The importance of discs large homolog 5 and microRNA-34 expression to the development of the mosquito *Aedes aegypti*

By

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A thesis submitted to the Department of Biological Sciences, University of Manitoba, in partial fulfillment of the requirements for the course

BIOL 4100 (Honours Thesis)

for the degree of

Bachelor of Science (Honours)

Abstract

Understanding how the expression of particular genes and regulation of genes affects development in different organisms is key to advancing our knowledge in developmental biology. The epithelial-mesenchymal transition (EMT) process is commonly observed during the growth and development of various animal tissues. One gene involved in EMT regulation and directly related to development in humans is *discs large* homolog 5 (*dlg*5). If the role of this gene is conserved across species, it opens many medical application possibilities to help treat a multitude of medical issues, such as cancer. microRNAs are short, single-stranded non-coding RNAs that regulate gene expression by binding to the 3'UTR of complementary target mRNAs. microRNA-34 (miR-34) is a microRNA of interest with relation to *dlg5* as it is predicted to bind with *dlg* transcripts. miR-34 has also shown clinical potential in the past with regulating cell proliferation. This study looks at the *dlg*5 gene and miR-34 in the mosquito *Aedes aegypti* to see if the expression of this gene and microRNA is evolutionarily conserved to that of *Drosophila melanogaster*. A developmental expression qRT-PCR analysis, analyzing the relative *dlg* and miR-34 transcript levels in different temporal stages of *A. aegypti* development, was conducted to compare the relative transcript levels of *A. aegypti* throughout development. Then, a *dlg*-dsRNA RNAi-mediated knockdown by bacterial feeding was performed to see if knocking down *dlg* has an effect on development. The results of the qRT-PCR analysis and RNAi experiment were not significant enough to come to a justified conclusion. Therefore, further testing is required to produce meaningful conclusions regarding the developmental expression of *dlg* and miR-34.

Acknowledgements

I would like to acknowledge and thank my supervisor, Dr. Steve Whyard, for his guidance, advice, understanding and support throughout the course of my project. I would also like to give special acknowledgement and thanks to Aditi Singh for the enormous amount of help, knowledge and support she has provided me in the lab. An additional thank you to Alison Taylor and Nazanin Amanat for their technical assistance when I needed it. Finally, I would like to thank my thesis committee, Dr. Steve Whyard and Dr. Olivia Wilkins for their direction and support.

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Introduction

MicroRNAs

MicroRNAs (miRNAs/miRs) are single-stranded, short (~22 nucleotides long), non-coding RNAs that control gene expression by hybridizing to complementary target mRNAs. The hybridization can result in mRNA degradation or direct inhibition of translation (Zhang et al. 2015). miRNAs are also able to activate gene expression by interacting with complementary regions of the promoter and coding region, and the 3'UTR of mRNA targets (Zhang et al. 2015). miRNAs can regulate many genes, including genes associated with cell proliferation, and hence, abnormal expression of miRNAs can alter gene expression post-transcriptionally, leading to uncontrolled cell proliferation. Understanding miRNA function and knowing gene targets associated with particular miRNAs is useful to understanding how and when genes are expressed. Additionally understanding their function and how to control them is particularly useful and relevant to current cancer research as miRNAs can be classified as tumour suppressors or oncogenes due to the inhibition or enhancement of cell proliferation and differentiation of cancerous cells (Gajek et al. 2021).

Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is the process of epithelial cells becoming mesenchymal cells, most commonly observed during growth and development of various animal tissues. During the EMT process, epithelial cells lose their apical-basal polarity, cell-cell junctions, and epithelial markers. Thus, the epithelial cells take on characters of mesenchymal cells which have cell motility, a spindle-cell shape, and other mesenchymal markers (Lai et al. 2020).

discs large

One gene that is involved in EMT regulation and directly related to development in humans and other organisms is the *discs large* (*dlg*) homolog 5 gene. Some functions of *dlg* include inhibiting proliferation and epithelial invasion, and maintaining epithelial cell polarity (Zhao et al. 2008; Liu et al. 2017). In humans, *dlg* acts as a Hippo signaling pathway regulator, where in its absence, aberrant cell proliferation is possible by inhibiting the Hippo signaling pathway (Kwan et al. 2016; Liu et al. 2017). In *Drosophila*, the Hippo signaling pathway is involved in controlling cell proliferation, regulating tissue and organ growth, apoptosis, and cell fate (Rosales-Nieves and González-Reyes 2014; Kwan et al. 2016). When *dlg* is mutated and completely lost, epithelial cell invasion and premature EMT occurs (Zhao et al. 2008; Gandille et al. 2010). This can lead to morphological and phenotypic changes in the affected tissues.

miR-34 in EMT

Since microRNAs regulate gene expression, they may be capable of controlling genes involved in epithelial-mesenchymal transition and be able to alter cell proliferation when perturbed. If possible, microRNA research can be tested further and ultimately lead to clinical applications like diagnostics or therapeutics.

In humans, miR-34a is a microRNA from the miR-34 family that is located on chromosome 1p36 (Welponer et al. 2020). This miRNA is noteworthy as previous studies have shown miR-34a expression had a 100% decrease in cancers with the TP53 mutation; however, research surrounding its clinical potential is limited (Zhang et al. 2015). For this reason, observing how significant miR-34 may be to normal developmental genes and

pathways is intriguing. One study noted when miR-34a was absent, uncontrolled cell proliferation occurred and when reintroducing this microRNA to the aberrant cells, by ectopic overexpression, the apoptotic pathway is capable of being reactivated (Chen and Hu 2012). Although many microRNAs have been identified in a diverse array of eukaryotes, their individual gene expression effects are not all known as many mRNA targets are still waiting to be identified. Identifying these mRNA targets can be done through computer algorithm programs, which predict possible targets based on a combination of characteristics, and by *in vivo* verification. Using various algorithms, the binding of miR-34 to *dlg* transcripts was predicted in *Drosophila melanogaster* and using cell-based assays, miR-34 was observed to bind to the 3'UTR of *dlg* transcripts in *Drosophila* cultured cells (Whyard, unpublished results). An orthologue of *dlg* was identified in the mosquito *Aedes aegypti* (Steve Whyard, pers. comm). In studies conducted in their lab, knockdown of the miR-34 resulted in reduced fertility of the female mosquitoes, but it was unclear what genes were impacted by the loss of miR-34.

This study plans to focus on the typical relative expression of *dlg* and miR-34 throughout mosquito development and whether RNAi perturbation with the *dlg* gene, results in delayed or altered development of the insect. The study of miR-34 and *dlg* should lead to a better understanding of the gene's regulation in insects and possibly help us identify potential target molecular mechanisms associated development and tumourigenesis.

