highlighted referencing Figure 2. What did you want to be shown for this step?

Author response: We have highlighted the RNA extraction steps (step 7 and sub-steps) for filming, for a total of 2.75 highlighted pages. We highlighted Figure 2 with the intention that this would serve as a brief summary of the steps performed in the bioinformatics part of the protocol; the camera could pan over the figure while the narrator could say something similar to 'The remainder of the protocol outlines how to process the resulting next-generation sequencing data, from read preparation, assembly and annotation, and differential expression analysis. See the accompanying paper for methods and sample command line examples".

5. Please revise the Discussion to cover the following in detail:
a) The significance with respect to existing methods
b) Any future applications of the technique

Author response: We have re-organized the first paragraph of the discussion (l. 480-492) to include new statements explaining the significance of the outlined protocol with respect to existing methods, and potential future applications of the technique. Because the modifications to this paragraph were substantial, we decided to move the text devoted to discussing the variation in diapause response to the Results section (l. 435-439).

6. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then *et al.* Please abbreviate all journal titles with the appropriate abbreviation.

Author response:
We have corrected the references to show only abbreviated journal titles, and removed the second author entry from references with more than six authors.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is a nice paper outlining a bioinformatic workflow for the processing of RNAseq data.

Major Concerns:
No Major concerns

Minor Concerns:
It might be nice for people reading this article to have some discussion of the various options given to the various programs. No one using this is going to use the same parameter set as you did, at least they probably shouldn't. Some discussion of the major parameters and what they mean might add a nice dimension to the experimental outline.

Author response: We have added additional explanations/discussions of the command parameters to the following steps:

- Digital normalization (step 10). We have added more explanations to the command parameters of normalize-by-median.py in the supplemental file 1.
- Trinity *de novo* assembly (step 11). We have added more explanations to the command line parameters of the Trinity.pl script in the supplemental file 1.
- Annotation of the transcriptome (step 13). We have added an example of how and when to modify the e-value cutoff for the blastx command in the supplemental file 1.
- The parameter choices for EdgeR are numerous, and will depend on the particulars of the dataset and the question asked. In the supplemental file 1, we now emphasize that the reader should review the EdgeR users' guide prior to performing any analysis to familiarize themselves with the software's many options.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
Polecuau et al. describe an experimental protocol involving A. albopictus mosquitoes in the context of experiments that analyze the genes involved in the physiology of photoperiodic diapause. The topic is very timely, as there is evidence

that these mosquitoes are evolving to survive in more temperate climates, allowing populations to spread further north. A. albopictus is a vector of the viruses that cause St. Louis encephalitis, dengue, yellow fever, West Nile and other human diseases. Investigating the set of genes that may be promoting its northerly spread will certainly be the focus of research beyond the Armbruster lab. An additional significance of this paper is that it serves as an important bridge from researchers that are working in common model systems eg. Drosophila, Arabidopsis and C. elegans, to non-model systems. Working with this particular vector involves special considerations, and a video tutorial that highlights some of its idiosyncrasies would be very valuable. However, the way that this manuscript bridges practical work with biological specimens to the workflow strategies of bioinformatic analysis is relevant to an infinite array of additional biological systems. The manuscript is well written contains a minimum of terms that would not be encountered by a typical mosquito researcher. The work is original and important. I am not aware of any similar videos that show mosquito rearing methods as applied to the analysis of A. albopictus or mosquitoes in general. A generalized primer on how to get from biological samples to usable bioinformatics data will also be a worthwhile step forward, especially for those who have been reluctant to enter a new and complex area of research.

Major Concerns:
None

Minor Concerns:
Capitalization in the name: Asian tiger mosquito should be consistent in the title and the text.

Author response: We have changed 'tiger mosquito' to lower-case throughout the text (specifically, on lines 2 and 38).

* Do you think the steps listed in the procedure would lead to the described outcome?
Certainly.

* Are the steps listed in the procedure clearly explained?
Yes, at least from the mosquito side of things. I am not as familiar with the bioinformatics, but it looks solid. The manuscript provides a stepwise guide for how to approach the bioinformatic analysis and does not attempt to serve as a user manuals for any particular application. In this context, I think they have done an admirable job of providing enough detail to be meaningful, but keeping it sufficiently broad to be still relevant as the software advances.

