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Author(s):	**Aaron M. Tarone**
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PD/PI Name, Title and Contact Information:	Aaron M. Tarone 2475 TAMU Department of Entomology Texas A&M University College Station, TX 77843-2475
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Project Design: In forensics, entomologists estimate the age of immature insect, primarily flies, in order to provide meaningful information for timelines in death investigations (Byrd and Castner 2010). At the present, forensic entomology seems to be accurate and precise with early developmental stages and accurate but imprecise with the later developmental stages (Tarone and Foran 2008, VanLaerhoven 2008, Tarone and Foran 2011). The later developmental stages are associated with longer periods of time: up to 25% in postfeeding third instars and 50% in intrapuparial development of *L. sericata.* Therefore, without additional information, there is little that can be done to increase precision in estimates of age for postfeeding third instars and pupae.

The central dogma of biology posits that the DNA of an organism's genome encodes genes, which are transcribed into messenger RNAs, which then are translated into proteins that perform all of the functions that help an organism survive. Accordingly, any of these molecules are potential markers of fly developmental progress. This concept applies to developing larvae and pupae of carrion-feeding flies and can be very useful in evaluating how one might use molecular markers to predict fly age. Much focus has been evaluating mRNA levels (Tarone et al. 2007, Tarone and Foran 2011, Sze et al. 2012, Boehme et al. 2013) and the ability to develop statistical models to predict blow fly age and increase precision in larval and pupal age estimation (Tarone et al. 2007, Tarone and Foran 2011, Boehme et al. 2013). Unfortunately, mRNA is widely known to be less chemically stable than DNA (Parker et al. 1991, Peltz et al. 1991) and is also targeted by numerous organisms that express RNAse, further risking degradation of mRNA in casework (Regnier and Arraiano 2000). Since forensic casework is often collected in non-ideal conditions for RNA work, markers that are less likely to degrade in typical preservative solutions (like 70% ethanol), but are equally informative of developmental progress as mRNA, are warranted.

Ideally, a molecular marker of age would require little additional expertise that a crime lab does not already possess, be stable in casework conditions, be used on site at a death scene or require no additional treatment, and be insensitive to environmental or genetic changes. Two types of molecules could potentially expand upon previous findings and address many of these requirements. The first, microRNA (miRNA). These short (~22bp), hairpin shaped RNAs expressed by cells are used to regulate gene expression. These miRNAs are partially double stranded and short, which seems to impact their insensitivity to degradation, making them more robust than mRNA. For this reason, these molecules may be more ideal targets for forensic analyses than mRNA (Courts and Madea 2010, 2011). They are already studied in forensics for this reason, and have recently been cataloged in *L. sericata* (Blenkiron et al. 2015).

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Another class of molecule that is likely to satisfy criteria required of a molecular marker of fly age is proteins, which are also more stable than mRNA. Until recently, it was not possible to screen for large numbers of differentially expressed proteins in a non-model organism, with much of a hope of faithfully identifying the targets – as *de novo* proteomics is notoriously difficult and is improved with reference sequence data (Waridel et al. 2007). These molecules as markers of age are exciting as it is easy to imagine an immunological assay for protein expression that mirrors the well-known pregnancy test (Wide and Gemzell 1960, Butler et al. 2001). Such a test, targeted toward proteins found to be expressed only in a specific portion of pupation (or other time points in development), could be used on-site during a death investigation (for instance in the style of a pregnancy test) or could easily be implemented by a forensic analyst.

Purpose and Goal: This project attempts to catalog miRNAs and proteins which are differentially expressed during intrapuparial development of three blow fly species and a flesh fly species of forensic relevance. These stable markers will ideally be employed in actual casework to increase precision of age estimates of entomological evidence more effectively than current molecular options for predicting insect age across a range of environments and genotypes.

Methods:

Colony Care: All species were reared in insect cages maintained in the Forensic Laboratory for Investigative Entomological Sciences (FLIES) facility. Flies were reared at controlled temperatures and maintained on a diet of beef liver, sugar, water, honey, and powdered milk.

Sampling Scheme*:* Mature flies were provided liver in four hour windows to promote egg laying. Eggs were mixed to promote heterogeneity and then separated into aliquots of approximately 200-250 eggs for development studies. Egg aliquots were put into 1qt mason jars with 200 g of sand. This was performed in four separate cages with enough jars for unique sampling and undisturbed development time measurement (~ 15-20 jars per cage). Jars were maintained in Percival incubators at 25^{o}C, 14:10h Light:Dark cycle and 50% relative humidity, unless otherwise specified. Once pupae were observed, samples were collected daily until adult flies were observed. Samples were collected into labelled 15 mL tubes and flash-frozen. Samples were stored at -80^{o}C until further analysis.

Development percentage for each sampling point was calculated (Table 1). This was done by dividing the amount of time passed from egg laying to collection by the time from egg to adult for each cage. Development points of “Early”, “Mid”, and “Late” were determined for each species

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miRNA extractions*:* RNA extractions of whole pupae were completed using Qiagen miRNeasy kits according to manufacturer protocols with the additional Qiagen DNase I. Samples were treated with Superase In to inhibit RNase that may be present in the sample. Sample extraction quantity and quality were measured with a Nanophotometer. The only deviation from this protocol was for sex identification of *L. cuprina* and *B. plinthopyga*. In these species, approximately 10% of the pupae homogenate was removed before addition of Qiazol for sex identification through genome size estimation. Sex was identified in *C. macellaria* and *C. rufifacies* through a PCR sex identification assay optimized in the Tarone Laboratory throughout this grant. This PCR was performed using a small aliquot of the Qiagen RNA extract which was subsequently converted to cDNA for analysis.

miRNA sequencing*:* Only samples identified as females were submitted for sequencing, unless otherwise noted. In an effort to account for natural variation in expression levels between individuals, each library preparation was a pool of three samples (2 samples for *B. plinthopyga*). Each time point (Early, Mid, Late) of interest was replicated 4 times (Cage A, B, C, D). For each sample, approximately 2 ug of RNA suspended in RNAse free water was submitted to Texas A&M Agrilife Sequencing (*C. macellaria*) or Scripps Institute (*C. rufifacies, L. cuprina, B. plinthopyga*). Samples were prepared with a standard 2S rRNA depletion for Diptera before being sequenced. 50bp read NextSeq (Illumina) sequencing was completed for a maximum of 5 million reads per pool of samples.

In addition to sequencing wild-type pupae (reared at 25ºC, in 14:10 LD), pupae reared in different environmental conditions (light:dark cycles, temperature) and pupae with different genotypic backgrounds (developmental rate selection, sexes) were collected for sequencing. *L. cuprina* raised at 12:12 LD and 16:8 LD cycles for the early puparial stage, *C. macellaria* selected to be fast and slow developers at the early post-feeding and "mid pupae 2" stages were also collected for sequencing. Males and females for each developmental stage of *B. plinthopyga* have been submitted for RNAseq, while *C. macellaria* reared at 20ºC, 25ºC, and 30ºC are being collected for sequencing.

Sequence Analysis: Raw sequence files were uploaded to the Brazos Cluster Supercomputer for processing (brazos.tamu.edu). Adaptor sequences were trimmed from sequences before read length, read counts for each length, and number of unique sequences per length distributions were plotted (Figure 1). Sequences ranging from 20-25nt were mapped to the miRbase and FlyBase miRNA databases for identification. Once sequences were identified, raw count data was further analyzed using DeSeq2 in R 3.3.0 to normalize expression values and identify significantly differentially expressed miRNA. This expression information was then mapped on heat maps for visualization. Normalized expression data was

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used in Principal Component Analysis (PCA) in order to identify to what extent miRNA expression in each species could separate samples by their development stage. In addition to these methods, normalized expression data was analyzed using Random Forest analysis (RFA) to determine which miRNAs contribute most to identification of development stage. miRNAs identified to be significantly differentially expressed and best fit to predict development stages were used for qPCR validation studies.

qPCR*:* Primers were designed based on these miRNA sequences of interest and ordered through Integrated DNA Technologies (IDT). Intrapuparial samples of identified age (collected as above) were extracted in the same manner as for RNA sequencing. RNA was converted to cDNA using Qiagen miScript II RT kits according to manufacturer protocols. Primer efficiency was established for best temperature protocols of each primer. qPCR of housekeeper genes and miRNA of interest was run on a Bio-Rad CFX96 RT PCR Detection System to measure Cq values.

DDCq and fold-change expression were calculated using Cq values. Fold-change values were analyzed with ANOVA and Tukey HSD to determine significant changes in expression between developmental time points. qPCR has been completed for four developmental stages of pupae for *C. macellaria* and *L. cuprina*. In addition to these species, qPCR has been performed on *B. plinthopyga* reared on different substrates (sand, carpet, and clothing).

Protein Extractions*:* Pupae were ground in a 250 µl of PBS containing a protease inhibitor cocktail (Pierce Chemical Co.) and subjected to centrifugation at 13000xg for 15 minutes. Clarified supernatant was removed and extracted with chloroform and methanol according to Wessel et. al, (1984).

DIGE: Difference gel electrophoresis (DIGE) was performed according to published procedures (GE Healthcare). Samples obtained from different age groups were randomized and differentially labeled with Cy5 or Cy3 dye, while a pooled sample was labeled with Cy2 as a normalization standard. (GE Healthcare). Two different samples and a portion of the pooled standard were mixed together and focused before being subjected to vertical gel electrophoresis. The gels were scanned using a Typhoon 3-Laser scanner (GE Healthcare) to create multiplex images of the three dye-labeled proteomes. The images were analyzed using DeCyder software (v. 6.5; GE Healthcare) to determine spots that changed intensity. Filters were set to create lists of Proteins of Interest (POI)

Acrylamide plugs that contained POI were robotically excised from the 2D gels using an Ettan Spot Picker and robotically digested with Trypsin. Peptides were extracted from the digested gel pieces and analyzed using nano-LCMSMS.

