Aedes aegypti Heat Shock 70 Genes and their Inducible Promoters

Tiffany Lauren Gross

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Entomology

Zach N. Adelman, Chair

Kevin M. Myles

Igor V. Sharakhov

Erin L. Dolan

Jinsong Zhu

July 11, 2011
Blacksburg, VA

Keywords: *Aedes aegypti*, heat shock, hsp70, inducible promoter

Aedes aegypti Heat Shock 70 Genes and their Inducible Promoters

Tiffany Lauren Gross

Abstract

Aedes aegypti is an important vector of the viruses that cause dengue fever, dengue hemorrhagic fever, and yellow fever. In depth genetic studies of vector species have been made possible due to the availability of genome sequences and techniques for producing stably transformed mosquitoes. These resources have also contributed to the establishment of new genetics-based approaches to the control of vector borne disease.

Genetic studies of *Ae. aegypti* have benefited from the ability to drive targeted transgene expression, however a ubiquitous inducible promoter has not been identified in this mosquito. The *Drosophila melanogaster* heat shock 70 promoter has been shown to drive inducible expression in heterologous systems; however, *DmHsp70* possesses significant basal activity in *Aedes aegypti*.

This study characterized the sequence and expression of the heat shock 70 genes of *Aedes aegypti. AaHsp70* genes were found to be organized in two clusters, each comprised of three divergent pairs. *AaHsp70* genes exhibited robust expression upon heat shock in larvae, pupae, and adults as well as in heads, salivary glands, midguts and ovaries.

Genomic regions upstream of *AaHsp70* genes were found to drive heat-inducible expression of a reporter in both cell and embryo assays. Deletion analysis of *AaHsp70*-derived promoters yielded two ~1.5 kb genomic fragments that maintained robust heat inducibility in these systems.

Aedes aegypti were transformed with *AaHsp70*-luciferase gene cassettes using the transposable element *Mos*1. *AaHsp70*-luciferase transcripts accumulated specifically after heat shock, and displayed a pattern of rapid induction and decay similar to endogenous *AaHsp70* genes. Heat-induced expression of luciferase was observed in transgenic larvae, pupae and adults as well as heads, midguts and ovaries but not salivary glands, with levels varying between transgenic strains.

The effect of heat shock on the endogenous RNAi pathway as well as the effect of blood feeding on the expression of *AaHsp70* genes was investigated, though reproducible results could not be obtained using the assays employed.

In conclusion, the heat shock 70 gene family of *Aedes aegypti* was identified and characterized. The *AaHsp70* promoters described could be valuable for gene function studies as well as for the precise timing of the expression of anti-pathogen molecules.

Acknowledgements

First of all, I would like to thank my advisor, Zach Adelman, for his constant patience and encouragement while I worked my way toward a goal I didn't seem to realize I had. Without your continuous challenges, I would never have come close to reaching my potential. In the nearly six years that I worked for you, I was always challenged, always encouraged, and always treated with respect. Thank you for realizing what I was capable of doing when I didn't see it myself.

I would like to thank my committee members, Kevin Myles, Igor Sharakhov, Erin Dolan and Jinsong Zhu for their advice and encouragement as well as for challenging me during my studies. To Nels Johnson, thank you for your help with my statistical analysis. Your patience was greatly appreciated.

I would also like to thank my fellow lab mates for their constant support, encouragement, and comedic relief when it was greatly needed. Michelle, I will forever be in your debt for all of the advice on techniques, the favors, and the chats across the bench. Sanjay, thanks for teaching me how to laugh when things get rough, and thank you for all the times you listened when I needed it. Mary Etna, thank you for your continuous optimism even when it seemed like things couldn't go more wrong. I am very lucky to count you all as friends.

To Azadeh, thank you for your hard work in establishing the lines I needed to complete my experiments, and for your happiness to help in the insectary. I have enjoyed working with you and learning about your culture. To John, thank you so much for your innumerable hours spent rearing, picking, sorting, and mating mosquitoes. I'm not sure I could have done it without you. To all of the Adelman lab undergraduates, past and present, thank you for doing all the things you do to make my life easier.

I would like to thank the members of the Myles lab, especially Mike and Elaine. It's been a long road we've walked together, and we're finally at the finish. Thanks for all the support and good times along the way.

Last, but certainly not least, I would like to thank my family. This work is the culmination of years of your support and encouragement, long nights doing homework at the kitchen table, and time spent crafting science fair projects. Thank you for always believing in me and pushing me to realize my potential. Thank you, Momma, for not scolding me when I brought you pockets full of caterpillars and six-legged wonders. I told you at 4 years old that I wanted to be an Entomologist, and here I am. I count myself as very lucky to have the family that I do, and I know you are all right behind me all the way.

Adam, I don't think you'll ever know just how much you are a part of this work. You have comforted me, encouraged me, and motivated me when I thought I couldn't keep going. Until I met you, I never dreamed that I could accomplish something like this. Your sacrifices did not go unnoticed, and our fun times are almost here. I knew from the moment I saw you that there was something special about you and I am so lucky to be spending the rest of my life with you.

I would like to dedicate this work to my grandmother, Connie Smith. You were my best friend and I miss you every day.

Table of Contents

List of Figures

List of Tables

Chapter 1

Literature Review

GLOBAL IMPACT OF *AEDES AEGYPTI*

Aedes aegypti is an important vector of multiple pathogens, including the viruses that cause yellow fever, dengue fever, and dengue hemorrhagic fever (Severson *et al.* 2004a). More than 2.5 billion people are at risk from dengue, with 50 million dengue infections worldwide each year, according to the World Health Organization (2008). Increased air travel and general globalization has catalyzed an increase in mosquito-borne disease by trafficking vectors across the globe and infected individuals (Tatem *et al.* 2006). Dengue hemorrhagic fever is also on the rise as increasing human populations place greater strain on inadequate infrastructure (Gratz 1999, Sutherst 2004).