RNAi

To knock down the *dlg* gene, RNA interference (RNAi) will be used. RNAi is the post-transcriptional gene silencing mechanism, inhibiting gene expression, in which small fragments of double-stranded RNA (dsRNA; i.e., siRNA or miRNA) can stop protein

translation. RNAi works by guiding small dsRNA molecules within protein complexes (e.g., RNA-induced silencing complex (RISC)) to target mRNAs. Proteins within RISC can then cut the targeted mRNAs, resulting in gene silencing or knockdown. In research, RNAi can target specific genes of interest to be downregulated by adding complementary exogenous dsRNA. RNAi can also be used in cancer research by knocking down protein synthesis inside cancer cells.

Research Objectives/Hypotheses

The specific questions for my project are:

1. Is *dlg* expressed throughout mosquito development or only during a short time span? I predict *dlg* expression to occur throughout development. A variety of tissues undergo significant developmental changes during insect development, particularly during larval to pupal transitions, and during pupal to adult transitions, *dlg* is likely to contribute to some of these developmental processes.

2. Is miR-34 expressed throughout mosquito development and does its expression negatively correlate with *dlg* expression? I predict that miR-34 will be expressed during development, and if its role is similar to that in *Drosophila*, it may down-regulate *dlg*, in which case, I predict miR-34 levels may be negatively correlated with the transcript levels of *dlg*.

3. Does RNAi-mediated knockdown of *dlg* affect development from larvae to eclosion? I predict that RNAi knockdown will also delay pupation and affect the sex ratio of adult mosquitoes.

Materials and Methods

Insect

The mosquito, *Aedes aegypti* (Liverpool strain), was used as the model organism for the following experiments. The mosquitoes were kept at 27°C, 50% humidity in an insectary with a 12h/12h light cycle. Larvae were fed ground rabbit chow and adults are provided 10% sucrose and rat blood.

Assessing the Developmental Expression of discs large and miR-34

The RNA of larvae (1^{st}, 2^{nd}, 3^{rd}, and 4^{th} instar), pupae, two-day-old and seven-day-old adult mosquitoes (male and female processed separately) was extracted using ThermoFisher Scientific's GeneJET RNA Purification Kit, then synthesized into cDNA. The extraction followed manufacturer's instructions. The mosquito tissue samples were placed into 1.5mL Safe-Lock microcentrifuge tubes with 150µL Lysis Buffer, 3µL β-mercaptoethanol, and a mix of RNase-free stainless-steel beads (0.2mm and 0.5mm beads). The tissues were disrupted by the Bullet Blender® Homogenizer at ~90% power for 5 to 7 minutes to homogenize the tissues. The tubes were then centrifuged to separate the supernatant and pellet. The supernatant was transferred to a new tube with 225µL EtOH and processed further by following the GeneJET kit protocol. The purified RNA was then eluted into 75-100µL (75µL for 1^{st} to 4^{th} instar and 100µL for pupae to 7-day-old adult mosquitoes) RNase-free water. About 2µL of each RNA sample was then placed on a BioTek Take3 Microvolume Plate and inserted into a BioTek Synergy H1 Plate Reader to determine concentration and purity of the RNA sample. TURBO DNase (Ambion) was used for DNase treatment of the RNA to remove any DNA contamination. Then, the pure RNA was reverse transcribed by adding qScript cDNA supermix (Quanta BioScience) to

synthesize cDNA. The conditions of the reverse transcription were 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. No-RT (reverse transcription) control RNA samples, which have no qScript cDNA supermix, were set aside to determine if there was any DNA contamination during the following PCR reactions. The PCR reactions were performed with new cDNA (and no RT-controls), EconoTaq PLUS GREEN PCR 2X Master Mix (Lucigen), and 10μM forward and reverse primers for the *S7* gene (used as an internal reference) to test quality of samples. The PCR reactions cycled under the following conditions: 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and then culminate at 72°C for 5 minutes. The products of the PCR reactions were then resolved on 1.5% agarose gel and imaged with EtBr under UV light using a Bio-Rad ChemiDoc Imaging System.

Next, using a Bio-Rad CFX Connect Real-Time PCR detection system, the synthesized cDNA was used for quantitative reverse transcriptase PCR (qRT-PCR) analysis to observe the relative expressions of *dlg* and miR-34 in different developmental stages. The qRT-PCR reactions contained the cDNA of interest, SsoFast™ EvaGreen® Supermix (Bio-Rad), and 10μM forward and reverse primers for *dlg*, miR-34 or *S7*. Primers were designed using Primer3 and Integrated DNA Technologies' (IDT) PCR design software. The cycling conditions used were 95°C for 30 seconds, 45 cycles of 95°C for 5 seconds and 57°C for 5 seconds. The qRT-PCR analyses had six replicates for each developmental stage, with 15 larvae per replicate, 10 pupae, and 5 adults.

dlg RNAi Experiment

Mosquito eggs were hatched and transferred to petri plates to observe the developmental effects of feeding mosquito larvae bacteria that express dsRNA targeting the *dlg* gene. To

prepare the dsRNA-expressing bacteria, two sets of primers with BglII and KpnI linkers were designed to amplify a 255bp dsRNA *dlg* target sequence (dlg 1) and a 348bp dsRNA *dlg* target sequence (dlg 2). The PCR product was resolved on an agarose gel and the *dlg* sequence band was excised and purified using the E.Z.N.A.® Gel Extraction Kit. The purified PCR product and a mini-prepped empty vector pL4440 plasmid were digested with 1.5µL 10x FastDigest buffer and 1µL FastDigest BglII and 1µL FastDigest KpnI restriction enzymes. Samples were incubated for 30 minutes at 37°C, resolved on a gel, the bands cut out, and purified using the same kit. Next, the *dlg* samples were ligated into the digested pL4440 using T4 DNA Ligase. The samples were then transformed into *E. coli* by adding 4µL of litigation mixture into 50µL of DH5α cells and leaving it on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42°C in a still water bath. The cells were then put back on ice for 2 minutes, after which 950µL of SOC broth was added to each tube. The tubes were then spun/shook at 37°C for a minimum of 30 minutes and then centrifuged at 5000 RPM for 3 minutes. 800µL of the supernatant was then discarded and the remaining supernatant was used to resuspend the pellet of cells. The remaining 200µL was then plated on an LB + amp plate, using the spread plate method, and left to incubate overnight at 37°C. The next day, five isolated colonies from each plate were picked off then used for colony screen PCR and streaked on a LB + amp grid plate. The colony screen used bacteria (tapped into the PCR tube), 5µL GoTaq, 0.4µL pL4440 forward primer, 0.4µL pL4440 reverse primer, and 4.2µL of nuclease-free water. The PCR cycling conditions were: one cycle of 95°C for 10 minutes, 38 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, then one cycle of 72°C for 5 minutes and 22°C for 3 minutes. The PCR was then run on a gel to verify the presence of the plasmid with the insert and then three colonies from each *dlg* sequence insert were

