Genetic Response to Desiccation Stress in Female *Aedes aegypti* Mosquitoes

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Abstract

Aedes aegypti is the primary disease vector for both Dengue and Yellow Fever. Climate change is affecting the natural environment of these mosquitoes, yet the extent to which individuals and populations can physiologically respond to environmental stressors is unknown. Understanding the mechanics of *A. aegypti* stress responses has important implications for predicting both mosquito and disease distribution patterns in the context of a rapidly changing global environment. In this research, the genetic response of adult female *A. aegypti* mosquitoes to dehydration stress is investigated using quantitative PCR. Homologs of four physiologically relevant genes (*Frost, Desat2, HSP70* and *Pepck*) are examined in *A. aegypti*. Mosquitoes were subjected to acute desiccation stress and then analyzed via qPCR to determine the extent to which these genes' expression patterns were altered. Altered gene expression in response to desiccation stress was observed for *HSP 70*, while evidence suggesting the evolutionary divergence of *Frost* was also uncovered. These results provide clues as to which physiological mechanisms are utilized by *A. aegypti* to mediate survival in desiccating environments. Genetic indicators of these mechanisms can be used in comparative studies against geographically distinct populations to generate an understanding of stress resistance mechanisms in *A. aegypti* as a function of geography and environment.

Introduction

Aedes aegypti is a tropically distributed species of mosquito. They are anthropophilic, implying that they thrive in urban centers and selectively blood feed on humans. *A. aegypti* is also a highly competent disease vector, being the primary carrier of both Dengue and Yellow Fever. These diseases combined affect more than

400 million people annually; current estimates put ~40% of the globe at risk for Dengue Fever infection (Hales et al, 2002). Its high vector competence in conjunction with its widespread cohabitation of human environments makes *A. aegypti* incredibly relevant from a public health perspective.

Climate Change and Disease Prevalence

Dengue and Yellow Fever distributions closely mirror the distribution of *A. aegypti*. As such, climatic fluctuations that affect the distribution of these mosquitoes will affect the distribution of the diseases themselves. Even seasonal effects can have drastic consequences for disease prevalence; studies have been able to predict the outbreak of dengue fever based on climate conditions during El Niño fluctuations (Linthicum et al, 2010). These predictions are useful for focusing disease prevention and treatment efforts.

If seasonal fluctuations in climate can have measureable effects on disease distributions, it is impossible to ignore the implications that a rapidly changing global environment could have on the spread of these diseases. In the context of global climate change, the potential ranges of many disease vectors will be altered, allowing previously unaffected areas to become endemic for the diseases they carry. Studies examining this concept have predicted a marked expansion in disease vector distribution along with the increase of the diseases they carry (Hales et al, 2002).

The distribution of various insect species often coincides with physiological stress resistance traits, one of which is desiccation resistance. Both phenotypic and genotypic variability in desiccation resistance have been shown to change along

narrow longitudinal range lines (Telonis-Scott et al, 2011). As a result, understanding the physiological and genetic mechanisms behind desiccation resistance can have useful applications in predicting the distribution of various disease vectors in response to changes in humidity. Current disease prediction studies rely solely on the current population of disease vectors in conjunction with climate change predictions (Hales et al, 2002). They do not take into account the capacity for disease vectors to adapt to a changing environment. In an effort to better refine these predictions, this study focuses on the genetic response of female *A. aegypti* mosquitoes to environmental desiccation stress.

Stress Tolerance Plasticity

There are many ways in which insects can be affected by desiccation stress. The haemolymph of their open circulatory systems requires tight regulation of ion homeostasis to maintain functional solute concentrations (Telonis-Scott et al, 2011). As such, genes involved in the regulation of this homeostasis are interesting targets for study in response to desiccation stress. This regulation can take the form of altered metabolic patterns, changes in membrane properties and even atypical osmoregulatory patterns (Telonis-Scott et al, 2011; Gibbs et al, 2012).

Many studies have examined desiccation resistance on an evolutionary time scale, observing emerging expression patterns after multiple generations of lab controlled stress selection (Hoffmann et al, 2003; Telonis-Scott et al, 2012; Wang et al, 2011). While these studies shed important light on the evolutionary potential of

desiccation resistance, they do not allow for a nuanced understanding of desiccation response at the organismal level.

This study focuses on individual genes in an effort to increase the understanding of desiccation stress responses as they currently exist. Four genes found to be differentially expressed in response to desiccation stress in various organisms are examined in the *A. aegypti* system. These genes were selected to cover a wide array of possible desiccation responses in an effort to enhance the focus of future studies.

Frost

The first gene identified for study was *Frost*. This gene codes for a mucin like protein containing a secretory sequence that facilitates secretion into the haemolymph (Goto et al, 2001). Gene ontology reports indicate that it has a chitin binding domain and as such may be involved in the modification of cuticular hydrocarbons or their precursors. *Frost* was originally implicated in cold stress recovery in *Drosophila melanogaster* but has since been shown to be up-regulated during desiccation recovery as well (Sinclair et al, 2007). On a cellular level, cold and desiccation stress are very similar in at least one respect: water is either absent or locked up in ice, making it inaccessible. As such, genes that function in response to cold stress may very well be utilized in response to desiccation as well. This dual capacity makes *Frost* an attractive candidate gene, as it may exhibit high levels of conservation due to its cross-utilization.