Aedes aegypti mosquitoes are closely associated with human populations and preferentially bite humans (Harrington *et al.* 2001). Urban environments provide ideal breeding sites for mosquitoes with excess artificial larval habitats including flower pots, garbage, and used tires (Monath 1994, Gratz 1999). In areas without sufficient water supply, rainwater collection and storage provide breeding sites for mosquitoes. As a result of the movement of troops during World War II, hyperendemic dengue infection arose in Southeast Asia. The shuttling of viremic soldiers led to the mixing of four serotypes of dengue viruses in this area, and a concomitant increase in the number of dengue hemorrhagic fever cases. The risk of dengue hemorrhagic fever is increased by sequential infection by more than one dengue virus type (Monath 1994).

Although an effective vaccine against yellow fever virus exists to help control the disease, no vaccine is yet available to protect against dengue viruses. The phenomenon of antibody dependent enhancement of infection precludes vaccination against all four serotypes

individually (Halstead 1988). Research is currently underway to develop a safe and effective vaccine (reviewed in Murphy and Whitehead 2011). With no vaccine available, protection against dengue viruses, responsible for more morbidity and mortality than other any mosquito borne virus, is limited to vector control strategies (Harrington *et al.* 2001, Severson *et al.* 2004a).

The most effective method of controlling *Aedes aegypti* populations is through destruction of larval habitat (Halstead 1984, Monath 1994, Gubler 1998). Urban environments provide numerous breeding habitats for *Aedes aegypti*, and it is nearly impossible to identify all of them. Eradication programs were put into place in 1947 by the Pan American Health Organization. However, by 1972 funding and enthusiasm for the implementation of control programs was lacking and mosquito control efforts waned. *Aedes aegypti* was then re-introduced into areas where the species had been eradicated (Halstead 1984, 1988).

The use of insecticides and habitat destruction has proven inadequate in eradicating the global population of *Aedes aegypti* so far. The combination of a lack of resources for surveillance and control programs, along with public complacency about vector borne diseases, has resulted in a resurgence of diseases such as dengue fever and a global increase in dengue hemorrhagic fever (Gubler 2002). The reduction of morbidity and mortality due to dengue viruses and other arboviruses must be approached from a different angle. To that end, molecular strategies for reducing disease transmission have been proposed (consult Terenius *et al.* 2008, Hay *et al.* 2010). A better understanding of the genetic basis of vector competence is necessary to determine ways of disrupting pathogen transmission (Blair *et al.* 2000, Severson *et al.* 2004a).

GENETIC TRANSFORMATION OF *AEDES AEGYPTI*

Genetic transformation provides a means of stably expressing anti-pathogen genes and other means of altering mosquito immunity (reviewed by Wimmer 2003). *Aedes aegypti* mosquitoes were first transformed in 1998 (Coates *et al.* 1998, Jasinskiene *et al.* 1998). *Aedes aegypti* was stably transformed using the *Hermes* element derived from the housefly and the transformation was stable and heritable through ten generations. This species was also transformed using Mos1, a modified *mariner* element (Coates *et al.* 1998). Additionally, *Aedes aegypti* have been successfully transformed using the piggyBac element (Kokoza *et al.* 2001).

The *mariner* element is a Class II transposable element derived from *Drosophila mauritiana*. Heterologous *mariner* activity was described and validated as a means of transposition *in vivo* in *Lucilia cuprina*, the Australian sheep blowfly, and *Bactrocera tryoni*, the Queensland fruit fly (Coates *et al.* 1997). Subsequently, stable transformation of *Aedes aegypti* was demonstrated using a modified *mariner* element (Coates *et al.* 1998). *Mos1*, a modified *mariner* element, has demonstrated highly stable integrations in the *Ae. aegypti* genome. *Mos1* exhibits a canonical cut-and-paste mechanism with integration at the 3' end of a –TA dinucleotide and high levels of post-integration stability (O'Brochta *et al.* 2003, Wilson *et al.* 2003).

piggyBac, another Class II transposable element, has also been used to transform *Aedes aegypti* (Kokoza *et al.* 2001). Although *piggyBac* exhibits canonical cut-and-paste transposition in *D. melanogaster*, it was sometimes found to integrate in a non-canonical manner in *Aedes aegypti*. Non-canonical insertions included large amounts of flanking plasmid DNA (Pinkerton *et al.* 2000) as well as large arrays of donor and plasmid DNA that were easily lost within one generation (Adelman *et al.* 2004).

With dengue fever and dengue hemorrhagic fever accounting for a large portion of worldwide morbidity and mortality, the ability to genetically modify *Aedes aegypti* through genetic transformation is of special interest.

PROMOTER DRIVEN TRANSGENE EXPRESSION

With sequence data available for three major disease vector mosquito species, *Anopheles gambiae* (Holt *et al.* 2002), *Culex quinquefasciatus* (Arensburger *et al.* 2010), and *Aedes aegypti* (Nene *et al.* 2007), scientists have been able to approach aspects of vector competence from a molecular standpoint (Lawson *et al.* 2007). Comparative genomics has allowed for identification of genes important to the mosquito immune response and vector competence in addition to providing information on the structure, function, and location of other genes (Severson *et al.* 2004b, Severson *et al.* 2004a, Waterhouse *et al.* 2008). The vast amount of sequence and genomic information available has made more informed attempts at modulating vector competence possible.

By expressing anti-pathogen effector molecules in specific tissues, it may be possible to reduce vector competence (Moreira *et al.* 2000). Various promoter elements exist that can be used to target effector molecule expression to specific tissues, with specific timing, and in response to different physiological phenomenon (Zhao and Eggleston 1999). These promoters include the *D. melanogaster* hsp70 and actin5C promoters as well as the immediate early IE1 promoter from the *Bombyx mori* baculovirus among others.