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Results from the mass spectrometry were used to search the SwissProt database for protein ID using MASCOT and !Xtandem. The search results were compiled and validated using Scaffold.

***Western Blot**:* Protein extracts for samples equally loaded into each well of a 10% SDS Agarose gel. After electrophoresis, proteins were transferred to a PVDF membrane. Membranes were blocked overnight with a PBS Nonfat Dry Milk (NFDM) mixture. After blocking, primary antibodies were added to the PBS + NFDM in order to mark POI. After at least 4 hours with primary antibodies, the membranes were washed at least 3 times with NFDM. After washes, a mixture of secondary antibodies with PBS+NFDM were introduced to the membrane for at least 2 hours. The membrane was then subsequently washed three time for 20 minutes with NFDM. After washes, the membrane was exposed to PICO SuperSignal for at least 20 minutes before chemiluminescence imaging.

Detection of miRNA when Traditionally Preserved: Intrapuparial samples of known age were collected with traditional collection methods (parboiling water and stored in 70% ethanol), labelled, and stored at room temperature for approximately one year. After storage, RNA was isolated from samples for further validation of miRNA. Results are pending.

Data Analysis:

***RNAseq**:* RNAseq has resulted in the identification of 255 miRNAs in three of the four species of interest (152 in *C. macellaria*, 179 in *C. rufifacies*, and 231 in *L. cuprina*). 144 of these miRNA have been identified in all three species; whereas 58 are shared between *L. cuprina* and *C. rufifacies*, and 16 between *L. cuprina* and *C. macellaria*, and 4 between *C. macellaria* and *C. rufifacies* (Figure 2). Of these identified miRNA, 11 have been identified to be significantly differentially expressed in intrapuparial stages of wild type *C. macellaria* (25ºC, 14:10 LD) using traditional DeSeq analyses (Figure 3). 23 were identified to be significantly differentially expressed in *C. rufifacies* (Figure 4), and 5 were found to be significantly differentially expressed in *L. cuprina* (Figure 5). Three of these significantly differentially expressed miRNAs were shared between *C. macellaria* and *C. rufifacies*, two were shared between *C. rufifacies* and *L. cuprina*, and three were shared between *C. macellaria* and *L. cuprina* (Figure 6).

PCA found that stages of intrapuparial development could be differentiated through normalized expression of miRNA. 36.6% of variation in *C. macellaria* could be explained by two components, while 57.5% could be explained by two components in *C. rufifacies*, and 41.9% in *L. cuprina* (Figure 7). These results support the conclusion that expression of miRNA can be used to differentiate stages of intrapuparial development in these flies of forensic interest.

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RFA of RNAseq data from *C. macellaria* intrapuparial miRNA could correctly identify the stage of intrapuparial development with an out of box error rate (OOB) of 16.67% (1 error in 12 samples, Mid 1 mistakenly identified as Mid 2). This same analysis in *C. rufifacies* found and OOB of 8.33% (1 error of 12, late pupae called as mid). RFA of *L. cuprina* RNAseq data found an OOB of 33.33% (4 errors in 12, where 3 early pupae were identified as mid; 1 mid was identified as early). When comparing the top 25 ranked miRNA for each species analyzed, 3 miRNA were identified in all three species (Figure 8). Six were shared between *C. rufifacies* and *C. macellaria*, six between *C. macellaria* and *L. cuprina*, and two between *L. cuprina* and *C. rufifacies* (Figure 8).

In addition to intrapuparial samples, three larval stages (feeding 3rd instar, early post-feeding 3rd instar, and late post-feeding 3rd instar) were sampled and sequenced for *C. macellaria.* PCA was able to explain 31.8 % of the variation of these seven stages in two components, suggesting miRNA expression can differentiate larval and intrapuparial stages (Figure 9). Differential expression analyses found 47 significantly differentially expressed miRNA (Figure 10). *C. macellaria* from different developmental selection regimes were found to have 22 in early post feeding third instar larvae (Figure 11) and 36 significantly differentially expressed miRNA in the mid-late intrapuparial stage (Figure 12). Seven of these miRNA were found to be significantly differentially expressed in wild-type (25ºC, 14:10 LD) and selected (slow, fast, and control development) *C. macellaria*, 17 were found to be shared between wild-type and selected mid-late intrapuparial stages, and 6 were found to be shared between wild-type and selected early post-feeding *C. macellaria* (Figure 13). These results suggest that 17 miRNA are not sensitive to selection of developmental genotypes.

When investigating differences in miRNA expression of early intrapuparial samples in *L. cuprina* samples reared at different light cycles (14:10 LD, 12:12 LD, 16:8LD), no miRNA were found to be significantly differentially expressed between 12:12 LD and 16:8 LD; yet 74 miRNA found to be differentially expressed between 12:12/16:8 and the 14:10 LD cycle samples (Figure 14). Since there is higher error in differentiating early and mid intrapuparial stages of *L. cuprina*, early and mid stages were combined for DeSeq comparison of early and late intrapuparial stages (Figure 15) 19 of these miRNA were found to be shared with the 29 significantly differentially expressed miRNA between early and late intrapuparial stages of *L. cuprina* at 14:10 LD (Figure 16), indicating that 10 of these miRNA are not sensitive to light cycle differences.

Male and female *B. plinthopyga* samples for all developmental time points (early, mid, and late intrapuparial stages) were submitted for RNA sequencing in January 2019. Early samples for 20ºC, 25ºC, and 30ºC in *C. macellaria* are currently being collected for extraction and submission for RNA sequencing.

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qPCR analyses: Sixteen miRNA of interest were identified from RFA and DeSeq analyses of *C. macellaria* results for qPCR validation. While there are potentially biologically relevant patterns in miRNA expression levels in *C. macellaria*, only two miRNA were found to have significantly differences in development validated with qPCR (Figure 17). Eight of these miRNA were also used for validation of miRNA expression in *L. cuprina*. Significant differences were found in four miRNA (Figure 18).

Four of these miRNA were investigated with qPCR in *B. plinthopyga* development on different substrates. Significant expression patterns were found to be exhibited in 3 miRNA for development from early larvae through late pupae on carpet (Figure 19). When investigating early vs late larvae reared on carpet or clothing, no significant differences were found in expression levels. Two miRNA were found to exhibit significant differences in expression patterns in puparial development of *B. plinthopyga*, with significant differences between rearing substrates (Figure 20).

DIGE: DIGE analysis identified 227 potential protein isolates in *B. plinthopyga*, 38 potential protein isolates in *C. rufifacies*, and 26 potential protein isolates in *L. sericata*. When uncharacterized, hypothetical proteins, and different units of the same proteins were removed, there were 109 putative proteins identified in *B. plinthopyga*, 24 in *C. rufifacies*, and 16 in *L. sericata*. One of these proteins was identified in all three species, three were shared between *B. plinthopyga* and *C. rufifacies*, 1 between *C. rufifacies* and *L. sericata* and three between *B. plinthopyga* and *L. sericata* (Figure 21). *C. macellaria* samples have been submitted to the Proteomics Core Facility and await analyses.

Western Blot analysis: Preliminary Western Blot analyses were completed on *B. plinthopyga* and *L. sericata* early, mid, and late pupae samples. Five different primary antibodies have been tested on these species (Ecdysone receptors A & B (EcR-A, EcR-B), Enolase, Broad, and Ecdysoneless (ECD)). Antibodies for EcR-B and Broad were able to identify proteins in Western blot analyses, and preliminary data suggests that there are consistent expression patterns which differentiate early and late pupae samples in both species (Figure 22). Western blot analysis validation of proteins is continuing in the Protein Chemistry Lab at Texas A&M University.

Casework validation: miRNA extractions of traditionally collected samples were found to yield quantifiable and acceptable quality samples for further validation with miRNA qPCR.

Findings:

Throughout this grant we have successfully identified over 200 miRNAs present in the development of three different species of flies of forensic relevance, with flesh fly data forthcoming. We have found that expression of these miRNAs is

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differential through intrapuparial development and that statistical analysis (PCA, RFA, DeSeq) of these expression patterns can be used to differentiate the amount of time that has passed since pupariation has begun. Many of the miRNAs that are weighted (based on RFA importance scores) more heavily in these predictions are conserved between species within the same family. In addition to wild-type development (25ºC, 14:10 LD), we have been able to identify miRNAs which may be differentially expressed between samples reared in different environmental and genotypic conditions. This information on variation in environmental and genotypic conditions is important to determine which of the identified miRNAs are most reliable and stable in their ability to identify the amount of puparial development which as occurred.

Beyond traditional studies of development in sand, we have also completed developmental profiles of *B. plinthopyga*, a species commonly found in indoor death cases in Houston, reared on different substrates (clothing, carpet, and sand). We have been able to identify differences in developmental between puparial samples reared on difference substrates and have been able to identify differences in miRNA expression between developmental stages and developmental substrates. Conduct of these grant activities also required some studies on the basic biology of the fly (oviposition rates, plasticity, and general developmental patterns), which are being developed into manuscripts.

Of the70 miRNA that are identified to be significantly differentially expressed or in the top 25 weighted from RFA, 21 are identified to be involved in development and metamorphosis in Drosophila and mosquitoes. In addition to these developmental pathways, 7 are identified to be involved in metabolic pathways, 16 in nervous system development and maintenance, 4 in muscle development, and 4 involved in “clock” pathways and developmental timing. All of these processes are known to be highly important in the development and metamorphosis of a dipteran pupae to its mature, adult stage. These results are promising in regards to finding a mostly universal marker for development in these species, due to the conservation of developmental pathways across the order.

Proteomic analyses have been able to identify proteins which have developmental patterns of change and expression and are conserved between the species of interest tested here. At least four of these proteins are involved in muscle development, at least six are involved in metabolism and energy processing, and many involved in cell function and protein maintenance. This information will be further validated with the goal of achieving an efficient, cost-effective method for quickly identifying the developmental stage of pupae in a forensic laboratory setting – such as an ELISA assay for fly developmental progress.