Desat 2

Another gene involved in the modification of cuticular hydrocarbons is *Desat 2*, which codes for a fatty acid desaturase protein. Fatty acid desaturases are important in regulating the saturation levels of fatty acid chains by introducing a double bond into normally single bonded fatty acid chains. By desaturating fatty acid chains found in cell membranes, the fluidity and permeability of the membrane can be altered to respond to cellular stress. Fatty acid desaturases have been shown to be differentially regulated in response to environmental stressors in many species (Los and Murata, 1998), and *Desat 2* specifically has been shown to be down-regulated in response to desiccation stress in *D. melanogaster* (Sinclair et al, 2007).

HSP 70

Environmental stress results in atypical cellular conditions, which can have a myriad of undesirable consequences. Improper protein folding and protein aggregation frequently occur under stressful conditions, and having mechanisms to deal with these symptoms is crucial to an organism's survival under stressful conditions. Heat shock proteins are chaperone proteins expressed in large numbers in response to various cellular stressors (Herve et al, 2010). They assist in preventing protein aggregation, promoting proper protein folding and in the solubilization and refolding of aggregated proteins. One particular heat shock protein, HSP 70, has been specifically implicated in desiccation stress responses in mosquitoes (Benoit et al, 2010). HSP 70 interacts with key regulators of important

cellular signal transduction pathways that control many cellular processes, including cell homeostasis, proliferation, differentiation and apoptosis (Mayer and Bukao, 2005). RNAi induced knockdown of *HSP 70* mRNA has been shown to decrease resistance to desiccation in *A. aegypti* (Benoit et al, 2010). These traits make *HSP 70* a strong candidate for further study.

Pepck

The last aspect of desiccation stress responses in *A. aegypti* that is examined in this work is central metabolism. Studies looking at energy metabolism in insects have found that patterns of energy utilization are altered during desiccation stress (Marron et al, 2003). There are two main cellular energy sources, lipids and carbohydrates. Although lipids contain twice as much energy per unit weight than carbohydrates, studies have shown that desiccation selected populations of *D. melanogaster* store fewer lipids and more carbohydrate than control populations (Marron et al, 2003). Additionally, it was found that these flies resort to carbohydrate metabolism instead of lipid metabolism under direct desiccation stress.

Structurally, carbohydrates have a higher water binding capacity, so when metabolized, this bound water becomes available for cellular use (Marron et al, 2003). This pattern is reflected in stress response studies on desiccation and central metabolism. Desiccation stress in *D. melanogaster* was found to result in the up-regulation of a particular central metabolism gene, Pepck (Matzkin and Markow, 2009). This gene codes for the protein that catalyzes the first concerted step in

gluconeogenesis, whose action begins the process of de novo synthesis of carbohydrates. Due to this metabolic importance, Pepck was chosen as a target for further study.

Expectations and Significance

In this research, the above four genes are examined via qPCR to determine their relative expression patterns in response to desiccation stress. Expected patterns of expression mirror those found previously in other organisms; *Frost, Pepck* and *HSP 70* are hypothesized to be up-regulated while *Desat 2* is hypothesized to be down-regulated. Knowledge of the temporal expression patterns of these four genes in response to desiccation stress can be used to better understand the nuances of the dehydration stress response in *A. aegypti*. This knowledge can be used in comparative studies between geographically distinct populations, with the goal of increasing the understanding of the adaptive potential of *A. aegypti* in a rapidly changing global environment

Materials and Methods

Mosquito breeding

Mosquitoes were raised in a 95% relative humidity environment at 28°C. Eggs were the product of lab populations derived from individuals captured in Goudiry, Senegal. Eggs were placed in containers containing 1L deionized water and allowed to hatch. Larvae were separated from these containers and fed daily with a

50/50 brewer's yeast/fish flake mixture until pupation. Upon pupation, individual pupae were transferred to emergence cages until emergence. Upon emergence, mosquitoes were fed on sugar water and allowed to acclimate for 2-5 days before being used in stress trials. Only female mosquitoes were chosen for stress tolerance tests, due to their public health relevance and greater survival capacity in comparison to males.

Primer Design

Primers were designed for use with *A. aegypti* by first identifying the protein sequences of the target genes from *D. melanogaster*. These protein sequences were compared against the *A. aegypti* genome using the Ensembl.org BLAST program. Once *A. aegypti* sequences were obtained, primers were designed using IDT's primer quest software, while primer sequences for the S7 ribosomal housekeeping gene were taken from Xin et al. (2008).

Primers were verified using previously synthesized *A. aegypti* cDNA libraries as template. qPCR reactions for these primers were run over a dilution curve to determine amplification efficiencies. Standard curve reactions were run in triplicate at 1000, 100, 10 and 1 ng/μl concentrations. Each primer was also examined via melting curve analysis to determine product specificity. Primer sequences and efficiency values can be found in Table 1. Efficiency curves can be found in Figure 1 while melt curves can be found in Figure 2.

Desiccation Stress

At 2-5 days post emergence, adults were subjected to a brief CO_2 knockout. During this time, groups of 10 females were placed in glass vials and covered with foam stoppers. Roughly 2 grams of Drierite desiccant was added to experimental vials, while control vials received no desiccant. All vials were then sealed with parafilm. Control and experimental vials were left for either 4 or 8 hours and then placed in a -80°C freezer for flash freezing and storage.

Heat Stress

To verify the function of the HSP 70 primer pair, a positive control was run wherein one group of 10 mosquitoes was stressed for 1 hour at 37° C and then allowed to recover for 1 hour before freezing and storage.