Multiple promoter elements have been described that drive expression in response to blood feeding. The *Aedes aegypti* carboxypeptidase (*AeCP*) promoter drives robust expression of a reporter in response to a blood meal (Moreira *et al.* 2000). This expression is sex and stage

dependent, as only adult female mosquitoes take blood meals. Vitellogenin (*Vg*) is another gene activated by blood feeding. The *Vg* promoter drives fat body specific expression in response to a blood meal, and was used to drive expression of a major immune factor (Kokoza *et al.* 2001, Kokoza *et al.* 2010). The timing of midgut expression was ideal for expression of immune factor genes to protect against ingested pathogens. CecropinB is an antimicrobial peptide expressed in response to bacterial challenge in mosquitoes (Lowenberger *et al.* 1999). A portion of the regulatory region of *Aedes aegypti* CecropinB has been used to drive expression of transgenes in response to injected lipopolysaccharides (Isoe *et al.* 2007).

Multiple genes have been identified in *Aedes aegypti* that are expressed specifically in salivary gland tissues. These genes include *Maltase-like I* (*MalI*) (James *et al.* 1989), *Apyrase* (*Apy*) (Smartt *et al.* 1995) and *30K* (Mathur *et al.* 2010). The regulatory region of *MalI* has been shown to drive expression mainly in the proximal lateral lobe of the salivary glands, while the *Apy* promoter drives expression mainly in the distal lateral and medial lobes (Coates *et al.* 1999). *Apy* expression is further limited only to female mosquitoes as this gene is associated with blood feeding. The *30K* promoter drives divergent expression localized to the distal-lateral lobes of the female salivary glands (Mathur *et al.* 2010). These promoters allow for targeted expression of effector genes directly to the salivary glands, which are the last stop for pathogens before being transmitted to a new host.

Certain promoter elements target transgene expression to the reproductive organs. The *Aedes aegypti nanos* and vitellogenin recepter (*VgR*) promoters drive expression specifically in the ovaries (Cho *et al.* 2006, Adelman *et al.* 2007), while the β2 tubulin promoter targets expression to the testes (Smith *et al.* 2007).

In addition to driving expression of anti-pathogen molecules, specific promoter elements are necessary for the expression of transgenes as markers for genome integration. Marker genes are necessary to indicate successful genomic integration events. The synthetic 3xP3 element (Horn *et al.* 2000) has been used to drive eye-specific expression of marker genes such as EGFP. In addition to tissue specific marker expression, full body expression of fluorescent protein driven by the *polyubiquitin* promoter has been utilized (Anderson *et al.* 2010). This allows for quick and easy screening of large numbers of larvae for transformation events. Other promoters utilized for general expression of transgenes include the *Drosophila* actin5C promoter (Pinkerton *et al.* 2000) and the *Drosophila melanogaster hsp70* promoter (Ramos *et al.* 2006). Actin5C drives constitutive expression of transgenes throughout *Drosophila* with expression limited to the midgut, ovaries and fat body in *Aedes aegypti* (Pinkerton *et al.* 2000), whereas *DmHsp70* drives heat shock regulated expression of transgenes. However, *DmHsp70* acts constitutively in *Aedes aegypti* with some level of increased expression under heat shock (Morris *et al.* 1991, Zhao and Eggleston 1999). Constitutive expression makes the *DmHsp70* promoter unsuitable to drive expression of genes that require highly specific regulation. Overall, many promoter elements are available to drive both targeted and ubiquitous expression of transgenes, however identification and characterization of a tightly regulated inducible *Aedes aegypti* promoter would be beneficial.

DROSOPHILA HEAT SHOCK 70 GENES

Heat shock proteins are molecular chaperones that are responsible for preventing the cytotoxic aggregation of denatured proteins and for the refolding of denatured proteins (Bettencourt and Feder 2002). Heat shock protein synthesis is induced by heat stress, anoxia,

exposure to ethanol, and chemical treatments including sodium arsenite, cadmium chloride, and sodium salicylate (Li and Werb 1982, Craig 1985, Tanguay 1988, Liu *et al.* 1994).

The heat shock 70 genes of *Drosophila melanogaster* have been very well characterized and have been used for comparative studies of *hsp70* in other organisms (Severson *et al.* 2004b, Waterhouse *et al.* 2008). The *D. melanogaster* genome contains six highly conserved copies of the heat shock protein 70 gene (hsp70). The first two copies, Aa and Ab, are arranged as a divergently transcribed pair at the 87A locus of chromosome 3R (Gong and Golic 2004). The next two copies, Ba and Bbb, are arranged as a divergent pair with the fifth, Bb, and sixth, Bc, copies arranged in the forward orientation. *DmHsp70Aa* and *DmHsp70Ab* are separated by 1.7 kb including a transposable *S* element insertion. It is thought that *S* element insertions are associated with the duplication of two ancestral *DmHsp70* genes into six (Maside *et al.* 2002). *D. melanogaster* possesses a relatively small genome in comparison to *Aedes aegypti*, 180 million base pairs with approximately 44 percent repetitive sequence compared to 1376 million base pairs and approximately 50 percent repetitive sequence (Adams *et al.* 2000, Nene *et al.* 2007). It is likely that six copies remain in the comparatively small genome due to subfunctionalization of each gene copy (Lynch and Force 2000). Research in *D. melanogaster* has suggested that each copy of the Hsp70 gene may be individually regulated and may respond to various heat stress conditions differently (Krebs and Feder 1997). Additionally, the duplication of heat shock genes increased the thermotolerance of *D. melanogaster* allowing for increased range and niche expansion (Bettencourt and Feder 2001).

DmHsp70 genes can be induced in larvae, pupae, and adults (Feder *et al.* 1996, Dahlgaard *et al.* 1998). Expression has been documented in multiple larval tissues including the

brain, salivary glands, midgut, hindgut, caeca, fat body, malphigian tubules, female gonadal disks, and imaginal disks (Krebs and Feder 1997).