Implications for Criminal Justice Policy and Practice:

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The results we find here suggest there are stable and conserved molecular markers for determining age of pupae and differentiating feeding and post-feeding third instar larvae. In addition to identifying potential markers of aging pupae, we have been able to optimize a sex identification assay for three species of carrion flies. Since there is known to be differences in development of immature males and females, the optimization of this assay will prove to be beneficial in increasing the precision and accuracy of these age estimates. Confirmation analyses that were blind to sex (as would be currently implemented) may also explain why some qPCR results were not as informative as sex-specific analyses. We have also been able to find significant differences in development of *B. plinthopyga*, a fly found in many indoor cases in Houston, TX, on different substrates, which opens the door for increased precision for estimates with that species in the future.

Scholarly Products:

Theses:

1. Thesis for Honors Undergraduate Research. Texas A&M University. Michelle Jonika. "Genes as Markers of Sex for Forensic Entomology".—Currently Pursuing PhD in Genetics at Texas A&M University.
2. Thesis (MSc) in Forensic Science. Strathclyde University, Glasgow, Scotland. Amanda Purcell (U.S. Citizen) "Analysis of miRNA expression during pupal development of *Lucilia sericata* and *Cochliomyia macellaria* (Diptera: Calliphoridae) for use in criminal investigations".—currently pursuing a career in Forensic Pathology.

Visiting Researchers:

1. Ahmed El Hefnawy. PhD research on ISSR identification of Sarchophagidae Species/Development Studies of *B. plinthopyga*. Benha University, Egypt—Currently finishing PhD
2. Anika Sharma. PhD research. Fulbright Scholar. Punjabi University, India—Currently Finishing PhD

Book Chapters:

1. M.R. Sanford and A.M. Tarone. Is PMI the hypothesis or the null hypothesis? Final draft submitted to editors Jason Byrd and Jeffery Tomberlin for publication in third edition of Forensic Entomology: The utility of arthropods in legal investigations (CRC Press). This chapter focuses heavily on assumptions in casework and this grant identifies some potential markers of environmental / genetic conditions that would could confirm assumptions.
2. A.M. Tarone and J.B. Benoit. Insect development as it relates to forensic entomology. Final draft submitted to editors Jason Byrd and Jeffery Tomberlin for publication in third edition of Forensic Entomology: The utility of arthropods in legal investigations (CRC Press). This chapter summarizes various aspects of developmental biology of forensically important insects.

Presentations:

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1. Identification of small RNA markers of fly age in *Cochliomyia macellaria*. Yuan Y, Parrot JJ, Srivasta S, Pimsler ML, Sze S-H, Tarone AM. North American Forensic Entomology Association Annual Meeting, Davis, CA 2017 (oral)
2. Let's Talk About Sex: Identifying Female and Male Markers in Blow Flies. Jonika MM, Faris AM, Hjelmen CE, Tarone AM. Department of Entomology Mentorship Symposium, Texas A&M University 2018 (oral)
3. Let's Talk About Sex: Identifying Female and Male Markers in Blow Flies. Jonika MM, Faris AM, Hjelmen CE, Tarone AM. Undergraduate Research Presentation, Student Research Week, Texas A&M University 2018 (oral)
4. Let's Talk About Sex: Identifying Female and Male Markers in Blow Flies. Jonika MM, Faris AM, Hjelmen CE, Tarone AM. Ecological Integration Symposium 2018, Texas A&M University (oral)
5. Genes as Markers of Sex for Forensic Entomology. Jonika MM, Faris AM, Hjelmen CE, Tarone AM. Undergraduate Research Scholars Symposium, Texas A&M University 2018 (oral)
6. Markers of Sex Determination in Blow Flies. Jonika MM, Faris AM, Hjelmen CE, Tarone AM LAUNCH Undergraduate Research Summer Poster Session, Texas A&M University 2018 (poster)
7. Transcript-Based Sex Determination for Forensic Entomology. Jonika MM, Faris AM, Hjelmen CE, Tarone AM. Southeast Texas Evolutionary Genetics & Genomics Symposium, Rice University 2018 (poster)
8. Transcript-Based Sex Determination for Forensic Entomology. Jonika MM, Faris AM, Hjelmen CE, Tarone AM. International Association for Identification, San Antonio, TX 2018 (poster)
9. Rates of viviparity in *Blaesoxipha plinthopyga* (Diptera: Sarcophagidae) have forensic implications. AM Tarone. North American Forensic Entomology Association Annual Meeting 2018, Orlando FL. (oral)
10. Differential expression of proteins in species of forensically relevant Diptera. Hjelmen CE, Srivastav S, Parrott JJ, Dangott LJ, Tarone AM. Annual North American Forensic Entomology Association Annual Meeting 2018, Orlando, FL (oral)
11. Developmental plasticity of *Blaesoxipha plinthopyga* (Diptera: Sarcophagidae) Flesh Fly on different substrates. Ahmed El Hefnawy. North American Forensic Entomology Association Annual Meeting 2018, Orlando FL. (poster)
12. Aaron M. Tarone, Christine J. Picard, Sing-Hoi Sze. Genomic tools to reduce error in PMI estimates derived from entomological evidence 2012-DN-BXK024, Proteomic and microRNA markers of fly development: Stable correlates of insect age to improve forensic entomology casework 2015-DN-BX-K020. Invited webinar. Forensic Technology Center of Excellence. October 2018.
13. Differential expression of proteins in species of forensically relevant Diptera. Hjelmen CE, Srivastav S, Parrott JJ, Dangott LJ, Tarone AM. Entomological Society of America Annual Meeting 2018, Vancouver, Canada (oral)
14. Transcript-based sex determination for forensic entomology. Jonika MM, Faris AM, Hjelmen CE, Tarone AM. Entomological Society of America Annual Meeting 2018, Vancouver, Canada (oral)

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Appendix:

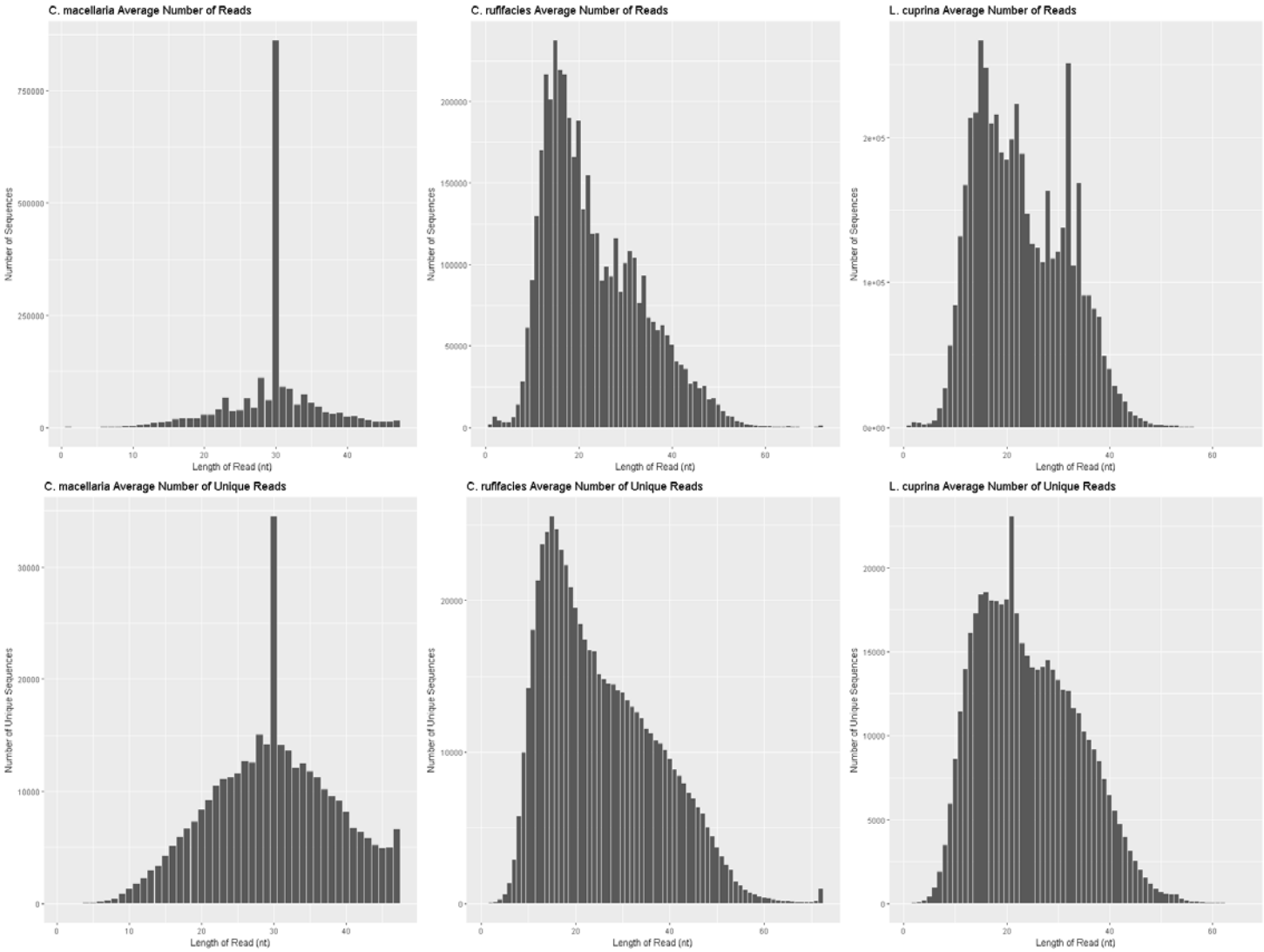

Figure 1: Distribution of average number of reads by length (top) and unique reads by length (bottom) from RNAseq of *C. macellaria* (left), *C. rufifacies* (middle) and *L. cuprina* (right). C macellaria samples (left) include read information for larval samples