RNA extraction and cDNA synthesis

Frozen mosquitoes were transferred to a 1.5 ml centrifuge tube. They were then homogenized in 500 μl Trizol until no anatomical parts were identifiable. Vials were then centrifuged at 4°C and 12,000 rcf for 10 minutes. The resulting supernatant was transferred to a second centrifuge tube. To this new tube, 100 μl of chloroform was added, followed by vigorous shaking and centrifugation at 4°C and 10,000 rcf for 15 minutes. The resulting upper aqueous layer was transferred to a third centrifuge tube, wherein 250 μl of isopropanol was added to precipitate the RNA. These tubes were inverted to mix and allowed to incubate at room temperature for 10 minutes. Tubes were then centrifuged for 10 minutes at 4°C and

12,000 rcf. The supernatant was removed (leaving only an RNA pellet), and 1 ml of 75% ethanol was added, followed by centrifugation at 4°C and 7,500 rcf for 5 minutes. The resulting supernatant was removed and the final pellet was allowed to air dry. Pellets were then re-suspended in 100 μl of RNAse-free water. Concentration was checked spectrophotometrically using a nano-drop. cDNA was synthesized using 250 ng of the extracted RNA and the Quantitech reverse transcription kit (Qiagen, California, USA). cDNA concentrations were again determined spectrophotmetrically using a nano-drop. cDNA libraries were then diluted to 100ng/μl for use in qPCR.

Quantitative PCR

qPCR was performed in triplicate using the Qiagen SYBR Green qPCR master mix. Each reaction consisted of 1μl 1:1 primer mix (50pmol/μl each primer), 1μl cDNA template (100ng/μl cDNA), 8 μl RNAse free water and 10 μl SYBR Green Master Mix. qPCR was performed on a Rotor-gene Q-Series using the following program: 10 min 95°C, 40 repeats of 15s at 95°C, 15 s at 54.6°C and 20 s at 72°C followed by a melt curve from 54.6-95°C. The greatest difference in threshold cycle (Ct) values between technical replicates for this study was 3.51 with an average difference amongst replicates of 1.0.

Relative expression levels were determined using the Pfaffl method for relative expression analysis (Pfaffl, 2001). This method standardizes relative expression values to a housekeeping gene (in this case an S7 ribosomal protein) and the efficiency of the qPCR primer pairs being used. These relative expression values

are then compared via a one sample t-test to determine whether they are significantly different than 1. A relative expression value of 1 indicates no change between the experimental and control treatments. Relative expression values greater than 1 indicate and up-regulation while values less than 1 indicate a down-regulation.

The sample size for 4 hours of desiccation stress consisted of 5 stress/control pairs, while the sample size for 8 hours of stress was 6 stress/control pairs. Results were then analyzed via the Grubbs test to determine outliers.

Results

Primer verification

Standard curve analysis indicated linear amplification over a standard curve for *HSP 70, Desat 2*, *Pepck* and *S7*, while the standard curve for *Frost* indicated no difference between standard concentrations (Figure 1). Primer pair efficiencies were extrapolated from these standard curves (Table 1). Melt curve analysis indicated the presence of single melt peaks for *HSP 70*, *Desat 2*, *S7* and *Frost*, with two peaks apparent for *Pepck* (Figure 2).

To verify these results, efficiency reaction products were run on a 2.0% TBE agarose gel. The expected amplicon for *Frost*, *Desat 2, Pepck* and *HSP 70* was ~100bp in length, while the expected amplicon for *S7* was ~250 bp. *Desat 2, HSP 70* and *Frost* all produced single bands at ~100 bp whereas *S7* produced a single band at ~250 bp. *Pepck* produced two bands, both <100 bp in length (Figure 3).

Stress Response

qPCR reactions were run comparing the relative expression of *HSP 70* and *Desat 2* between various stress treatments. Primer pairs for *Frost* and *Pepck* were not used in desiccation stress experiments because they were exhibiting aberrant amplification patterns.

HSP 70 expression in response to desiccation was an order of magnitude lower than *HSP 70* expression in response to heat stress (Figure 4). *HSP 70* expression varied at different desiccation stress intensities (Figure 5). After 4 hours of desiccation stress, *HSP 70* expression was found to be up-regulated 2.5 fold in comparison to an untreated control. This up-regulation was not statistically significant (p=.145). After 8 hours of desiccation stress however, *HSP 70* showed a .5 fold down-regulation in comparison to an untreated control. This down regulation was nearly statistically significant (p=.059).

Desat 2 expression was found to be up-regulated in response to desiccation stress (Figure 6). After 4H of desiccation stress, a 1.7-fold up-regulation was observed in comparison to an untreated control. This up-regulation was not statistically significant (p=.388). After 8 hours of desiccation stress, *Desat 2* expression was found to be up-regulated 1.9-fold in comparison to an untreated control. This up-regulation was not statistically significant (p=.329).

Discussion

Primer Verification

Primer verification data indicated functional primer pairs for *S7*, *Desat 2* and *HSP 70*. These pairs had linear efficiency curves that indicated a decrease in CT with an increase in concentration (Figure 1). In addition, the melt curves for these primer pairs contained a single peak, indicating amplification of a single PCR product (Figure 2). These findings were further verified by gel electrophoresis, with each primer pair producing one distinct band of the expected length when run out on a gel (Figure 3). Use of these three primer pairs was deemed valid, so they were used in further experiments.