Induction of *DmHsp70* expression is rapid following exposure to elevated temperatures (Lindquist 1980). Treatment at 37°C induces transcription of heat shock messages within four minutes, and results in translated protein within eight to 12 minutes. Detectable induction occurs after exposure to temperatures ranging from 26 to 37°C, with the highest levels of induction at 37°C. Messenger RNA turnover is more rapid at 33°C than 37°C because of the 3' untranslated region of each gene, which confers instability to messages under non-stressed conditions. Other cellular processes are slowed or stopped while heat shock genes are transcribed and translated exclusively under severe heat shock (Lindquist 1980). Less severe heat shock allows for very fast recovery of cellular processes, and sometimes a lack of complete repression (Craig 1985). The *D. melanogaster* heat shock response has been extensively reviewed by Craig (1985) and Nover (1991).

DmHsp70 gene sequences are free of introns and contain a conserved sequence in the 5' untranslated region (UTR) necessary for heat induction (Pelham 1982, Tanguay 1988). The conserved heat shock element was originally identified as being 14 basepairs in length with the consensus sequence CTnGAAnnTCCnAG. Subsequently, the heat shock element was found to be a conserved five basepair unit, nGAAn, organized in trimers (Perisic *et al.* 1989). Heat shock transcription factor (HSTF) binds equally well to head-to-head or tail-to-tail repeats of the heat shock element, as reviewed in Sorger (1991). Multiple copies of heat shock element trimers result in stronger heat inducible expression, with increased HSTF affinity for upstream elements upon binding to the most downstream element (Topol *et al.* 1985, Amin *et al.* 1988, Perisic *et al.*

1989). Additional regulatory sequence from *DmHsp70* is required for heat inducible expression in heterologous systems (Amin *et al.* 1985).

Regulation of *DmHsp70* transcripts has been tied to the 3' UTR. During recovery from heat shock conditions, *DmHsp70* 3'UTRs target transcripts for degradation (Petersen and Lindquist 1989). Expression of heat shock genes under normal conditions is detrimental to cell growth, therefore the abundance of transcripts during and after heat shock is highly regulated (Feder *et al.* 1992). The more extreme the heat shock, the longer messages are stable, as the half-life of heat shock messages is at least 20 fold higher at 37°C as compared to 25°C (Pauli *et al.* 1992). All *DmHsp70* messages are synthesized with a long, 120 bp poly-A tail, and messages examined after severe heat shocks were found to be fully poly-adenylated while those arising from more mild heat shocks exhibited truncated or absent poly-A tails due to rapid, preferential deadenylation (Dellavalle *et al.* 1994). Deadenylated transcripts are not efficiently transcribed and are targeted for degradation.

DmHsp70 upstream regions are occupied by paused transcriptional machinery when not under heat shock conditions (Rasmussen and Lis 1993, Tang *et al.* 2000). This promoter-proximal pausing of transcriptional machinery diminishes upon heat shock after the recruitment of heat shock transcription factor, RNA polymerase II, and other transcription factors (Lebedeva *et al.* 2005). This organization of transcriptional machinery allows for quick transcription of *hsp70* messages under heat shock conditions. The binding of transcription factors under normal conditions also maintains an open chromatin conformation at the heat shock loci allowing for fast induction (Karpov *et al.* 1984).

Due to the highly regulated nature of heat shock genes, *hsp70* is used as a marker for stress in *D. melanogaster* (Mukhopadhyay *et al.* 2003). Heat shock proteins provide an

important marker for detrimental effects of environmental chemicals. Specifically, transgenic *D. melanogaster* have been used to examine the effects of effluent from the chrome plating industry and its potential for detrimental environmental effects (Mukhopadhyay *et al.* 2003).

Regions upstream of *Drosophila melanogaster hsp70* genes have been identified as heat inducible promoters. The *Drosophila melanogaster hsp70* promoter has been widely used in heterologous systems for gene function studies and to drive transgene expression. This promoter can control transgene expression both temporally and spatially. The *DmHsp70* promoter has been used to drive tightly controlled expression of transgenes in *Danio rerio* and *Bicyclus anynana* using a modified soldering iron and a laser, respectively (Ramos *et al.* 2006, Hardy *et al.* 2007). These methods allow transgene expression to be controlled at the level of small groups of cells, in the case of the soldering iron in the *Danio* study, and at the single cell level in the case of the laser in the *Bicyclus* study. These methods allow researcher to test the function of transgenes in any tissue accessible to heat treatment (Ramos *et al.* 2006). Additionally, the *DmHsp70* promoter has been used to drive whole-body expression of transgenes in heterologous systems such as *Bombyx mori*. The heat shock promoter has been used to establish an inducible RNAi system and to drive whole-body inducible transgene expression in *B. mori (Uhlirova et al. 2002, Dai et al.* 2007).

The *DmHsp70* promoter has also been used to drive ectopic expression of endogenous genes to study gene function in transgenic *D. melanogaster*. For example, studies have examined phenotypic consequences of the heat induced ectopic expression of genes important to embryogenesis and mating (Aigaki *et al.* 1991, Read *et al.* 1992). The heat-induced ectopic expression of *Drosophila* sex peptide in virgin adult females altered mating behavior in the presence of courting males, with heat induced expression of sex peptide leading to the refusal of

courting males by transgenic females (Aigaki *et al.* 1991). An increase in the frequency of ovulation in heat shocked virgin transgenic females was also noted. Therefore, Aigaki *et al.* concluded that the sex peptide is responsible for the post-mating behavior of *Drosophila* females (1991). Similarly, the putative embryonic segmentation function of the *D. melanogaster ttk* protein was confirmed by induction of ectopic expression of both isoforms of the protein through heat shock and examination of the heat shocked embryos (Read *et al.* 1992). The study of embryogenesis relies heavily on the specific timing of ectopic expression, and other genes important to *Drosophila* embryogenesis have been identified in this manner, including *hairy* and *tailless* (Read *et al.* 1992). Heat inducible gene knockdown using the RNA interference pathways has also been useful for functional genomics studies in *Drosophila*. Lam and Thummel established that inducible expression of dsRNA against a gene of interest was a valuable method of gathering information on gene function (2000). This method circumvented the issues of mosaic activity of injected dsRNA, as well as the issue of dominant lethal phenotypes arising from loss of function mutants. The ability to regulate the timing of transgene expression makes the *DmHsp70* promoter ideal for studies of this type.