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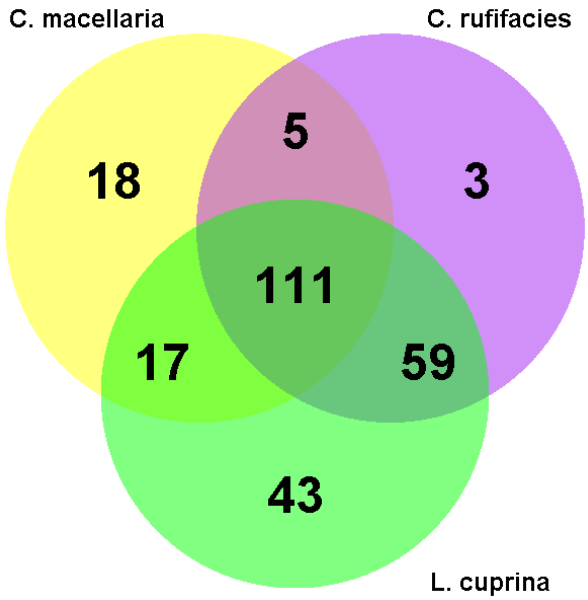

Figure 2: Number of miRNA identified in RNAseq analysis

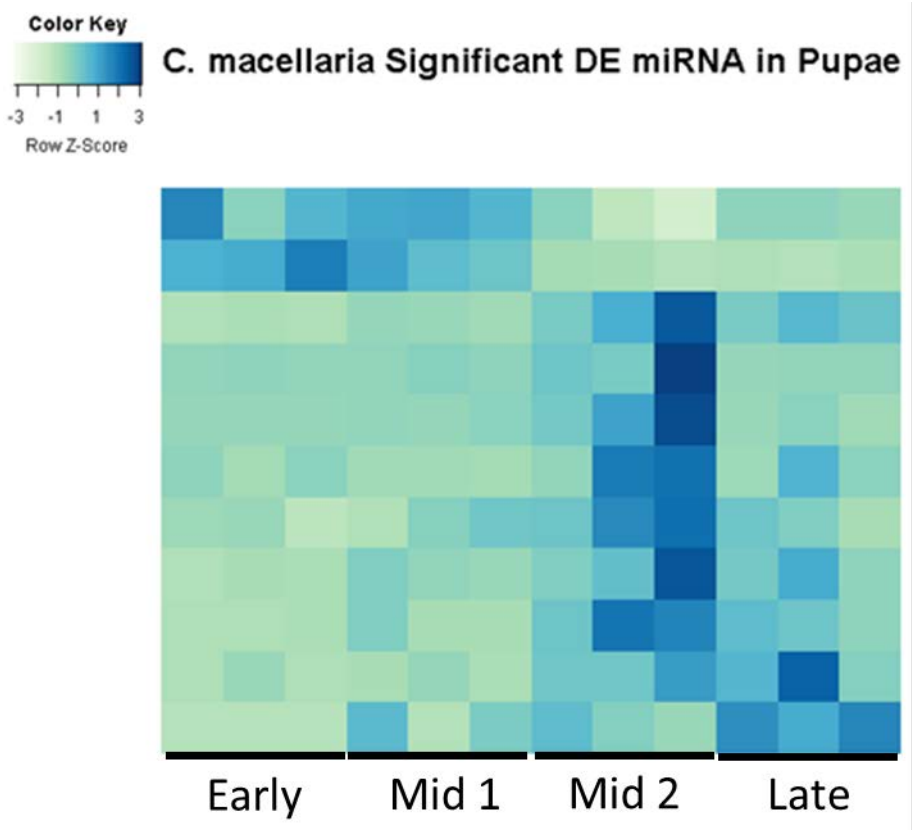

Figure 3: Heatmap of significantly differentially expressed miRNA during pupariation in *C. macellaria*

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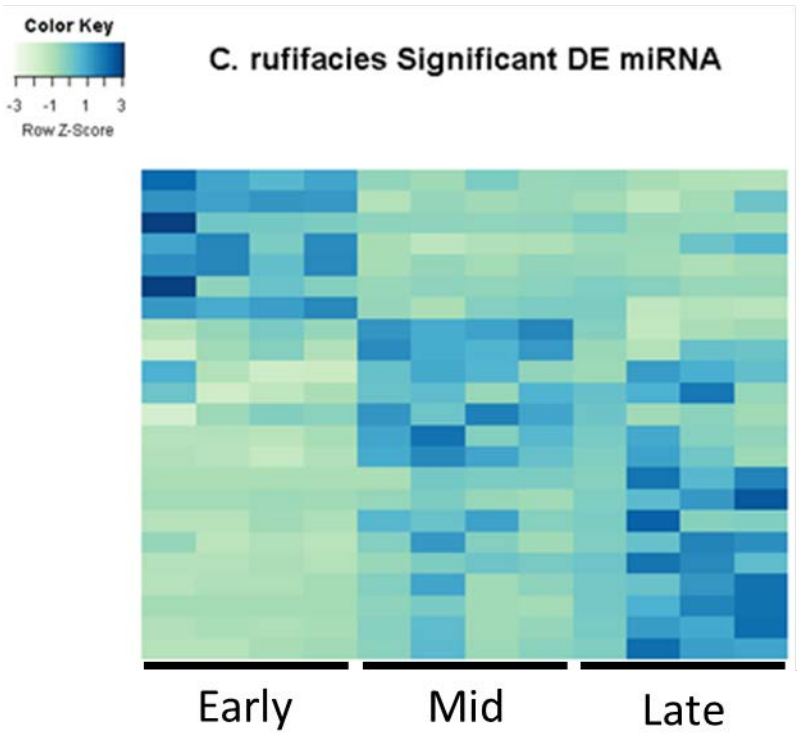

Figure 4: Heatmap of significantly differentially expressed miRNA during pupariation in *C. rufifacies*

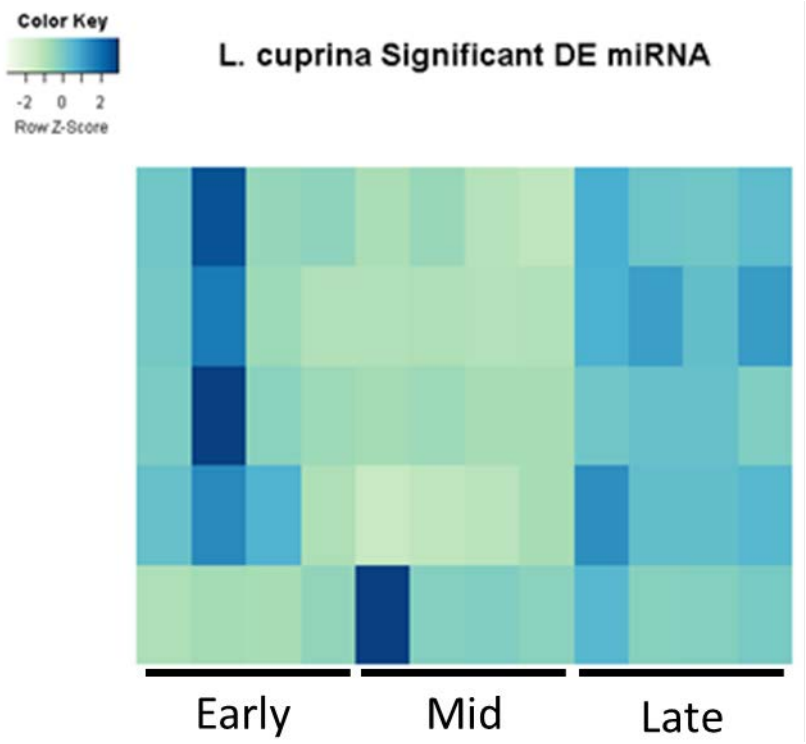

Figure 5: Significantly differentially expressed miRNA identified during pupariation by DeSeq in *L. cuprina*

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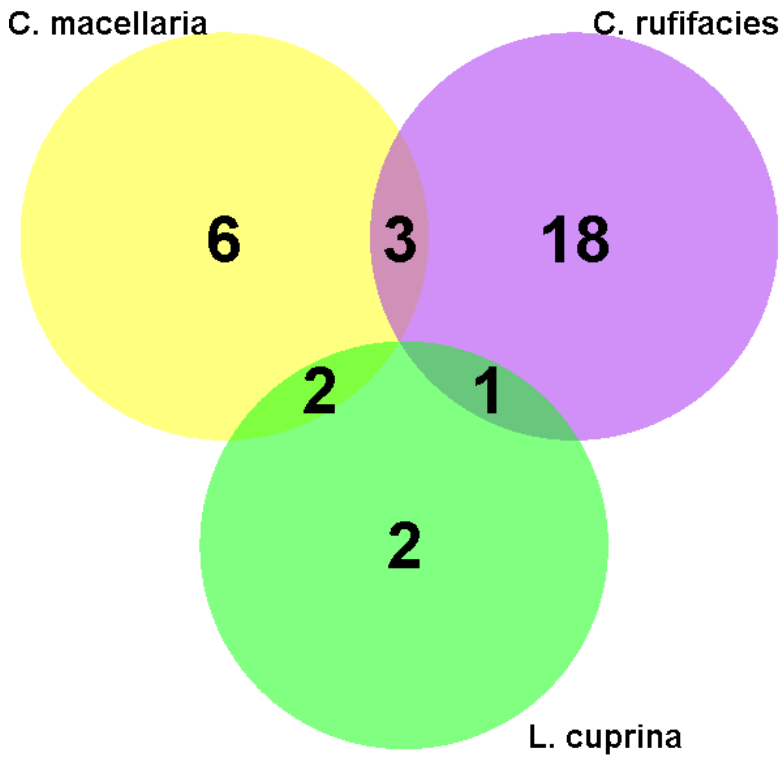

Figure 6: Number of shared significantly differentially expressed miRNA between early, mid, and late puparial stages identified by DeSeq