chosen for to be transformed into HT115 cells. The chosen colonies were mixed in a culture tube with 4mL LB broth and 4µL of 50mg/mL ampicillin. Culture tubes were placed spun/shook in a 37°C incubator overnight then miniprepped using the E.Z.N.A.® Plasmid DNA Mini Kit. The liquid in the culture tubes was spun down in a centrifuge to collect the DH5α cell pellet and discard the supernatant. Then the miniprep followed the E.Z.N.A.® kit protocol. A final volume of 55µL was eluted and transformed into HT115 cells similarly to the previous transformation and plated on an LB + amp + tet plate then incubated overnight at 37°C. The next day another colony screen PCR and streak grid plate was done. The transformants were then grown in an overnight culture. Four tubes of 6mL of LB broth, 6µL 50mg/mL ampicillin, and 6µL 12.5mg/mL tetracycline were prepared then two of them were inoculated with one of the colonies from the first *dlg* sequence (dlg 1) grid plate. The other two tubes were inoculated with one colony from the second *dlg* sequence (dlg 2) grid plate. Then an additional two tubes were prepared with 6mL LB broth and 6µL 12.5mg/mL tetracycline and a scrape from a chemically competent HT115 aliquot. Then a final two tubes were prepared with 6mL LB broth and 6µL 50mg/mL ampicillin and inoculated with one of the colonies from the DH5α dlg 1 plate. The chemically competent HT115 aliquot culture serves as a negative control with no plasmid insert and the DH5α dlg 1 culture serves as an additional negative control. The next day four baffled flasks were filled with 150mL of LB broth, 1µL to 1mL of the ampicillin and tetracycline, and the two overnight culture tubes for each treatment. Flasks were covered with tin foil and shook at 225 RPM at 37°C. Once an OD600 reading of 0.6-0.8 is achieved, 650µL 100mM IPTG is added to each flask. Then the flasks were returned to the 37°C incubation while shaking at 225 RPM for an additional 4 hours. Afterwards, the cells were spun down in 50mL Falcon tubes at 3650xg for 5 minutes, decanting the

supernatant. This was repeated until all the media has been spun down. To the Falcon tube with the pellet, 300mg of Brewer's Yeast and 3mL of dH_2O were added and vortexed. 12mL of molten 1% agar was then added to each falcon tube and vortexed. The tubes were then placed into a 75°C - 80°C hot water bath for 10 minutes while two 10mL syringes were prepared, with ends cut off, for the agar pellets. After the 10 minutes, the tubes were cooled down slightly (to avoid Brewer's yeast settling at the bottom after being poured) while vortexing regularly. Then the agar pellet mixture was poured into the syringes and allowed to solidify. The syringes were kept at 4°C unless needed to cut 0.5mL pellet disks. Three replicates of each treatment were set up with larvae being fed the 0.5mL pellet disks continuously until pupation. Larvae in petri dishes were left on the lab bench at room temperature, then after pupation were collected in vials and placed in the incubator at 28°C to speed up eclosion time. After eclosed, the sex for each eclosed mosquito was noted (the sex of the mosquitoes is not visibly apparently until they are adults). Each replicate contained 20 newly hatched larvae.

Data Presentation and Processing

All the gene expression data is presented as bar graphs, with standard errors, to visualize the levels of transcript abundance in different treatments. Using the $2^{-\Delta\Delta CT}$ method, the relative expression of different genes in the cDNA was analyzed. The internal control, *S7* gene, was used to compare the level of expression of each transcript using the equation: $\Delta CT = (\Delta CT_{(dlg \text{ or miR-34})} - \Delta CT_{S7})$. Then to check the relative mRNA transcript expression levels, the $2^{-\Delta\Delta CT}$ values were calculated. Pupation timing differences noted in the RNAi experiment are illustrated as bar graphs, to visualize proportion of males/females reaching pupation on a specific day.

Statistic Analysis

ANOVA tests were conducted to analyze the statistical significance and statistical difference of the gene expression and RNAi data.

Results

Developmental Expression of discs large and miR-34

The relative expression of *discs large* (with S7 as the reference gene) in *Aedes aegypti* was measured at different temporal stages using qRT-PCR analysis (Figure 1). The highest expression of *dlg* was found in 2-day-old adult male mosquitoes. The lowest expression of *dlg* was found in the 1st instar and 4th instar larval stages.

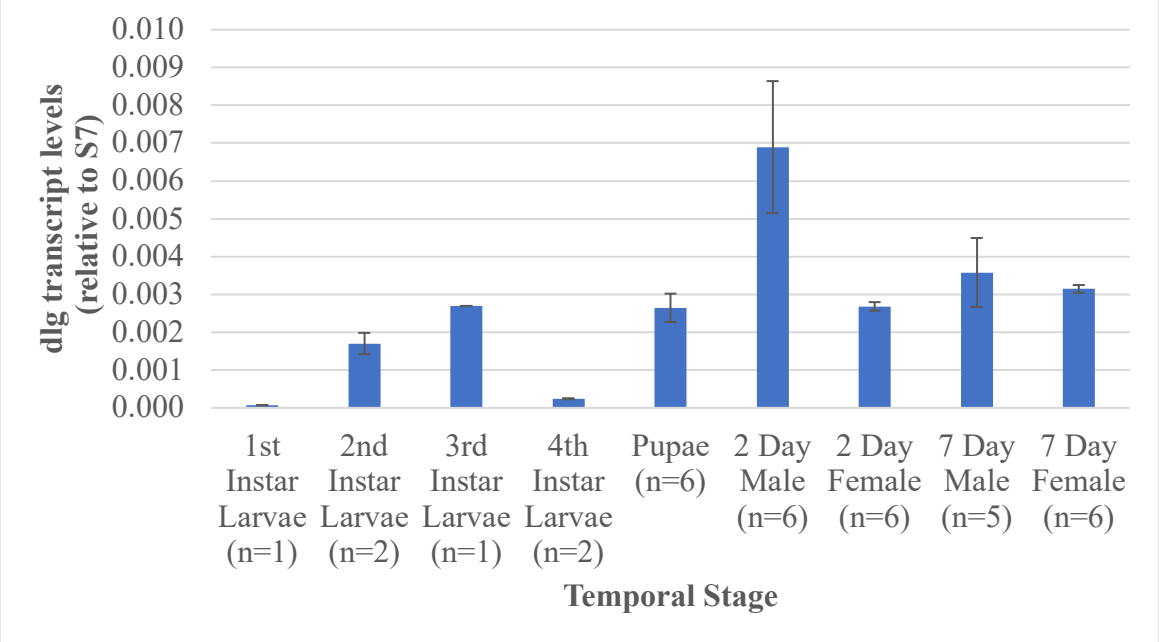

Figure 1. The relative expression of the dlg transcript in different A. aegypti temporal stages, measured via qRT-PCR analysis. Six biological replicates for each temporal stage were analyzed in technical duplicates, measuring the dlg and S7 cycle threshold values for each cDNA sample. Sample size (n) is provided for each temporal stage as some biological replicates could not detect the dlg or S7 transcripts, and were

therefore not included in the analyses. Error bars show the standard error for each temporal stage.