STRESS RESPONSE AND VIRUS SUSCEPTIBILITY

Viral infection has been associated with the stress response and an increase of heat shock gene expression in human, chicken, mosquito, and *Drosophila* cells (Collins and Hightower 1982, Phillips *et al.* 1991, Kampmueller and Miller 2005, Sim *et al.* 2005). However, the relationship between the heat shock stress response and viral infection replication is not well understood. The heat shock stress response is associated with the replication of numerous viruses including adenoviruses, alphaviruses and flaviviruses (Glotzer *et al.* 2000, Reyes-Del

Valle *et al.* 2005, Sim *et al.* 2007). Some studies indicate a positive correlation between increase heat shock gene expression and viral replication, some suggest a negative relationship, and still others offer evidence to support both positive and negative effects (Kampmueller and Miller 2005, Sim *et al.* 2007, Weeks *et al.* 2010).

In the yeast model, deletion of certain members of the hsp70 family resulted in increased flock house virus RNA accumulation, while deletion of others had a suppressive effect (Weeks *et al.* 2010). Additionally, the hsp70 cochaperone *YDJ1* of yeast is found to facilitate the accumulation of flock house virus RNA polymerase (Weeks and Miller 2008). Hsp70 proteins are also associated with tombusvirus replicase and are found to enhance viral replication in yeast (Serva and Nagy 2006).

Other heat shock proteins, including hsp90 are involved in virus-induced stress response. Both hsp90 and hsp70 are components human cellular receptor complexes of dengue viruses (Reyes-Del Valle *et al.* 2005). In addition to acting as molecular chaperones, these proteins are found on the surface of human cells. Cells treated with hsp90 and hsp70 inhibitors are refractory to dengue virus type 2 entry (Reyes-Del Valle *et al.* 2005). Hsp90 has also been identified as a member of the flock house virus replication complex in *Drosophila* S2 cells and is thought to be necessary for assembly of the complex (Kampmueller and Miller 2005).

Heat shock cognate proteins, which are constitutively expressed, are also associated with modulating viral infection. Heat shock cognate 70 (Hsc70) was found to impede o'nyong-nyong virus (ONNV) replication (Sim *et al.* 2007). Sim *et al.* proposed that *hsc70B* regulates ONNV titers to prevent harmful effects in *Anopheles gambiae*. *A. gambiae hsc70B* expression is also induced by West Nile and La Crosse viruses (Kang *et al.* 2008).

Temperature and insecticide stress may make *Aedes aegypti* more susceptible to viral infection (Mourya *et al.* 2004, Yadav *et al.* 2005). *Aedes aegypti* adults reared from larvae that had been heat shocked for 10 minutes at either 42°C or 44.5°C were found to be more susceptible to chikungunya virus (Mourya *et al.* 2004). Larvae reared at elevated temperatures ranging from 42°C to 46°C were also found to survive an average of 5-6 days longer as adults than larvae reared at 28°C (Mourya *et al.* 2004). The authors hypothesized that increased viral susceptibility in addition to increased life span would increase the vectorial capacity of *Aedes aegypti* exposed to increased temperatures as larvae. Further studies indicated that *Aedes aegypti* mosquitoes subjected to heat shock were also more susceptible to infection with dengue 2 virus (Yadav *et al.* 2005). However, mosquitoes subjected to both DDT exposure and heat shock were found to be less susceptible to viral infection (Yadav *et al.* 2005). There is little other research available on the subject of heat shock and virus susceptibility in *Aedes aegypti* and further study may elucidate precise interactions between heat shock and susceptibility to viral infection. Vectorial capacity is a complicated interplay of vector, pathogen, and environmental factors requiring a good understanding of each factor to be able to explain arboviral epidemiology.

The following study describes the sequence and expression pattern of *Aedes aegypti* heat shock 70 genes. In addition to establishing the expression profile of native *AaHsp70* genes, promoter constructs were designed based on regions upstream of the native heat shock genes. These promoters were characterized in cells, embryos, and transgenic individuals to demonstrate that *AaHsp70* promoter elements drive heat inducible expression. The interaction of heat stress and the RNA interference pathway was also investigated.

Chapter 2

Identification and characterization of heat shock 70 genes in the yellow fever mosquito, *Aedes aegypti*

Tiffany L. Gross, Kevin M. Myles, and Zach N. Adelman

*as published in the Journal of Medical Entomology, May 2009

ABSTRACT

Heat shock genes are highly evolutionarily conserved and are expressed to varying degrees in all organisms in response to stress. Heat shock 70 genes (*hsp70*) have been well characterized in a number of organisms, most notably *Drosophila melanogaster*, but not as yet for any of the major arboviral mosquito vectors. In order to identify *hsp70* genes in the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae), basic local alignment searches of the *Ae. aegypti* genome were performed using *Drosophila melanogaster* Hsp70 protein sequences as query. Two clusters of six previously unannotated *AaHsp70* genes were identified and found to be organized into three pairs of nearly identical open reading frames which mapped to two genomic scaffolds. Consistent with a designation as heat shock genes, no detectable level of expression of *AaHsp70* genes was observed under normal rearing conditions (28°C), with robust expression observed with a heat shock of 37°C -39°C. Northern analysis revealed heat-inducible expression of putative *AaHsp70* genes at all life stages and in all tissues tested in a time and temperature dependent manner. Monitoring of *AaHsp70* gene expression levels in field caught *Ae. aegypti* may serve as a general marker for stress. In addition, promoter sequences from *AaHsp70* genes may be used to control the expression of transgenes in an inducible manner.