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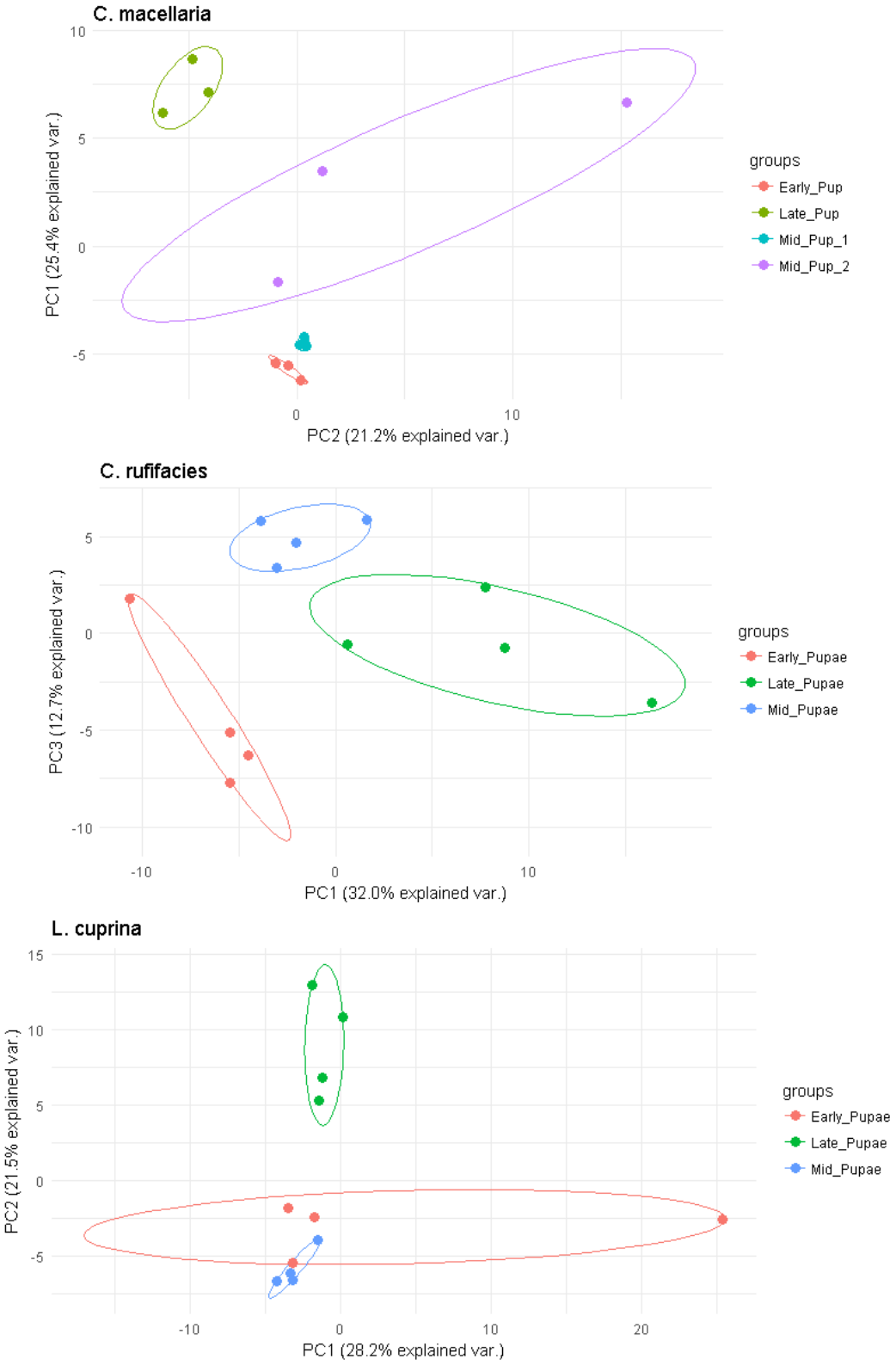

Figure 7: Principal Component Analysis of normalized RNAseq data

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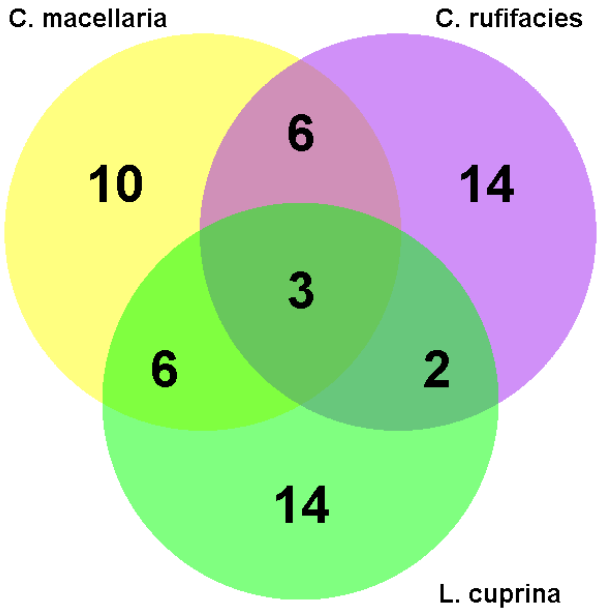

Figure 8: Shared miRNA identified by Random Forest Analysis

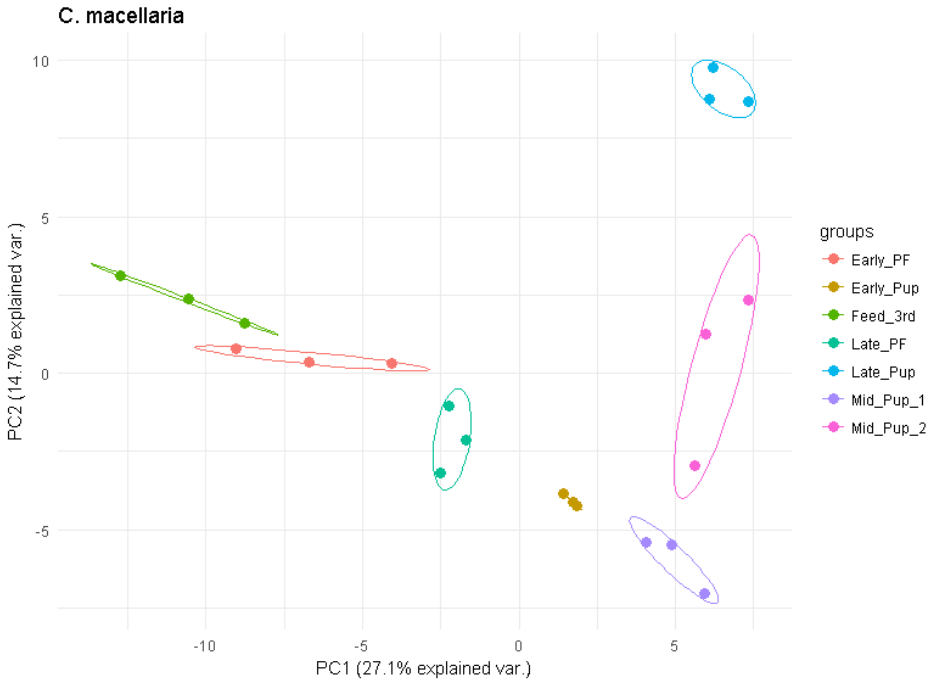

Figure 9: Principal Component Analysis of *C. macellaria* third instars and puparial stages

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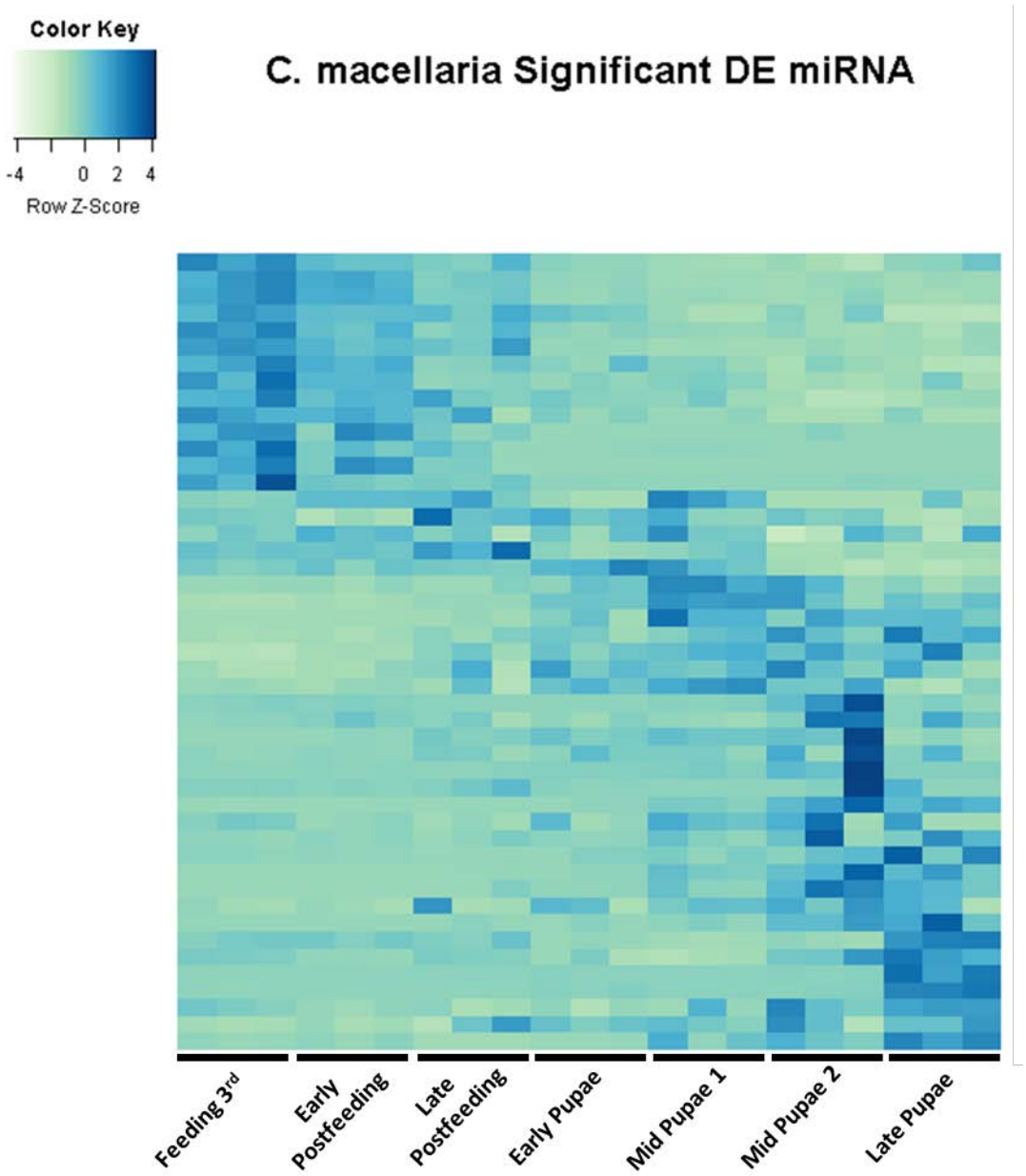