Verification data for *Pepck* was less ideal. Although the standard curve indicated a concentration dependent depression of CT (Figure 1), the melt curve indicated two distinct melt peaks over a temperature gradient (Figure 2). This finding was further verified by gel electrophoresis, wherein the *Pepck* primer pair produced two distinct bands (Figure 3). The presence of two products implies one of two possibilities. The first is that the *Pepck* primer pair was binding to two different places in the *A. aegypti* transcriptome. This would result in two separate gene products being amplified, each creating a different peak on the melt curve. The other possibility is that the primer pair is dimerizing, and amplifying itself. The occurrence of either of these scenarios does not allow for the accurate reading of gene specific regulatory patterns, so use of the *Pepck* primer pair was discontinued.

Verification data for the *Frost* primer pair yielded confusing yet potentially very interesting results. Although the melt curve and gel analysis indicated that a single PCR product was being amplified (Figures 2 and 3), the standard curve indicated that there was no concentration dependent depression of CT (Figure 1). The most likely explanation for this occurrence would be if the original transcript was being expressed at such low concentrations that even a 1000-fold dilution had a negligible effect on starting transcript concentration. This hypothesis was supported by gel electrophoresis; the *Frost* primer pair produced a single band at the expected size, however it was incredibly faint (Figure 3). Due to the aberrant standard curve, use of the *Frost* primer set was discontinued for further trials.

Although no further experiments were done with *Frost*, the data gleaned from its verification experiments is compelling. We are observing the production of the Frost transcript, however at such low concentrations as to make dilution functionally irrelevant. This does not reflect the amplification of *Frost* genomic DNA because the primer pair was designed flanking a ~1000 bp exon, and the amplified PCR fragment was ~100 bp, indicating amplification based on template derived from the *Frost* mRNA. Thus, we are seeing incredibly low levels of Frost mRNA expression.

Previous studies have shown that tropically distributed species exhibit lower levels of resistance to both cold and desiccation stress than do more temperate species (Kellermann et al, 2009). In addition, some of these more specialized species cannot evolve higher resistance to these stresses even after generations of intensive lab selection (Hoffmann et al, 2003). The mosquitoes used in this

experiment were derived from individuals collected in Goudiry, Senegal, where the lowest monthly average temperature does not drop below 19.4 °C (Anonymous, 2014). In such a warm environment, there would not be evolutionary pressure to conserve a cold resistance gene such as *Frost*. Insignificant levels of *Frost* expression in conjunction with a lack of evolutionary context for its conservation could indicate a loss of gene function for *Frost* over time. Sequence similarity analyses support this hypothesis; the protein sequences for all of the other genes in this study had a 70-80% sequence similarity between *D. melanogaster* and *A. aegypti* protein sequences, whereas *Frost* only had a 35% similarity (Table 2).

Low levels of *Frost* expression in conjunction with low sequence similarity values could indicate the loss of *Frost* function in *A. aegypti* over evolutionary time. Low expression levels of *Frost* could be due to a wide range of factors. A mutation in a promoter sequence (Bookstein et al, 1990) or DNA methylation events (Li et al, 1993) could severely reduce the levels of *Frost* expression. In genes under very little selective pressure, it is common to see coding mutations that can result in an early stop codon. These early stop codons result in the translation of truncated proteins, which can have many deleterious effects in the cell. To mitigate this possibility, mRNAs containing these early stop codons are tagged for degradation during splicing, resulting in degradation of the mutated transcript (Chang et al, 2007). High levels of targeted mRNA degradation could explain the low expression levels observed for the *Frost* transcript. Studying the genetic and epigenetic factors influencing the *Frost* gene could offer further insight into the nature of this seeming loss of function.

Desiccation Dependent Expression Patterns

Although up-regulation was observed for *Desat 2* after both 4 and 8 hours of desiccation stress, neither of these values was statistically significant (p= .388 and .329 respectively). After 4 hours of desiccation stress, *HSP 70* showed an up-regulation, however this value again lacked statistical significance (p=.145). Small sample sizes (n=5-6) may have contributed to the lack of statistical significance found in this study. The observed down-regulation of *HSP 70* after 8 hours of desiccation stress was nearly significant (p=.056) however, and as such warrants further discussion.

HSP 70 is a general stress response gene, however its expression pattern runs counter to the logic of cellular stress responses. The up-regulation of *HSP 70* was observed after 4 hours of desiccation stress, followed by a down-regulation after 8 hours (Figure 5). At first glance, this seems as if it would be detrimental to insect survivability. The eventual down-regulation could result in lower levels of HSP 70 protein after prolonged stress, followed by the subsequent loss of acute stress tolerance. *HSP 70* expression studies in response to heat stress have shown however that once HSP 70 protein levels become high enough, *HSP 70* transcription is turned off by a negative feedback loop (DiDomenico et al, 1982). This does not result in the loss of transcript or protein; *HSP 70* mRNA is stabilized at high temperatures, where it continues to be translated (Petersen and Lindquist, 1988). Desiccation stress does not involve heating, so the stabilizing effect of heat stress on *HSP 70* mRNA is not a factor in this study. After 4 hours of desiccation stress, HSP 70 protein levels may not be high enough to activate the negative feedback loop and

turn off transcription. However by 8 hours of stress, this HSP 70 threshold may have been reached, turning off mRNA transcription and allowing for the degradation of *HSP 70* mRNA without its replacement. This pattern would account for the observed down-regulation after 8 hours of desiccation stress.

This result is interesting, especially in the context of *A. aegypti* desiccation resistance. It has been shown that *A. aegypti* mosquitoes can survive identical desiccation stresses for far longer than 8 hours (Gray, unpublished data), despite this eventual down-regulation of *HSP 70*. Perhaps some of the proteins that are involved in desiccation resistance require HSP 70 for folding. If these proteins were otherwise stable and long-lived, *HSP 70* would only be necessary until these proteins were abundant, at which point its transcription could be turned off without harming the organism.