INTRODUCTION

Aedes aegypti is a significant vector of disease agents, capable of transmitting the viruses that cause yellow fever, dengue fever, and the more severe dengue hemorrhagic fever (Tatem *et al.* 2006). According to the World Health Organization (2008), 2.5 billion people are at risk from dengue with 50 million dengue infections worldwide each year. In 2007 in the Americas alone, there were 890,000 dengue infections, 26,000 of which were dengue hemorrhagic fever cases (WHO 2008).

Ae. aegypti is one of the most highly studied arthropods, not only for its importance as a vector of disease, but also because of its ease to rear in the laboratory (Severson *et al.* 2004b). With sequence data available for both *Ae. aegypti* and *Anopheles gambiae*, comparative genomics is an important tool for understanding vector biology. Comparative analysis with *Drosophila melanogaster* will also lead to better understanding of each of the mosquito genomes (Waterhouse *et al.* 2008).

Heat shock protein 70 (*Hsp70*) genes are members of a highly conserved gene family, with proteins related to *D. melanogaster hsp70* found in prokaryotes as well as higher and lower eukaryotes (Craig 1985, Mukhopadhyay *et al.* 2003). The chaperone proteins encoded by these genes are responsible for preventing the cytotoxic aggregation of denatured proteins, as well as for the re-folding of denatured proteins (Bettencourt and Feder 2002). Expression of *hsp70* genes is induced by various stresses including heat, anoxia, and exposure to ethanol and chemical treatments including sodium arsenite, cadmium chloride, and sodium salicylate (Craig 1985, Tanguay 1988, Li 1982, Liu 1994). *D. melanogaster hsp70* genes are well characterized and serve as a reference for comparative genomics studies of *hsp70* genes of other organisms (Severson *et al.* 2004b, Waterhouse *et al.* 2008).

DmHsp70 promoter sequences are commonly used to drive the expression of exogenous gene products, to induce transposition events, and also for gene function studies in *D. melanogaster* (Golic and Lindquist 1989, Wimmer 2003) and heterologous systems such as *B. mori* (Uhlirova *et al.* 2002, Dai *et al.* 2007). The *DmHsp70* promoter has also been shown to drive transgene expression in mosquito cultured cells (Zhao and Eggleston 1999). However, for some applications an endogenously-derived promoter may prove to be more useful than a heterologous promoter, particularly with respect to genetically modified mosquito strategies where limiting the amount of non-native DNA is critical.

Ae. aegypti is the best characterized species within the subfamily Culicinae (Nene *et al.* 2007). A joint effort between the BROAD institute and The Institute for Genomic Research (TIGR) resulted in 8x coverage of the approximately 1.3 Gb Liverpool genome (Lobo *et al.* 2007, Nene *et al.* 2007). Prior to the completion of the *Ae. aegypti* genome sequencing project, a putative *AaHsp70* gene was used by Fulton *et al.* (Fulton *et al.* 2001) as a marker for SSCP analysis based on an unpublished submission to GenBank. Isoe *et al.* (2007) also identified a putative *AaHsp70* gene. This gene, however, was found not to be heat inducible (Isoe *et al.* 2007), suggesting that it may be a heat shock cognate instead. Heat shock cognates are closely related to heat shock proteins, but are constitutively expressed and are not part of the stress response pathway. Lastly, a gene identified as *hsp70* was annotated on genomic scaffold 1.116 and mapped to the p-arm of chromosome three (Nene *et al.* 2007). However, uncertainty remains as to the nature and accuracy of these preliminary annotations. In this paper, we definitively identify and describe the expression pattern of novel *hsp70* genes from *Ae. aegypti*. We also present evidence that all three previous reports of *AaHsp70* genes were actually descriptions of a heat shock cognate gene, orthologous to *D. melanogaster Hsc70-4*.

MATERIALS AND METHODS

Mosquito strains and rearing

Aedes aegypti (Liverpool strain) mosquitoes were maintained at 28°C and 80% humidity with a photoperiod of 16 h of light and 8 h of darkness. Larvae were fed pulverized fish food and reared approximately 300 per pan in 4 L of reverse osmosis (RO) purified water until pupation. Adult mosquitoes were maintained on sucrose and were blood fed using artificial membrane feeders and defibrinated sheep blood (Colorado Serum Company).

Amplification and cloning of AaHsp70 genes

AaHsp70 genes were amplified from Liverpool strain genomic DNA using Platinum *Pfx* PCR (Invitrogen, Carlsbad, CA) [2X *Pfx* amplification buffer, 0.3 mM dNTPs, 1 mM $MgSO_4$, 0.3 μM primers, 1X enhancer solution, 1 unit Platinum *Pfx* DNA polymerase]. Genomic DNA was digested with either *Sac* II or *Bgl* II in order to separate inverted *hsp70* gene pairs. All attempts to amplify *hsp70* genes from undigested mosquito DNA were unsuccessful, presumably because the nearly identical open reading frames and 5' regions would be expected to form hairpin structures during the amplification process. Gene regions for putative *AaHsp70Ba*, -*Bb*, -*Ca* and -*Cb* were amplified using *Sac* II digested genomic DNA from male *Ae. aegypti* (94°C, 5 min.; 94°C, 30 sec., 60°C, 1 min., 68°C, 3 min., 35 cycles; 68°C, 10 min.) and putative *AaHsp70Aa* and -*Ab* were amplified using *Bgl* II digested genomic DNA from male *Ae. aegypti*. Amplified *AaHsp70* genes were cloned using *Kpn*I restriction sites added to the primers used for the original amplification. Primers used to amplify *AaHsp70* gene regions are listed in Table 2.1.

Table 2.1. Oligonucelotide primers used to amplify and clone *AaHsp70* paralogs.