Figure 10: Heatmap of significantly differentially expressed miRNA in larval and puparial development of *C. macellaria*

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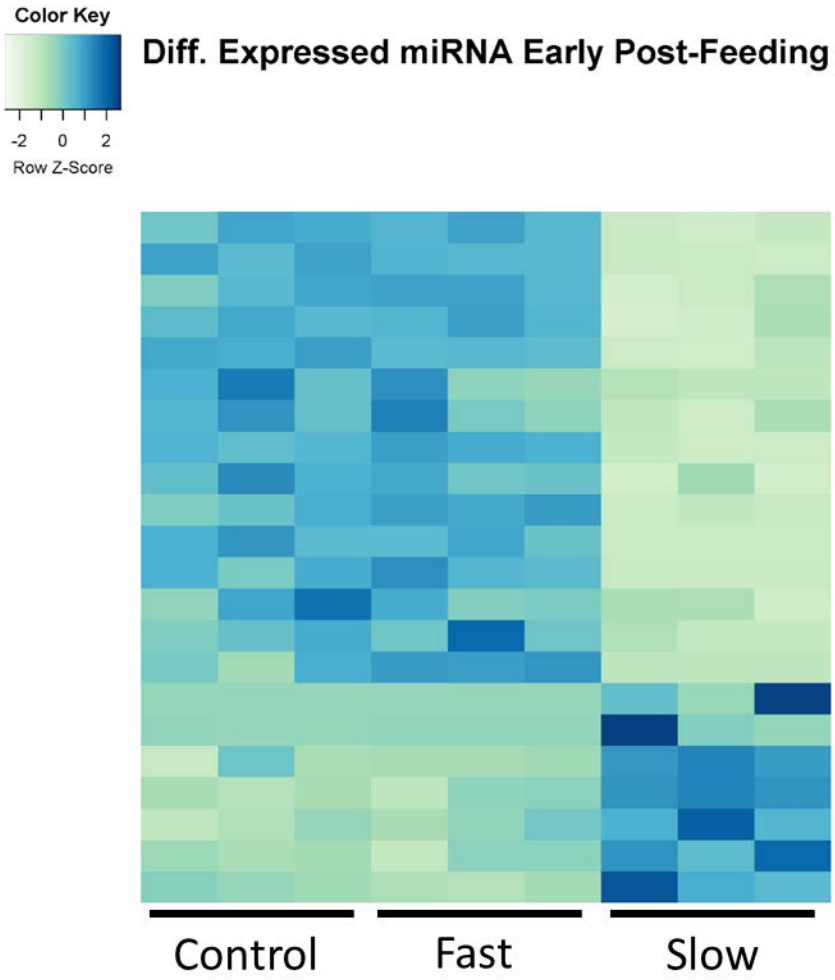

Figure 11: Heatmap of significantly differentially expressed miRNA in early post-feeding larvae in *C. macellaria* selection lines

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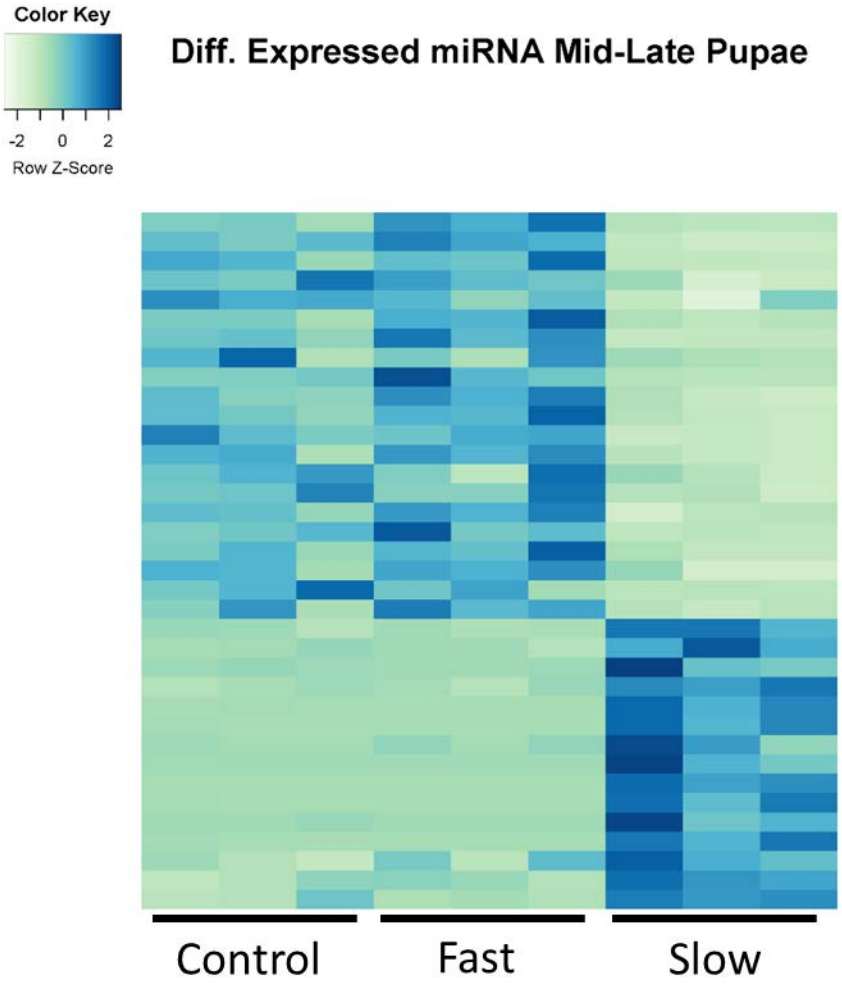

Figure 12: Heatmap of significantly differentially expressed miRNA in mid-late puparial stages in *C. macellaria* selection lines

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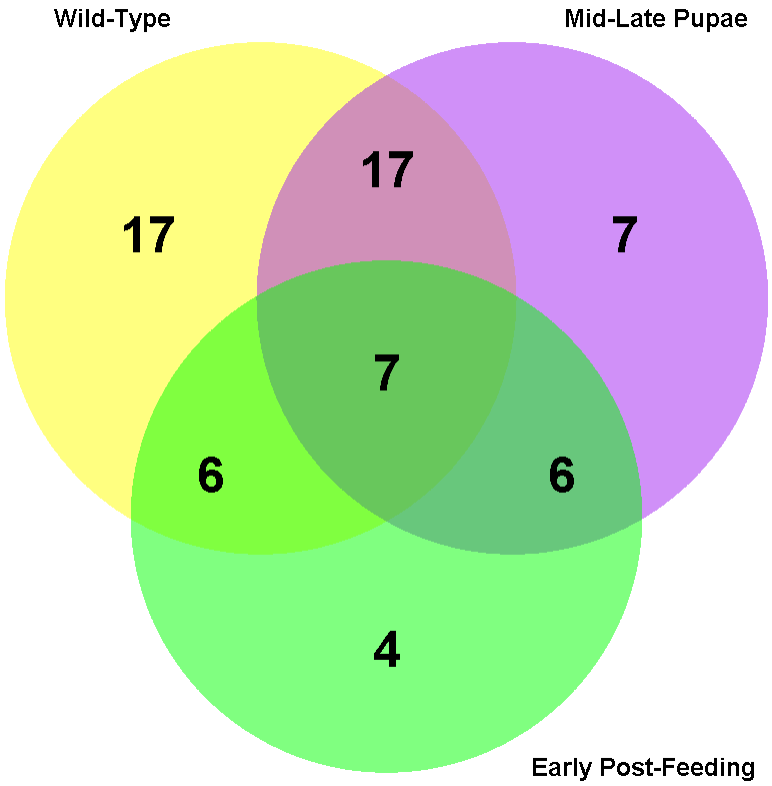

Figure 13: Significantly differentially expressed miRNA shared between wild-type development and development rate selected *C. macellaria*. 17 differentially expressed miRNA not sensitive to selection, yet 7 of these differentially expressed miRNA in puparial development are sensitive to selection for development rate.