Further studies aimed at increasing the resolution of the *HSP 70* temporal expression response should focus on a longer time period of observation in addition to quantification of protein concentration via western blot analysis. With the proposed regulation pathway, it would be expected that once HSP 70 protein has reached sufficiently low levels, the inhibition of *HSP 70* transcription would be reversed, resulting in an upwards oscillation of gene transcript levels. This oscillation could be altered based on the presence of other desiccation response proteins that require HSP 70 for proper folding. Testing this hypothesis could offer insight into how specific temporal patterns of climatic variation in a particular environment could affect mosquito survivability.

Experimental Constraints

Results for this research were generally lacking in statistical significance. There are many factors that could have contributed to this occurrence. The first involves the general quality of the sequence data available for the *A. aegypti* genome. The genome sequence for *A. aegypti* was published in 2007 (Nene et al, 2007). It has not been examined intensively and is lacking annotations for many well-studied and essential genes. In contrast, the *D. melanogaster* genome was published in 2000 (Adams et al, 2000), and has been extensively utilized as a model organism for genetic studies.

In addition, the *A. aegypti* genome sequence originates from a single, in-bred substrain of a wild isolate from Liverpool (Nene et al, 2007), whereas the mosquitoes used in this study were derived from wild isolates obtained in Goudiry, Senegal. Genetic differences between geographically distinct isolates could introduce error into sequence accuracy and thus primer design. This is markedly different from *D. melanogaster*, where all of the frequently utilized strains are derived from the same isolates, decreasing the chance for random sequence mismatches. As such, the transcripts being analyzed in *A. aegypti* may have a higher chance of containing small changes from the genome sequence in NCBI that could affect primer specificity. This error would then propagate during quantification reactions, reducing accuracy and precision. In this way, inconsistencies in sequence accuracy would ultimately impact the statistical significance of any results.

Another aspect of this research that pales in regards to other studies is its scale. cDNA libraries in this research were derived from groups of 10 mosquitoes.

The study by Sinclair et al (2007), in *D. melanogaster* used cDNA libraries derived from groups of 30 flies. This discrepancy could make already small differences in mRNA transcript concentrations even more difficult to detect, limiting the resolving power of this experiment. Uncontrollable errors introduced during the stresses themselves or during mRNA extraction and cDNA synthesis could have greater effects with this smaller sample size.

Future Directions

This research lays the groundwork for comparative studies between different populations of *A. aegypti*. Individual populations of related individuals can have different patterns of gene expression from others of the same species due to local climatic conditions. Studies looking at the genetic variability existing amongst adaptive traits have found that many of these traits vary over a species' range based on temperature and rainfall (Capy et al, 1993). Comparing the gene expression patterns from individuals derived from the Goudiry population against populations derived from other, climatically distinct areas can offer insight into how local climate affects the evolutionary trajectory of these globally relevant mosquitoes. Understanding the capacity of *A. aegypti* to adapt to different environments on a genetic level will be invaluable in determining the limits of their climate change induced expansion.

Tables and Figures

Table 1. Primer sequences, sources and efficiencies. Sequences were designed using IDT Primer quest software based on the template gene transcript designated under the source column. Efficiencies were determined via standard curve analysis.

Primer	Sequence	Source	Efficiency
Frost Forward	5'-TGGTCCTGATCACACAAATACC-3'	AAEL009219-RA	N/A
Frost Reverse	5'-TCGTATCGTGAAACAACCTCTG-3'		
Desat 2 Forward	5'-CTGCGGTTCCAGAGGAAATAC-3'	AAEL003203-RA	0.58
Desat 2 Reverse	5'-CCAGGTTTCGTTCCAGAAGTAG-3'		
Pepck Forward	5'-GCTTACGTCGTCAACTCCAT-3'	AAEL000006-RA	N/A
Pepck Reverse	5'-ACCGAGTGGAGGCATTTAAC-3'		
HSP 70 Forward	5'-GAATCGGCTGGAGACAAACT-3'	AAEL017974-RA	0.53
HSP 70 Reverse	5'-CCTTCTCGGCCATCGTATTT-3'		
S7 Forward	5'-GGGACAAATCGGCCAGGCTATC-3'	Xi et al, 2008	1.36
S7 Reverse	5'-TCGTGGACGCTTCTGCTTGTTG-3'		

Table 2. Protein sequence similarities between *A. aegypti* and *D. melanogaster* gene transcripts.

Gene	*A. aegypti* Gene Transcript	*D. melanogaster* Gene Transcript	Protein Similarity
Frost	AAEL009219-RA	FBtr0082101	35.80%
Desat 2	AAEL003203-RA	FBgn0043043	70%
Pepck	AAEL000006-RA	FBgn0003067	69.27%
HSP 70	AAEL017974-RA	FBtr0082512	82.55%