Primers[a]	Sequence (5' to 3')	Expected Length (bp)
Aa_F	ATCAAGATTGTCGACCAACTAGAAGGACC	2398
Aa_R	TTTCTACTATATAAGCGCCCGGTTCG	
Ab_F	TCGTTTCCACTATATAAGCGCCCAGC	2346
Ab_R	CTGAGTTCGGTATGTTTGAGAATGAGAATG	
Ba_F	CGCGTCTTCCAACATTCCTTACTGAACCTA	2374
Ba_R	CCACTCACACAAAGCAATGAAATATGCGAC	
Bb_F	CGAATCACAAGCAAGAGCAATAAAGCGCC	2463
Bb_R	GGCAATTTCGTAGCTCTATTGACATGTCCAG	
Ca_F	GGTAGCAGTCGAGTAAGCCAAGACAACGAA	2334
Ca_R	AACAAGCTCAACATAATAGGAACATTAACAAACTTCC	
Cb_F	CCTGGCGTTTATATATAGGACCGATTCGAGC	2374
Cb_R	AAGCATTAGCTTGAGTCGTCAATCTTTAAGATTACTG	

[a] cloning primers contained the sequence *ttttggtacc* at the 5' end of each of the above oligonucleotides to introduce *Kpn*I restriction sites.

Plasmids containing open reading frames from each gene were sequenced using ABI BigDye Terminators v 3.0 (Foster City, CA).

Mosquito heat-shock regimens

Groups of adult female *Ae. aegypti* mosquitoes (n= 35-40) were heat shocked in a pre-warmed oven approximately 3-5 days post eclosion. Each heat shock lasted one hour at a predetermined temperature ranging from 35°C to 41°C in 2 degree increments with approximately 80% humidity (35°C ± 0.3, 37°C ± 0.6, 39°C ± 0.8, 41°C ± 0.4). Mosquitoes were permitted to rest at 28°C for varying amounts of time ranging from 30 minutes to 24 hours following the heat shock regimen. Once the predetermined resting period was over, mosquitoes were harvested into microcentrifuge tubes and snap frozen in liquid nitrogen to preserve RNA. Third and fourth instar larvae and pupae were heat shocked using a 37°C water bath for one hour and were permitted to rest for 30 minutes before being snap frozen. Groups of adult males were heat shocked for one hour at 39°C and allowed to rest for 30 minutes before being snap frozen. The temperature during heat shock was monitored and verified using a HOBO data logger (Onset Computer Corporation, Bourne, MA).

Northern analysis and RACE

Total RNA (5 μg) from each experimental group was electrophoresed in a 1.2 percent agarose, 1X MOPS (0.023 M MOPS, 0.3 mM NaOAc, 0.2 mM EDTA), 2% formaldehyde gel at 90 V. RNA was blotted onto a positively charged nylon membrane (Immobilon-NY^+, Milllipore, Concord, MA). Blots were prehybridized at 65°C in pre-warmed Church's buffer (0.25 M sodium phosphate buffer, 1mM EDTA, 7% SDS) using a Fisher Scientific Isotemp hybridization oven. Random primed probes were labeled with [α-^{32}P]dATP, specific activity 3000 Ci/mmol using the Amersham Megaprime DNA Labeling System (GE Healthcare, Buckinghamshire,

UK). The specific activity of the probes was determined using a Beckman-Coulter LS6500 Multi-purpose Scintillation Counter. Probes were purified using illustra NICK columns (GE Healthcare) and added to pre-warmed Church's buffer to hybridize overnight at 65°C. Blots were washed twice with 2X SSC, 0.1% SDS for 20 minutes each at 65°C and twice with 0.2X SSC (0.03 M sodium chloride, 0.003 M sodium citrate), 0.1% SDS for 20 minutes each at 65°C.

DNA templates to be radiolabeled as probes for Northern analysis were generated from putative 3' untranslated regions (UTR) of *AaHsp70* gene paralogs, and from sequence common to all 12 putative *AaHsp70* genes. The common probe was amplified using One Step RT-PCR (50°C, 30 min., 95°C, 15 min.; 94°C, 30 sec, 60°C, 30 sec., 72°C, 1 min., 35 cycles; 72°C, 10 min.) using primers (5'-TTGGTTGATGTGGCTCCACTCTCATTGG-3' and 5'-TTGTGCTCGAACTCGTCCTTCTCG-3') and optional Q solution (Qiagen, Valencia, CA). To generate DNA fragments specific for pairs of *AaHsp70* paralogs, a portion of each putative 3' UTR was amplified using One Step RT-PCR and the following primers *AaHsp70Aa* (5'-AAGTTGACTAAATTGAGTTGAGATACGAGACTGAATGAG-3' and 5'-TTGTTATAGTTTATTTTCGTGAAAACATTCTACTTATGATTAC-3'), *AaHsp70Ab* (5'-AGGAGAAGTGAATGAGACTGAATGTTTTAGTAGAG-3'and 5'-AACCTTATTCTCTAAGGCTTATGTCAGCAATTCC-3), *AaHsp70Ba* (5'-AATTGGGTTGACAAACGAGACTGAATGAG-3' and 5'-TACAACGATAATAATTGCAAATACGATCTACGAATCC-3'), *AaHsp70Bb* (5'-TTGAGGAAGTCGACTAAAGCGAATGGAGAGG-3' and 5'-TTCGTAAAAACAAGCTGTACACATTAAATAACTTTCTAAC-3'), *AaHsp70Ca* (5'-TTGAGGAAGTCGACTAAAGTGAATGGAGCG-3'and 5'-AACAAGCTCAACATAATAGGAACATTAACAAACTTCC-3'), and *AaHsp70Cb* (5'-