An ANOVA test was run, at a 95% confidence interval (p=0.05), against the results of the qRT-PCR analyses and found insufficient evidence of a statistical difference in *dlg* transcript levels across the larval to adult mosquito temporal stages.

The relative expression of miR-34 (with S7 as the reference gene) in *Aedes aegypti* was measured at different temporal stages using qRT-PCR analysis (Figure 2). No results are presented for the instar larval stages as no usable data was obtained. The highest expression of miR-34 was found in 2-day-old and 7-day-old adult male mosquitoes. The lowest expression of mir-34 was found in 2-day-old and 7-day-old adult female mosquitoes.

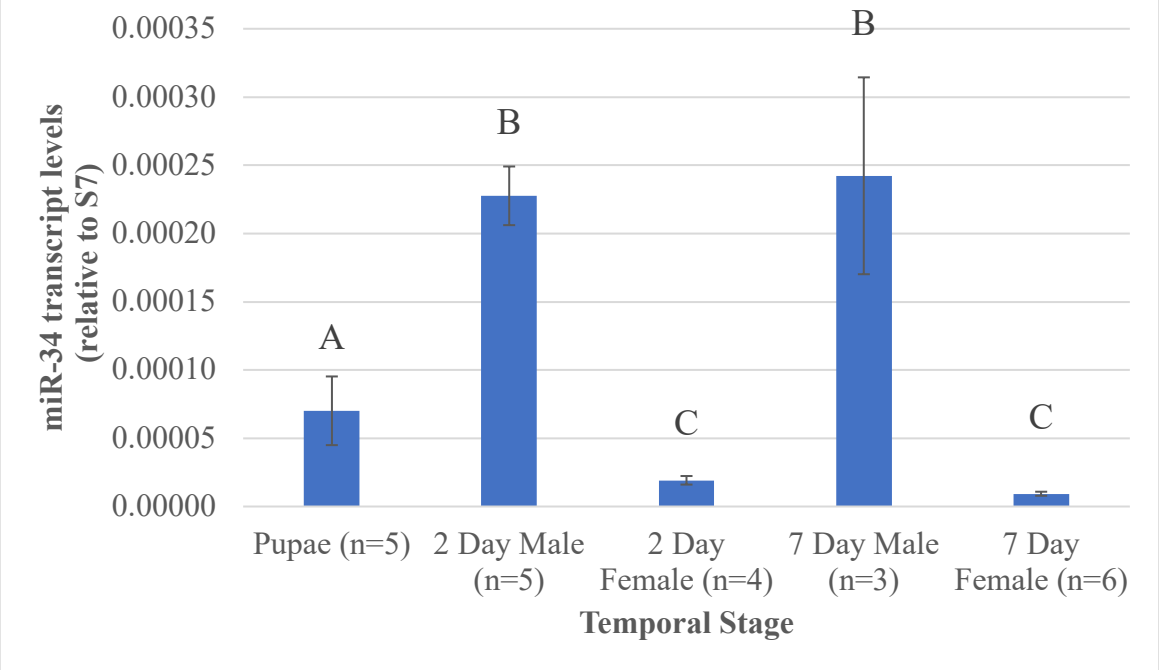

Figure 2. The relative expression of the miR-34 transcript in different A. aegypti temporal stages, measured via qRT-PCR analysis. Six biological replicates for each temporal stage were analyzed in technical duplicates, measuring the miR-34 and S7 cycle threshold values for each cDNA sample. Sample size (n) is provided for each temporal

stage as some biological replicates could not detect the miR-34 transcripts. Error bars show the standard error for each temporal stage. Samples that are statistically different from one another are labelled with different letters above each bar (ANOVA, $P<0.05$).

Statistical analysis of the results found sufficient evidence of a statistical difference in miR-34 transcript levels. The males showed significantly higher miR-34 transcript levels than that found in females. No difference was observed between 2-day-old and 7-day-old adults in either sex.

Discs large RNAi Experiment

RNA interference, RNAi, was used to observe the developmental effects of knocking down the expression of *dlg* in *A. aegypti* by feeding larvae on bacteria expressing *dlg* dsRNA, embedded in agar pellets. Data for the day of pupation, where day 0 = day of birth, and sex at eclosion were collected (Figures 3-5). The results of the *dlg*-dsRNA treatments, in comparison to the negative controls, do not show any significant difference in pupation timing, sex ratio, or survivorship.