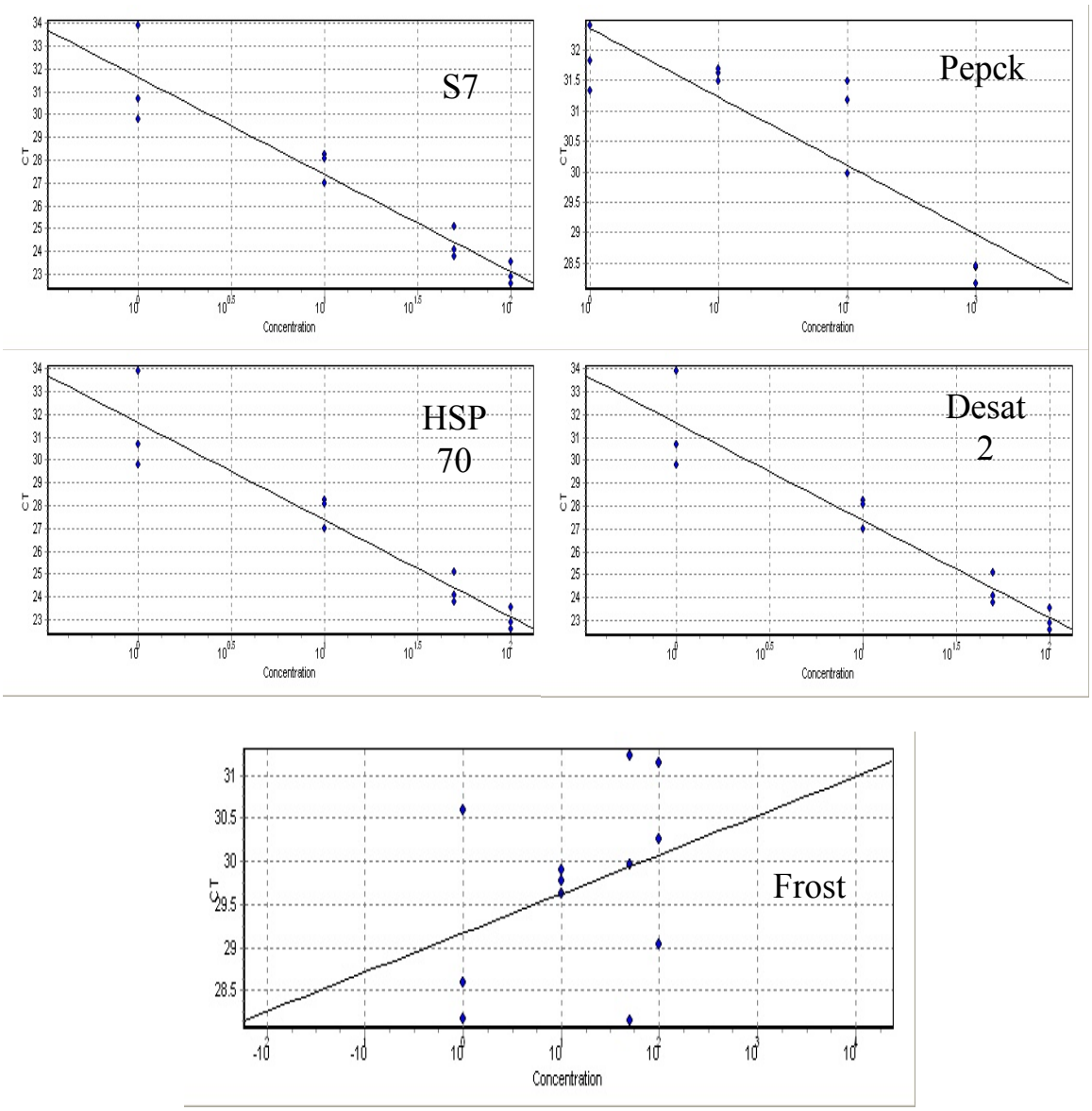

Figure 1. Efficiency curves for all primer pairs. Template concentration (ng/μl) is on the x-axis while CT (critical threshold) is on the y-axis. CT represents the number of cycles required to reach a given concentration threshold and can be used to determine relative initial concentration.