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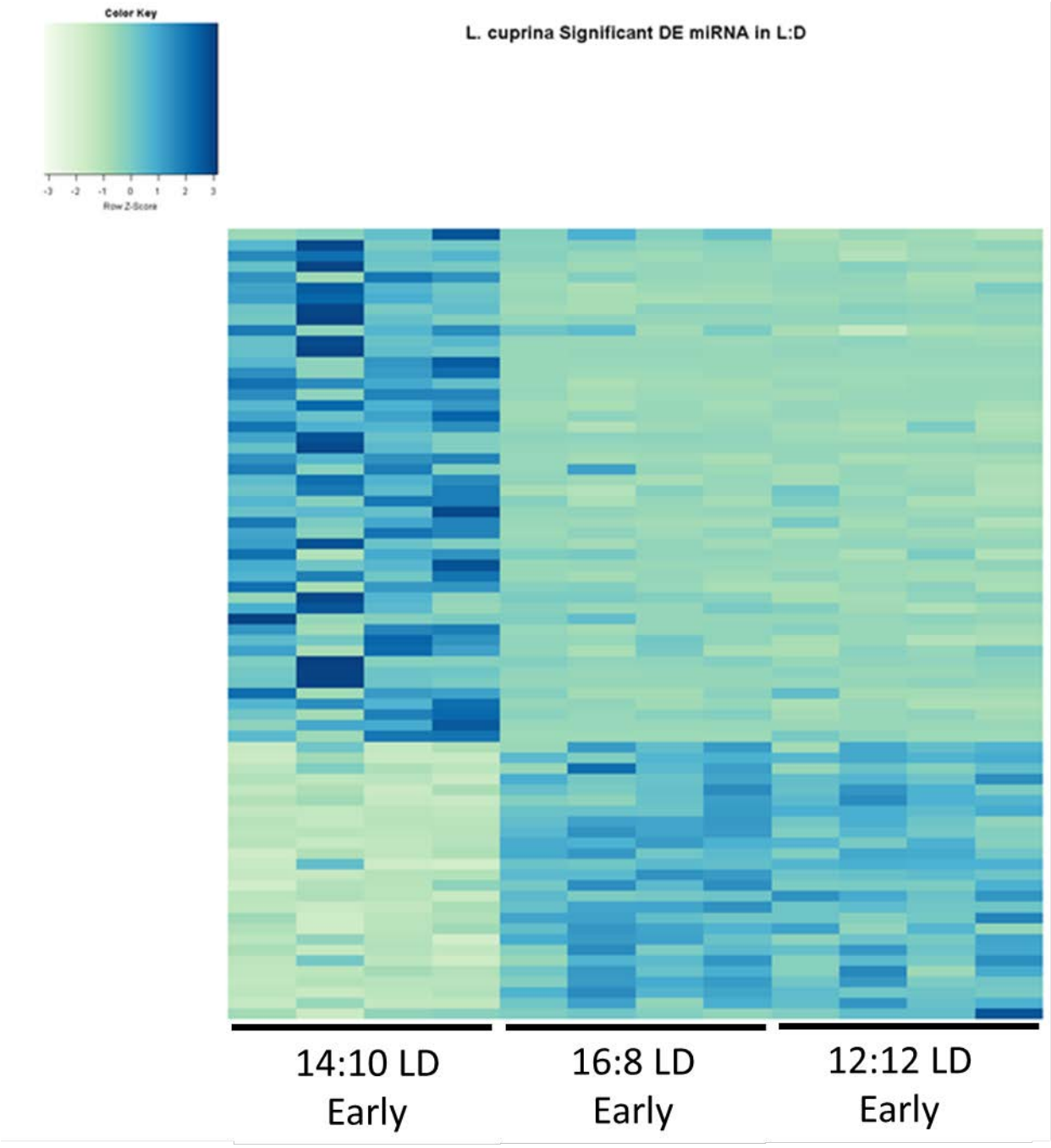

Figure 14: Heatmap of significantly differentially expressed miRNA in *L. cuprina* early puparial stage with differing light:dark treatments

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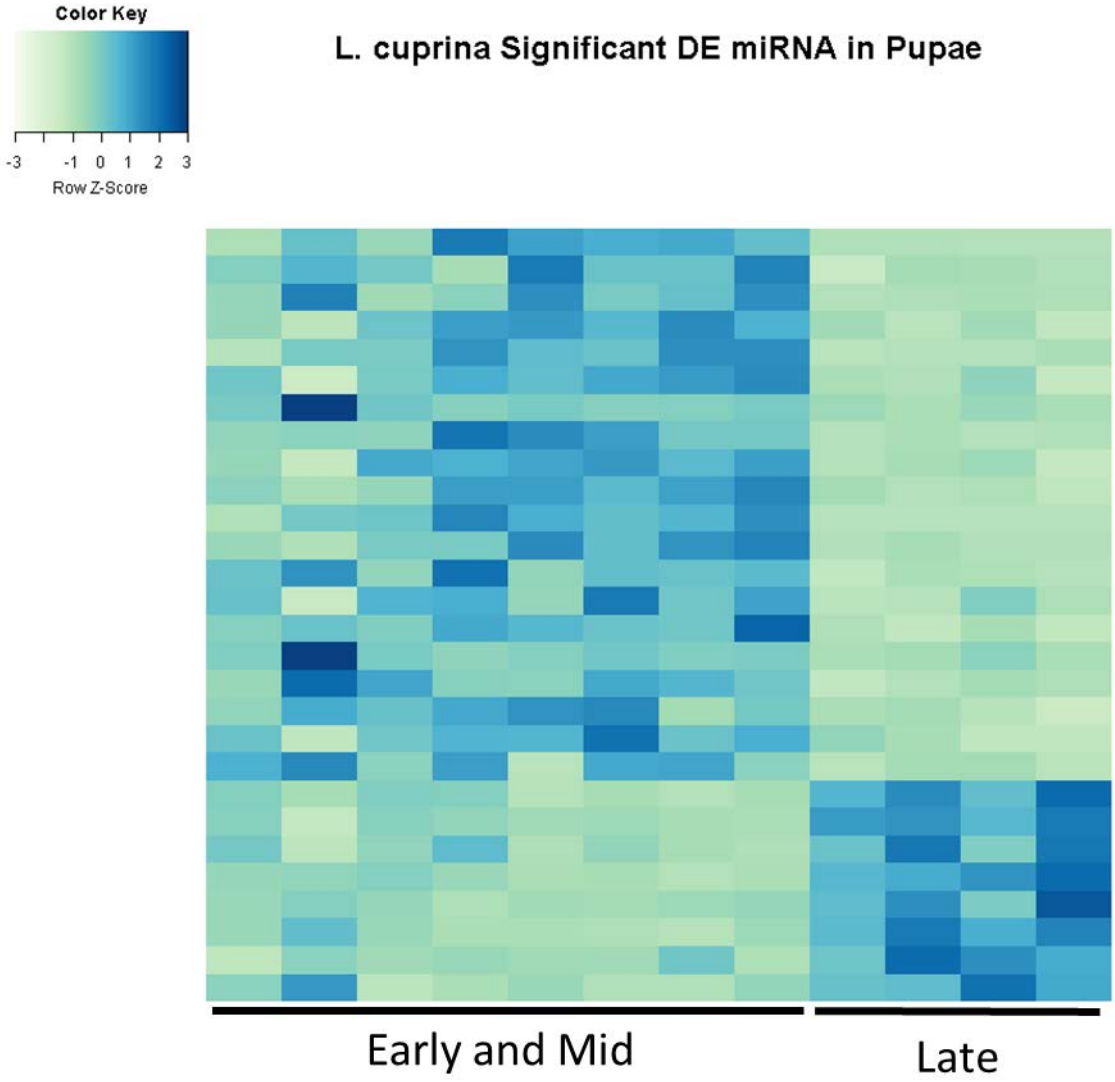

Figure 15: Heatmap of significantly differentially expressed miRNA in *L. cuprina* early vs late puparial stages with 14:10 light:dark treatment

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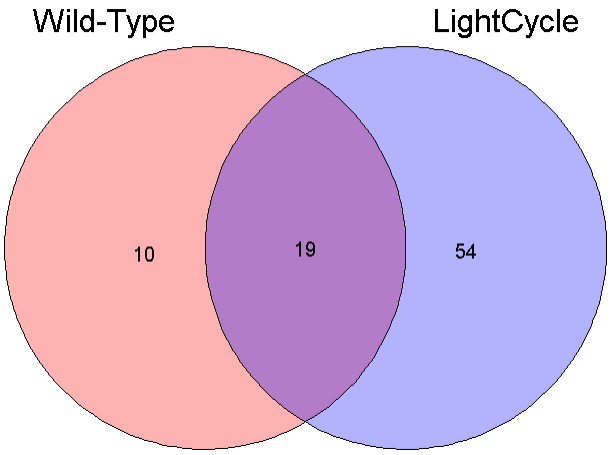

Figure 16: Number of shared differentially expressed miRNA between wild-type development significantly differentially expressed miRNA and miRNA found to be significantly differentially expressed between light cycles in early pupae. The 10 miRNA identified in "wild-type" are differentially expressed and are not sensitive to light cycle conditions. 19 of the differentially expressed miRNA in puparial development are sensitive to these light conditions.