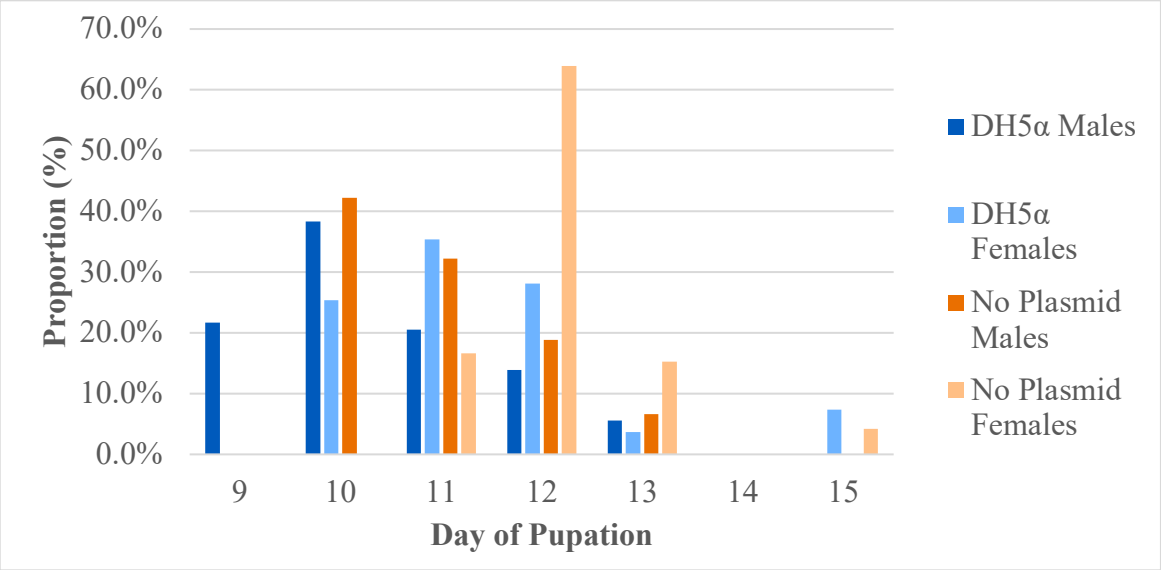

Figure 3. The proportion (%) of males/females pupated each day for negative control treatments. Three replicates of 20 larvae for each control treatment were observed and data was compiled to determine the total percentage of males/females pupated on each day since birth. The mean date of pupation was 10.45 days for DH5α males, 11.40 days for DH5α females, 10.90 days for no plasmid males, and 12.12 days for no plasmid females.

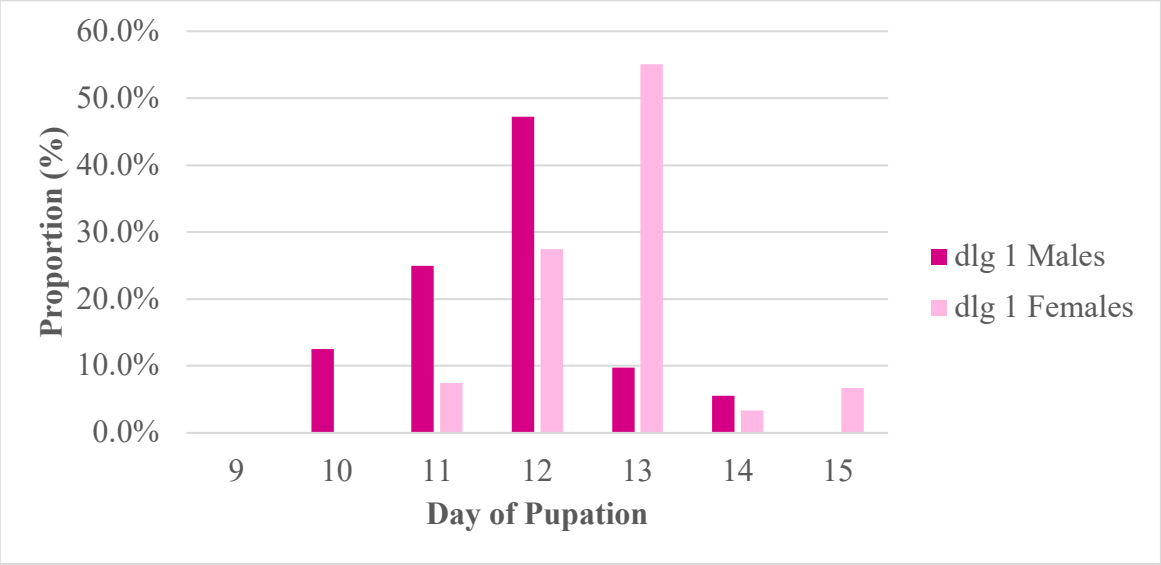

Figure 4. The proportion (%) of males/females pupated each day for dsRNA dlg 1 treatment. Three replicates of 20 larvae for the dsRNA dlg 1 treatment were observed and data was compiled to determine the total percentage of males/females pupated on each day since birth. The mean date of pupation was 11.71 days for dlg 1 males and 12.74 days for dlg 1 females.

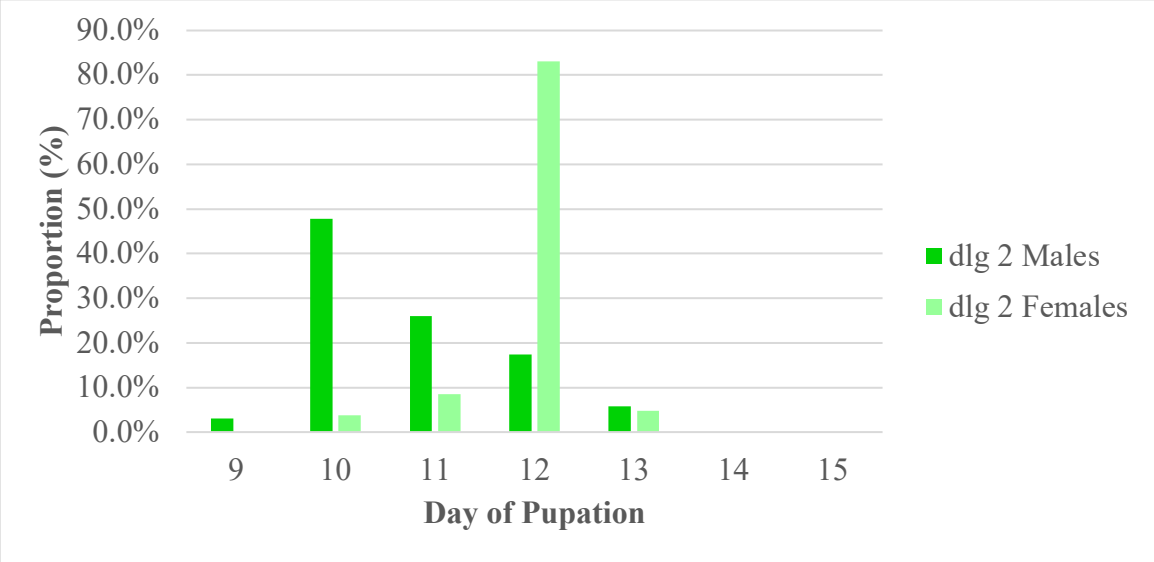

Figure 5. The proportion (%) of males/females pupated each day for dsRNA dlg 2 treatment. Three replicates of 20 larvae for the dsRNA dlg 2 treatment were observed and data was compiled to determine the total percentage of males/females pupated on each day since birth. The mean date of pupation was 10.74 days for dlg 2 males and 11.90 days for dlg 2 females.

Mortality rates for each treatment replicate is displayed in Table 1. The average rate of mortality in the DH5α negative control and HT115 no plasmid negative control was 11.7% before eclosion. The HT115 dlg 1-dsRNA treatment had an average of 5.0% deceased and the HT115 dlg 2-dsRNA treatment had an average of 16.7% deceased.

Table 1. Mortality observations for each treatment replicate.