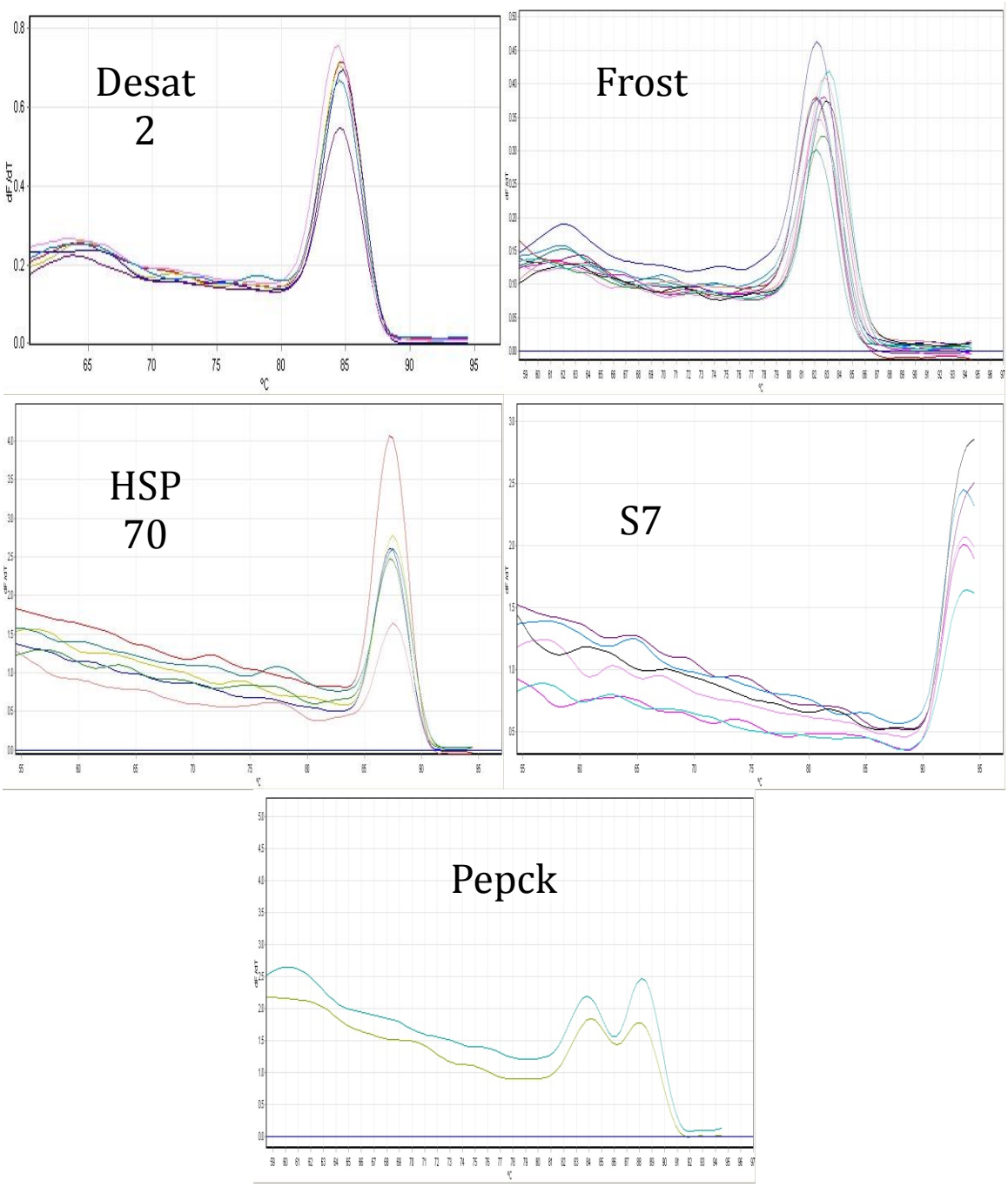

Figure 2. Melt curves for all primer pairs. Different fragments of DNA will melt at different temperatures depending on their length. By measuring the change in fluorescence over a temperature gradient, it is possible to observe whether or not multiple fragments have been amplified.

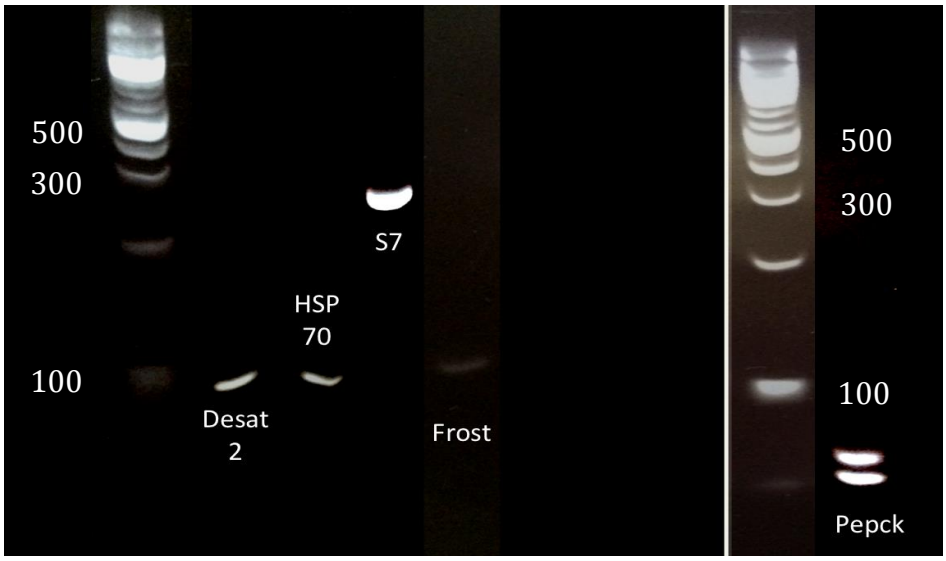

Figure 3. 2.0% TBE agarose gel loaded with PCR products from each primer pair. The *Desat 2*, *HSP 70* and *S7* lanes all contain a single, strong band at the expected length. *Frost* shows a single, weak band and *Pepck* exhibits two bands. This confirms the data gleaned from Figures 1 and 2.