* Are any important steps missing from the procedure?
The bioinformatics applications will always be changing, so I think they were sufficiently inclusive and included the appropriate caveats about understanding the assumptions that are being made by the software. That said, I think that a stepwise protocol of how to go about analyzing these data would be enormously helpful to

those entering the field and will reduce the barriers of entry.

* Are appropriate controls suggested?
Yes, especially with regard to mosquitoes.

* Are all the critical steps highlighted?
Yes, especially with experimental details that some might not think of, such as keeping the eggs at the same light cycle. As the eggs are opaque, this might not occur to some researchers studying egg diapause.

* Is there any additional information that would be useful to include?
Are there laboratory strains of A. albopictus? Where might these mosquitoes be sourced from? Not enough information was provided about the original source of the mosquitoes used in this experiment.

Author response: We have added text in the discussion (l. 495-500) that refers to *A. albopictus* habitat and container preferences, and where the strain used in this protocol was collected from:
"First, *A. albopictus* can be found in a wide variety of natural and artificial container habitats as described in previous papers[36, 37]. Used tire lots are a common source of larvae for establishing laboratory colonies. Populations collected above 32°N latitude in North America can be expected to exhibit a strong diapause response[13]. The *A. albopictus* strain used in this protocol was collected from Manassas, VA, and was reared in a laboratory setting for more than eight generations prior to experimental manipulation."

* Are the anticipated results reasonable, and if so, are they useful to readers?
No.

* Are any important references missing and are the included references useful?
No

Additional comments:
Some of the equipment required for this experiment is specialized and may not be available to all mosquito researchers. One suggestion would be to provide more specific information on where to purchase the HemoTek feeding system. This instrument is not sold through standard distributors and the company does not seem to have a functional website. Is it still available? Is the HemoTek system better than traditional glass membrane feeders? Glass membrane feeders are getting harder to find, so it might be good to suggest vendors or a reference for a design that can be made by a glass shop.

Author response: We have included the mailing address for the HemoTek feeding system in the Table of specific reagents/equipment. In the discussion (l. 506-509), we now refer to glass membranes as an alternative artificial feeding system, and cite papers that explain how glass membrane feeders may be used:

“Fourth, there are alternative methods to blood-feed adult female mosquitoes. Glass membranes are an alternative artificial membrane system[38, 39], although the HemoTek system performs better in the authors’ experience.”

I think it would be important to point out that if vertebrate animals are used for feeding, then proper animal care and use protocols must be in place. In some cases, researchers are required to use live animals, since not all mosquitoes are equally capable of membrane feeding. This issue should at least be addressed, even though it is certainly true that work that is described in this particular study does not require the use of vertebrate animals

Author response: We now address that live animals can be used to bloodfeed, and refer the reader to other manuscripts that outline how live animals can be used for bloodfeeding (l. 509-511):
“Live animals (usually chicken or rodent) can also be used[38]– in this case, it is essential to first obtain appropriate certification from your Institutional Animal Care and Use Committee (IACUC).”

A few small issues:
A "laminar flow hood" is not the same thing as a fume hood. I do not see the difference between using phenol in a laminar flow hood and any other well ventilated space. The purpose of a laminar flow hood is to protect the sample from contamination, not to protect the user from being exposed to harmful fumes. Does a mild bleach solution (to dechorionate eggs) really need to be used in a fume hood?

Author response: We have modified the text to state that leaving the bleach solution in the fume hood helps prevent the odor of bleach from spreading (see l. 271-274).

Line 115: … and is also more…

Author response: Corrected.

Line 142-3 Why mention specific days, MWF? Nowhere else are days mentioned. Were the eggs hatched on a particular day that not mentioned here?

Author response: We maintain a MWF schedule in our lab. We have generalized our recommendation to transfer the larvae every 48-72 hours, and suggest a M-W-F schedule to maintain this pattern (l. 145-146):
1.3.1) “Maintain ca. 30 larvae per dish. Transfer larvae to clean dishes every 48 – 72 hours, for example every Monday, Wednesday, and Friday (M-W-F)[24].“

Page 25. Collagen

Author response: We have corrected the spelling of ‘collagen’ in the Table of Materials/Equipment.

Revised ms with tracked changes (as requested by JoVE)

Click here to download Supplemental File (as requested by JoVE): revision_track_changes.docx

Supplemental File 1

Click here to download Supplemental File (as requested by JoVE): supplemental_file_1_mod.txt

Supplemental File 2 (as requested by JoVE)
Click here to download Supplemental code file (if applicable): supplemental_file_2.pl