Treatment	Replicate	Number of deceased larvae	Number of deceased pupae	% deceased
DH5α (negative control)	1	2	0	10%
	2	3	1	20%
	3	1	0	5%
HT115 no plasmid (negative control)	1	5	0	25%
	2	1	0	5%
	3	0	1	5%
HT115 dlg 1	1	2	0	10%
	2	1	0	5%
	3	0	0	0%
HT115 dlg 2	1	3	1	20%
	2	1	0	5%
	3	4	1	25%

Phylogenetic analysis of discs large coding sequence and protein sequence

dlg coding (Figure 6) and protein sequences (Figure 7) for closely related insects to *Aedes aegypti* were aligned using MAFFT and phylogenetic trees were constructed using MEGAX. Both phylogenetic trees confirmed that the predicted *dlg* coding sequence and the predicted Dlg protein from the mosquito *Aedes aegypti* was most similar to those of the other culicine mosquito, *Culex quinquefasciatus*, and then to the more distantly related mosquito *Anopheles gambiae*. Not unexpected, the mosquito clade of *dlg* sequences was more similar to that of the other dipteran (flies) insect, *Drosophila melanogaster*.

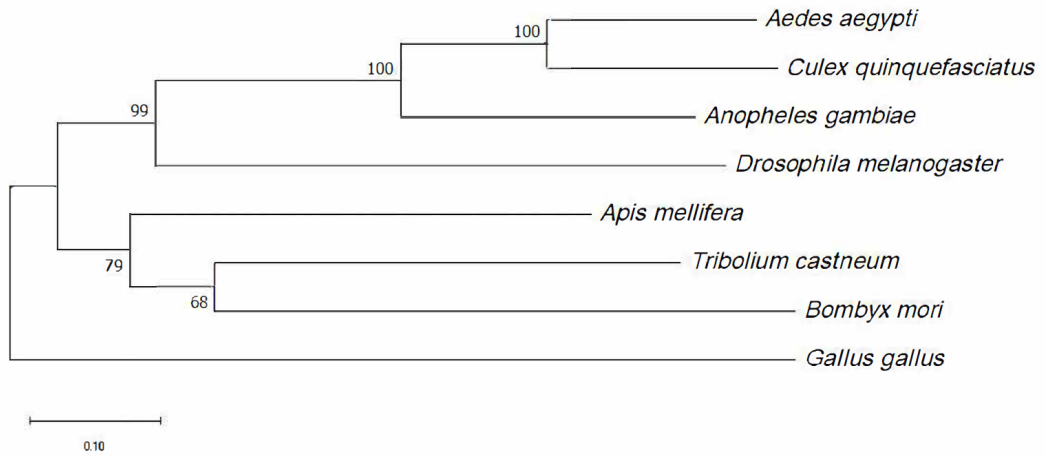

Figure 6. Maximum likelihood discs large coding sequence (CDS) phylogenetic tree. Constructed using MEGAX.

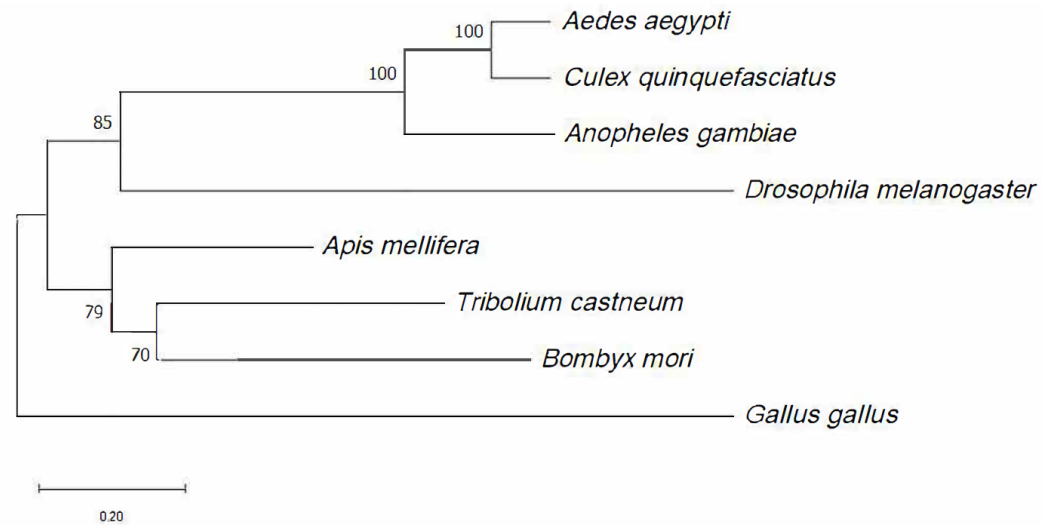

Figure 7. Maximum likelihood discs large protein sequence phylogenetic tree. Constructed using MEGAX.

Discussion

In previous research, *dlg* and miR-34 have shown their importance in development in the vinegar fly *Drosophila melanogaster* (Dugray, unpublished). This study aimed to build off those results to see if the importance of *dlg* and miR-34 throughout development is conserved amongst species by observing its expression in the mosquito *Aedes aegypti*.

Developmental Expression of discs large and miR-34

Based on the results of qRT-PCR analysis, *dlg* transcripts appear to be present in *Aedes aegypti* throughout development, although due to a small number of replicates no statistical difference in the transcript abundances could be discerned across the developmental stages of the insects. The highest transcript abundances were observed in some, but not all 2-day-old males, which may suggest that there is a narrow window of time during male maturation when higher *dlg* expression is required, although more replicates will be required to confirm this possibility.

In Dugray's study, *dlg* expression in *D. melanogaster* was found to occur throughout development, with relative expression levels ranging from approximately 0.02 – 0.07 (Dugray, unpublished). In this study, the results of the relative *dlg* expression calculations provided expression levels ranging from 0.00007 – 0.007. This overall showed the relative expression levels in *A. aegypti* could be 10-fold less than those in *D. melanogaster.* In both studies, the reference gene was the homolog of a single copy ribosomal protein (RP) gene, and if this RP gene's expression is similar in both species, this may suggest that fewer *dlg* transcripts are required in the mosquito than in the vinegar fly.

In addition, despite high total RNA concentrations for the instar larvae, qRT-PCR of the resultant cDNA failed to detect *dlg* transcripts in multiple samples, making relative expression calculations impossible. This leads to a possibly inaccurate representation of the relative expression at those stages. Although some troubleshooting was done to gain better cycle threshold values, further adjustments to the volume ratios, concentrations, and/or cycling conditions may yield better results.