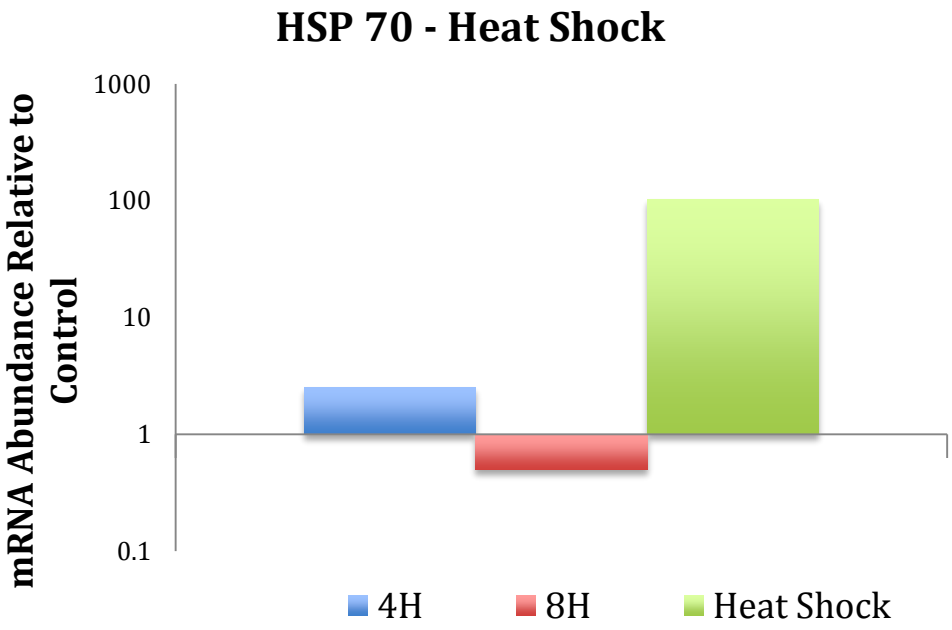

Figure 4. HSP 70 positive control. Relative expression of HSP70 mRNA relative to untreated controls after 4 hours desiccation stress, 8 hours desiccation stress, and 1 hour heat stress with 1 hour recovery. A relative abundance = 1 indicates no change, an abundance >1 indicates an up-regulation and an abundance <1 indicates a down regulation. HSP 70 response to heat stress is an order of magnitude greater than HSP 70 response to either 4 or 8 hours of desiccation stress.

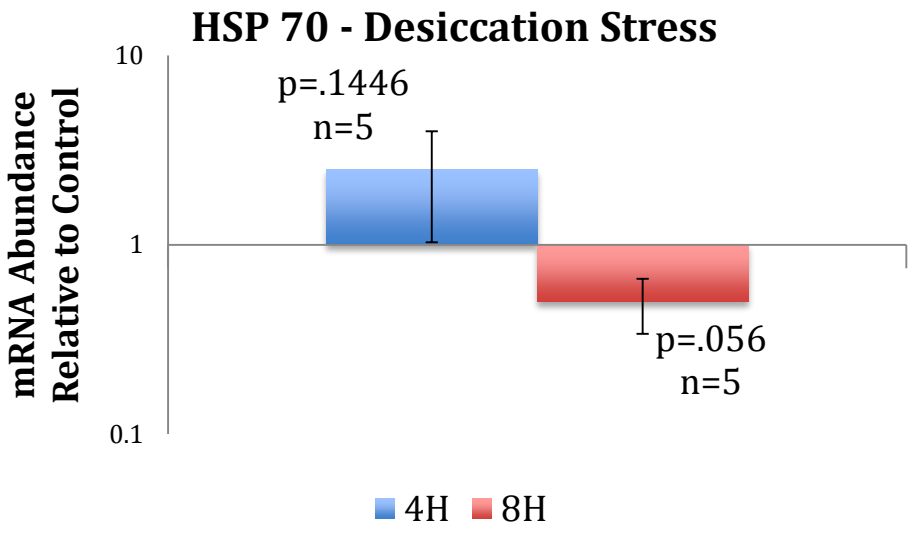

Figure 5. HSP 70 mRNA abundance relative to untreated controls after 4 and 8 hours of desiccation stress. Relative abundance = 1 indicates no change, abundance >1 indicates an up-regulation and <1 indicates a down regulation. After 4 hours of desiccation stress a statistically insignificant (*p*=.1446) 2.5-fold up-regulation observed. After 8 hours, a nearly significant (*p*=.056) .5-fold down-regulation was observed. Error bars = SE of the mean

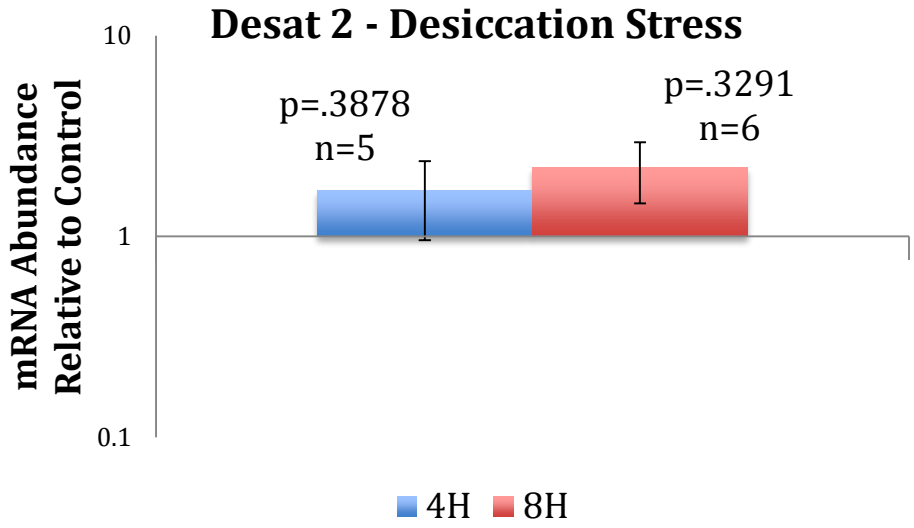

Figure 6. Relative expression of Desat 2 mRNA relative to untreated controls after 4 and 8 hours of desiccation stress. A relative abundance value = 1 indicates no change, an abundance >1 indicates an up-regulation and an abundance <1 indicates a down regulation. After 4 hours of desiccation stress, a statistically insignificant 1.7-fold up-regulation of Desat 2 was observed (*p*=.3878). After 8 hours of desiccation stress, a statistically insignificant 2.2-fold up-regulation was observed (*p*=.3291). Error bars = SE of the mean.

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