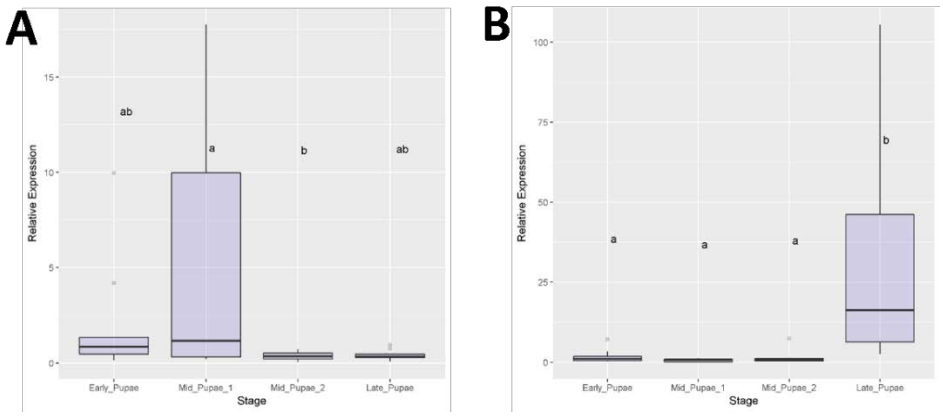

Figure 17: qPCR boxplots for significantly differentially expressed *C. macellaria* miRNA in puparial development

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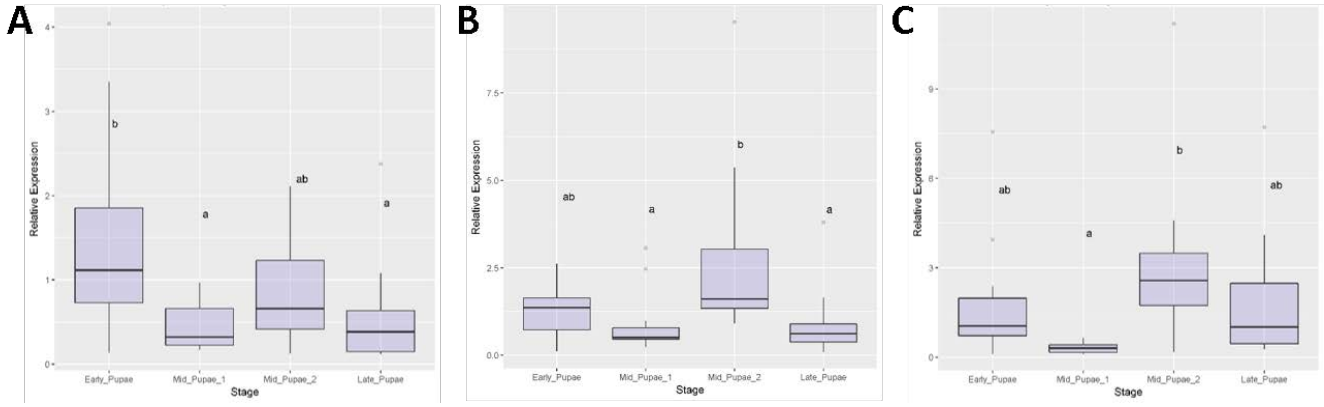

Figure 18: qPCR boxplots for significantly differentially expressed *L. cuprina* miRNA in puparial development

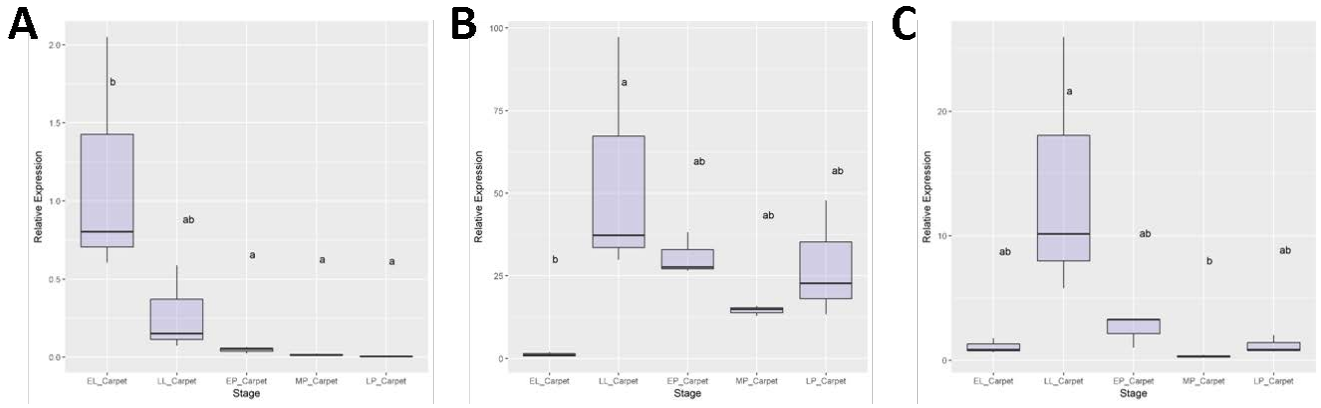

Figure 19: qPCR of miRNA in *B. plinthopyga* larval and puparial development on carpet

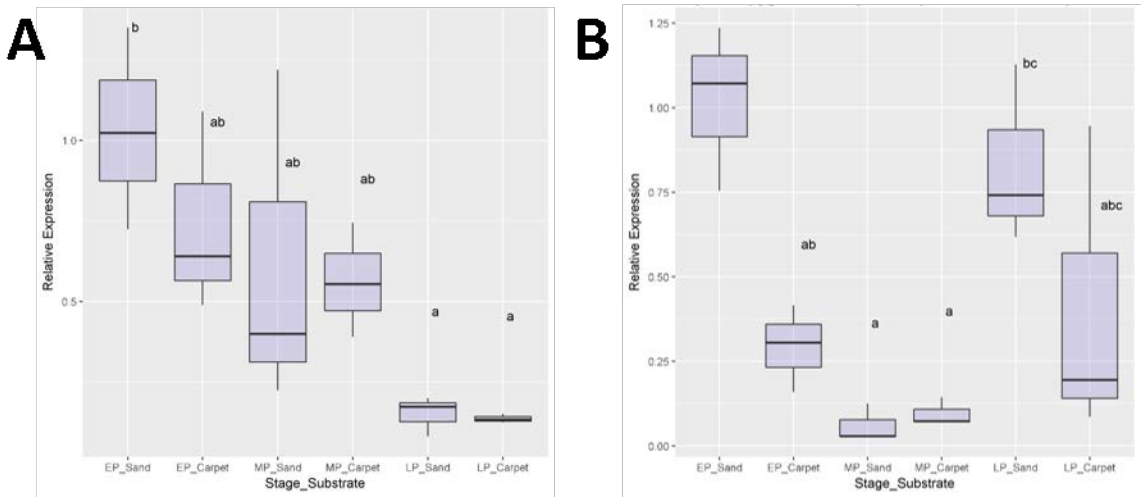

Figure 20: qPCR of miRNA in *B. plinthopyga* puparial development on sand or carpet

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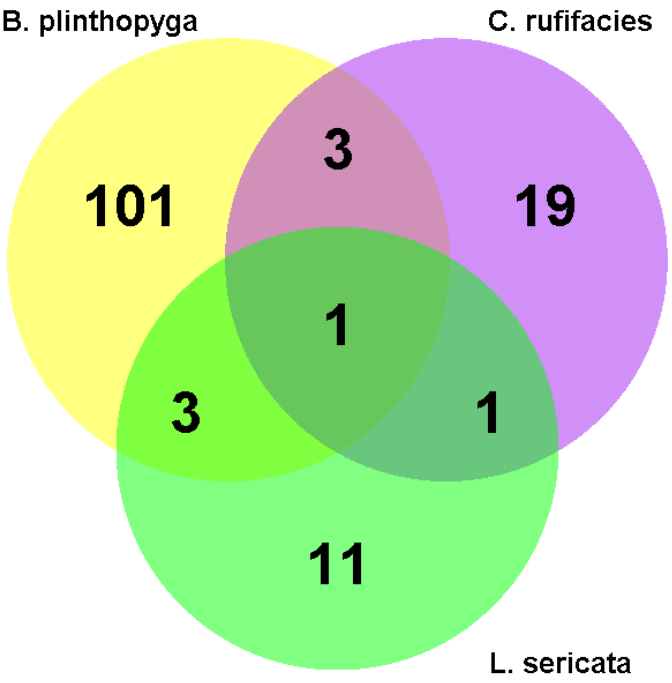

Figure 21 Number of unique putative proteins identified as differentially expressed in 2D-DIGE analysis

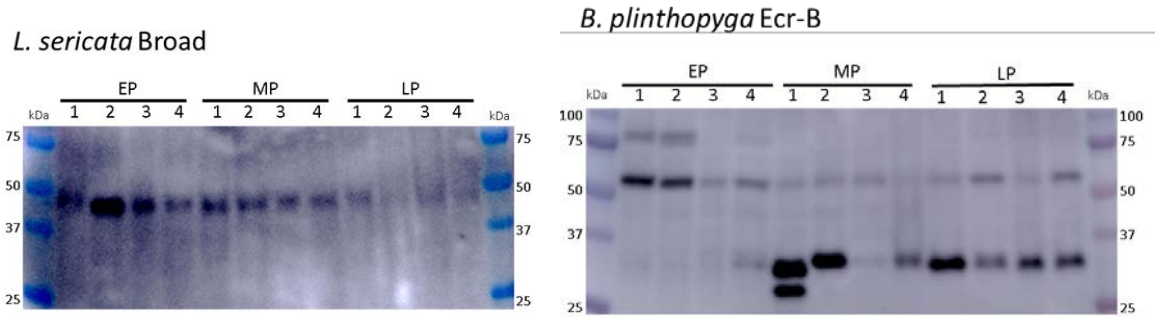

Figure 22: Western Blot example for Broad in *L. sericata* and EcR-B in *B. plinthopyga*

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Table 1: Development percentages of samples submitted for RNAseq

Stage	*C. macellaria*	*C. rufifacies*	*L. cuprina*	*B. plinthopyga*
Early Pupae-1	47.6%	68.0%	35.5%	33.4%
Early Pupae-2	47.1%	70.2%	34.6%	32.7%
Early Pupae-3	46.4%	64.5%	33.2%	31.3%
Early Pupae-4	-	62.5%	38.6%	-
Mid Pupae 1-1	63.2%	81.7%	55.4%	61.9%
Mid Pupae 1-2	63.4%	84.4%	54.1%	61.9%
Mid Pupae 1-3	64.4%	86.1%	51.9%	57.2%
Mid Pupae 1-4	-	85.4%	56.9%	-
Mid Pupae 2-1	82.4%	-	-	-
Mid Pupae 2-2	80.2%	-	-	-
Mid Pupae 2-3	84.1%	-	-	-
Mid Pupae 2-4	-	-	-	-
Late Pupae-1	93.0%	95.3%	93.4%	83.9%
Late Pupae-2	90.6%	91.5%	91.1%	91.1%
Late Pupae-3	91.2%	93.4%	91.1%	92.8%
Late Pupae-4	-	92.5%	97.7%	-

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