In Dugray's study, the impact *dlg* may have on male development in *Drosophila* was not assessed. However, previous studies have shown that *dlg* is important to male testis development (Papagiannouli and Mechler 2010; Papagiannouli et al. 2019). Therefore, the possible higher relative expression seen in 2-day-old males, in comparison to the other temporal stages, could indicate *dlg*'s expressional importance to *A. aegypti* adult male development. As male *Aedes aegypti* require a minimum of 24 hours maturation before they are capable of mating(OECD 2018), the increase in *dlg* transcripts could reflect a period of sexual maturation, and perhaps sperm production in the males. Further studies on the tissue specific expression of adult male and female mosquitoes would bring more insight to the possible importance *dlg* may have on reproductive development in *A. aegypti*. By studying the tissue specific expression, a more complete picture to determine if *dlg* expression is higher in tissue specific regions of the mosquito can be assessed. In Dugray's study 2-day-old fruit flies of both sexes had the highest relative expression in their carcasses, while expression in their gonads and guts was already beginning to wane, relative to pupae and 1-day-old adults. Observing if the same outcome is produced in *A. aegypti* will also help fill gaps surrounding *dlg*'s potential conservational functions across species as the methods used in this study cannot provide much comparison amongst species. Examining dlg transcript abundances in dissected tissues of different aged adult mosquitoes would specifically address some of these issues.

As for the results of the qRT-PCR analysis for relative miR-34 expression, while some of the biological replicates showed some expression, there was difficulty obtaining miR-34 cycle threshold values for the majority of samples. However, from the results of the calculations, miR-34 showed the most relative expression in the 2-day-old and 7-day-old

male mosquitoes and the least relative expression in the 2-day-old and 7-day-old female mosquitoes.

While it was anticipated that if miR-34 does down-regulate *dlg* expression, temporal stages with increased miR-34 levels would have decreased *dlg* levels and vice versa, this was not observed from this analysis. Despite there being increased *dlg* expression levels of 2-day-old and 7-day-old female where there was a decrease in miR-34 levels, the levels in the males are relatively high in comparison for both *dlg* and miR-34. Of course this miRNA may have multiple target genes, and hence, its body-wide abundances may not fully address its ability to regulate particular transcripts in different tissues. Future studies should examine the tissue-specific accumulation of both *dlg* and miR-34 to assess whether any negative correlations exist.

Through ANOVA testing, there was sufficient evidence to support statistical difference of miR-34 expression levels among the temporal stages; however, since the trend in the results does not completely follow what was expected, further testing would be beneficial. Additional tests should be conducted to gain a better perspective of the importance miR-34 may have, with relation to *dlg* expression, in development. Some future tests to conduct include: tissue specific qRT-PCR of relative miR-34 expression levels, a microRNA binding assay experiment, and a microRNA inhibition experiment. By performing tissue specific qRT-PCR for miR-34 expression and comparing it with tissue specific qRT-PCR for *dlg* expression, a negatively correlated association may be more clear. By performing a miR-34 binding assay experiment in epithelial mosquito cells like CCL-125, an actual determination if miR-34 does indeed bind to the 3'UTR of *dlg* in *Aedes aegypti* can be concluded. Lastly, performing a miR-34 inhibition experiment can

observe if inhibiting the miRNA has an affect on development, *dlg* expression, and morphology.

dlg RNAi Experiment

Based on the results of the dsRNA-expressing bacterial feeding assays, knocking down *dlg* does not have any obvious effect on delaying pupation, altering the sex ratio, or mortality from birth to eclosion. It is possible that the larvae did not ingest enough dsRNA to visibly impact their development or that *dlg* alone cannot produce developmental delay and/or sex change. However, the knockdown of *dlg* may impact fertility or fecundity in *Aedes aegypti* and requires further testing.

In Dugray's study, *Drosophila* was injected with dsRNA and the ovaries of injected females was observed for morphological differences. Dugray noted morphological changes in the *dlg* dsRNA-treated fruit flies. Based on the observed phenotypic changes Dugray observed, testing the same method of RNAi injections on *A. aegypti* may be beneficial.

Future testing to gain a more complete picture of the impact *dlg* RNAi has would include qRT-PCR to see if expression levels decreased after knockdown and dissections to compare morphology (specifically gonad morphology). Additionally, weighing the insects may give insight into internal developmental delays that affect rate of maturation.

Phylogenetic analysis of discs large

Since the phylogenetic trees constructed follow the expected phylogeny similarity, the *discs large* sequence used in my project was probably the correct ortholog of the gene for comparison to Dugray's research. This would support the assumption that what we know

as possible functions of *dlg* in fruit flies and other organisms (possibly including humans) possibly hasn't diverged substantially and likely has the same roles in mosquitoes. Therefore, similar expression patterns should have been observed. Deviations in these patterns may be due to the fact that fruit flies and mosquitoes are different insects which live in different habitats and may grow at different rates.

Overall, understanding the possible role *dlg* and miR-34 may have on mosquito development is still largely undetermined based on the results of this study. Conducting the future testing outlined throughout the discussion may help fill the gaps of understanding and lead to a clearer understanding of their importance.

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Appendices

Table 2. Primers used for qRT-PCR analysis.

qRT-PCR Target	Forward Primer Sequence	Reverse Primer Sequence
*dlg*5	5'-ACCGCAAAGAAGCTCATCAAG-3'	5'-TCCATCTTCACCGACATTTCCT-3'
miR-34	5'-CAAGGCAATACGCTATGGCAG-3'	5'-CCTGGCACCTCGCGGCGGCAGG-3'
S7 (reference gene)	5'-CCATTGAACACAAGGTCGACAC-3'	5'-GTAGGGCTCCGGGAATTCGA-'3

Table 3. Primers used for RNAi experiment.

RNAi Target	Primer Sequence
dlg 1 BglII (Forward)	5'-AAAAGATCTACCTGGCGATGCACTATACA-3'
dlg 1 KpnI (Reverse)	5'-AAAGGTACCAAGTGGAGCCTCGTCTTGTT-3'
dlg 2 BglII (Forward)	5'-AAAAGATCTAATATTGATTGTCGGGCCGC-3'
dlg 2 KpnI (Reverse)	5'-AAAGGTACCTCGGTAGAATGACGTGAATCCT-3'
pL4440 (Forward)	5'-CCACCTGGCTTATCGAA-3'
pL4440 (Reverse)	5'-TAAAACGACGGCCAGTGA-3'

Table 4. Individual treatment replicate data.

Treatment	Replicate	Number of eclosed males	Number of eclosed females	Number left to pupate	Number left to eclose
DH5α (negative control)	1	8	10	0	0
	2	5	9	1	1
	3	6	12	1	0
HT115 no plasmid (negative control)	1	6	8	1	0
	2	10	8	0	0
	3	10	9	0	0
HT115 dlg 1	1	8	8	1	1
	2	8	10	1	0
	3	6	11	3	0
HT115 dlg 2	1	12	4	0	0
	2	11	7	1	0
	3	4	9	2	0

Note: one adult mosquitoes from HT115 no plasmid (negative control) replicate 2 was intersex.

```
CLUSTAL O(1.2.4) multiple sequence alignment

A.aegypti          ------------------------------------------------------------  0
D.melanogaster     MAKMASGDLSLTSTSSQEEESSEYVGYDNTLRPPSNSSGSTTAANMSNNNGPSKGVSSGV  60

A.aegypti          --------------MIKKEFNQIKDKPGDALYIRVGFDRNCDLGESELPFNKDEVLYVDN  46
D.melanogaster     GVSGISGGNGANYDILQAQYKSALLELNTLRHQHANTKRRCDELATELTLYQEHYVA-DR  119
                              ::: :::.   : .   : :.. .*.**   :** : ::. :  *.

A.aegypti          TMFGGV---PGRWRAWKLDE-------------------------YGHKL----QCGIIP  74
D.melanogaster     NKFTDMVEESARFKRLLLETQNQQSQQAQNGNSQVPPVGSGNPYYFGKTQGCDGSCSEKL  179
                   . * .:    .*::   *:                           :*:.     .*.

A.aegypti          NKLKVEEELRLFGDIQDVETTRRG----STSARRSFFKRIKPQ-----RSSSRDSKELAS  125
D.melanogaster     AELKKERNMV------AVEREKYKKSYIELEKDRNYYRERGDENQKLKVLLSQESKNVLS  233
                    :** *.::        **  :      . .  *.:::.   :        *::**:: *

A.aegypti          FSNTHLSLYLETGN-------VGEDGFTSYQRVERLEYK---YRPILIVGPLSECVI---  172
D.melanogaster     LTEELNQLLSEKDNVLQEHQKMSDDLVLANKEIERLKKDEQLARAEIKVLQLANADLKKR  293
                   :::   .*  *..*       :.:* . : :.:***: .    *  : *  *::. :

A.aegypti          -------EKLCIDFPEHFHRSQQRQMRCTKEDMESGVQ---NNA-IADYRRRGSIFEYTT  221
D.melanogaster     DLLKSRDSSWSKEFPSGKELENSKELEKLRKSLEKALSEVERSSQDAEEAKRVRDWAISQ  353
                          .. . :**.  . .:.:::.  ::.:*..:.   ..:  *:  :*   :  :

A.aegypti          IQSILENKQHHYILDVCISAVERLQRNQIYPIVLLLRFKSSKQIKEIKDSRHST-DKI--  278
D.melanogaster     REKIVQER--DSVKTLCDKMR--HERDKAISD-SLMAIRDSEKIKKQKDEAQKKIDLLKE  408
                    :.*::::  . :  :* .     :*::      *: ::.*::**: **. :.. * :

A.aegypti          ----------S--AKA-----AKEMYEHALKLESDYRQYIS-----VVISGVNI------  310
D.melanogaster     QMEQQERQNLDSNASGSRRSFRPSSYEGEDLLEVELSGYEHTSDLGIILDDSNKRKLVCG  468
                              .  *..       . **    ** :   *       :::.. *

A.aegypti          ----AHMCTQIKS----------AVDSEQKK-------------LLWV------------  331
D.melanogaster     VTSSSPACGKLKINDVICKVNNLDCQSLSKRMVLDEIRACAPRSLLLVSRTRHSKRHAYS  528
                       :  * ::*             :* .*:             ** *

A.aegypti          ----------------------------------PVTTMA--------------------  337
D.melanogaster     VQLKTRDRDCPHGLQLDMGVFIAKIEQNSLAFYEPELDVGDRVLSINNKSMDSVQSIEEV  588
                                                    *   :.

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     MQLMNDPRSDGLNLFALKYVQDQLPPGMTTSSAQTDSIDSMQHVSSAGGGGGSGPSSATK  648

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     HPSRFAEFFFRKLKFSKPGTPEDNFEQEHDDAIAALDSVLSENSSEKSKENLFNRKKRTK  708

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     KEKEASKSMGTWPRTNISHENPTGTMRGNEKKRALMSLFTAGPINVDKDDELMGEVGVPE  768

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     KQPAALIQDQLPPPLPPQMSKPLAHIRGSNGGAIKTHPNRNSNPVSSGVSALFPPGPPNG  828

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     MPNTSYPTHPRHSLYGVTAEEINQKPIQRAPLMGANARVKMPSQERYGTRPHSNHRLSLN  888

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     ITPSGDFYQPKTSGQQAQQQVMNASSTSAGFGVGSGSMVGGTGPAAGEFPVRKQQVYDVF  948

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     HPPPLPKNSSGSNMFMPLHPTRGSHPPPVGQPPDVVSLKSQNSIESILSAKSPAISEYGM  1008
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A.aegypti          ------------------------------------------------------------  337
D.melanogaster     YAKRHVPQAVRHVPKYPSDSESIGSGVHGGYGGFLQSNHMPGNRHTQLFPTFGPGGRGNR  1068

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     RSSPLTLPSPPPQQQLQQLPAATAPHDSVGIPTDLDYHPHHHTQTNAMPHPHPHPHAPYL  1128

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     DYGHGLGIGPYMGVGGGVSGVGGAIYEGGTFPRKKDNQRLRIPSNPSVASKSSSMVKNSS  1188

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     GSIDHHYVTSTGPVSGGSMSASSSDRAPMSLMSSSIHNSYGANIAGGNGTGSSIGVGGGG  1248

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     GGGSGRGSPMPQVHVEVLSHGGGGSGKRNSNVPADFLCPGDLRRVTIDKRDKSLGITIQC  1308

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     NNNGGGIFVSTVADKSTAMRAGLQVGDQLLEVCGINMRAATQEIAANVLRQCGDSFTMLV  1368

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     QYNPEKFPSIEYEGAHNLEPESPINHSGSPTPRNSPRPPARNSLFPLPMQPQAPSTRPGS  1428

A.aegypti          ------------------------------------------------------------  337
D.melanogaster     RAPLSHQSIKDQSFTDSLENQSDISSSQDMPSSAATTTTTTASATSTVYDEEPKPSLPPP  1488

A.aegypti          --------- 337
D.melanogaster     PASVPAEST 1497
```

Figure 8. UniProt Align results for alignment of A. aegypti and D. melanogaster dlg protein sequences. Constructed using UniProt Align.