TTGAGGAAGTGGACTAAGTATATCAAGGCATTTAAACCC-3' and 5'-AAGCATTAGCTTGAGTCGTCAATCTTTAAGATTACTG-3'). Gene fragments were cloned using the same oligonucleotide primers with added *Kpn* I recognition sites in the same manner as the open reading frames. In order to generate cDNA for use in RACE reactions the Promega PolyATtract mRNA Isolation System was used to purify total RNA extracted from 60 female Liverpool *A. aegypti*. To perform 5' and 3' RACE, the Clontech (Mountain View, CA) SMART RACE amplification kit was used with gene specific primers: for 5' RACE (5'-CGATTTCCTTGGTCGTTGGCGATGATTTCC-3', 5'-CGATTTCCCTGGTCGTTGGCGATGATTTCC-3', 5'-CGATTTCCCTGGTCGTTGGCAATGATTTCC-3'); and for 3' RACE *AaHsp70Aa* (5'-TGCGGTACAAGCTGCCATCCTCAGTGGAGAC-3'), *AaHsp70Ab* (5'-CGATCATGACTCGATTGCATCAGGGTGG-3'), *AaHsp70Ba* (5'-ACGAAAAGCAACGCGAACGTGTCTCTGCC-3'), *AaHsp70Bb* (5'-CGATCATGACTCGTTTGCATCAAGGTGGAG-3'), *AaHsp70Ca* (5'-TTCGAGCACAAGATGCAAGAGCTGAGTCG-3'), and *AaHsp70Cb* (5'-ATCAGCTGGCCAGCAAGGAGGAAATGGACC-3').

Phylogenetic Analysis

AaHsp70 gene sequences have been deposited in Genbank and are available using accession numbers FJ177309-14 and are listed in Table 2.2. Other sequences used for phylogenetic analysis include *Anopheles gambiae Hsp70 genes* AGAP004582 and AGAP012891; as well as *AgHsc70B*, ENSANGP00000017398; and *D. melanogaster genes*: *Hsp70Aa*, CG31366; *Hsp70Ab*, CG18743; *Hsp70Ba*, CG31449; *Hsp70Bb*, CG31359; *Hsp70Bbb*, CG5834; *Hsp70Bc*, CG6489; *Hsc70-4,*CG4264 (Sim *et al.* 2007). ClustalW

parameters used to align Hsp70 protein sequences were: pairwise gap opening penalty of 10, pairwise gap extension penalty of 0.1, multiple gap opening penalty of 10, multiple gap extension penalty of 0.2 with negative matrix. Linearized neighbor-joining tree was produced using MEGA 4.1 (Tamura 2007).

RESULTS

AaHsp70 Gene Organization

In order to identify *hsp70* genes in *Aedes aegypti*, we performed tBLASTn searches of the *Ae. aegypti* genomic scaffolds using the *D. melanogaster Hsp70Aa* protein sequence. Eighteen putative genes were identified with an expected cutoff value of 0 (Table 2.2). The predicted protein sequences of 17 of the 18 genes (one sequence contained major gaps which prevented in silico translation) were used to back-query the *D. melanogaster* gene set, and the best match for each is reported in Table 2.2. Of the 18 genes, 12 were a best match for *D. melanogaster hsp70*. Six of the putative *AaHsp70* genes mapped to genomic supercontig 1.680 (Table 2.2, Fig 2.1), located on chromosome 1 (Nene *et al.* 2007), with a second cluster of six putative *AaHsp70* genes on the unmapped supercontig 1.824 (Table 2.2, Fig. 2.1).

Putative *AaHsp70* protein sequences were compared to *Anopheles gambiae* (*AgHsp70*) and *Drosophila melanogaster* (*DmHsp70*) genes and cognates using ClustalW and a neighbor-joining tree was produced (Fig. 2.2). The putative *AaHsp70* genes formed an independent set of clades from *An. gambiae* and *D. melanogaster hsp70* genes, consistent with the notion that heat shock 70 genes have undergone several independent duplication events in each of these organisms. In order to determine whether the sequences previously annotated as *AaHsp70* (Fulton *et al.* 2001, Isoe *et al.* 2007) corresponded to the same gene sequences we describe here, or to a novel set of genes, we performed BLASTn searches using Genbank deposited nucleotide

Table 2.2. tBLASTn search with the *D. melanogaster Hsp70*Aa gene product yields 6-12 *Ae. aegypti* orthologs

	Supercontig		Predicted protein			
Gene	Number	(Position)	# AA	MW (kD)	Best Dm[a]	Accession #
AaHsp70Aa	1.680	(378927-380840)	638	70.3	*Hsp70Bb*	FJ177309
AaHsp70Ab	1.680	(383454-385367)	638	70.3	*Hsp70Bc*	FJ177310
AaHsp70Ba	1.680	(402097-404010)	638	70.3	*Hsp70Bc*	FJ177311
AaHsp70Bb	1.680	(406760-408673)	638	70.0	*Hsp70B*	FJ177312
AaHsp70Ca	1.680	(416064-417977)	637	70.0	*Hsp70B*	FJ177313
AaHsp70Cb	1.680	(419055-420962)	636	70.0	*Hsp70B*	FJ177314
AaHsp70Aa'	1.824	(45244–47157)	638	70.2	*Hsp70B*	n/a
AaHsp70Ab'	1.824	(49384–51297)	638	70.3	*Hsp70B*	n/a
AaHsp70Ba'[b]	1.824	(61070–63023)	n/a	n/a	n/a	n/a
AaHsp70Bb'	1.824	(66155–68064)	638[c]	70.4	*Hsp70Bc*	n/a
AaHsp70Ca'	1.824	(86660–88569)	638	70.1	*Hsp70Bc*	n/a
AaHsp70Cb'	1.824	(89650–91384)	636	70.1	*Hsp70Bc/Bb*	n/a
AaHsc70-2	1.389	(987893-989673)	587	64.4	*Hsc70*-2	n/a
AaHsc70-4	1.28	(547680-549359)	593	65.2	*Hsc70*-4	n/a
AaHsc70-4	1.116	(1035564-1037399)	651	71.1	*Hsc70*-4	n/a
AaHsp68	1.752	(334685-336619)	624	68.8	*Hsp68*	n/a
AaHsc-3a	1.191	(572103-574019)	655	72.3	*Hsc70*-3	n/a
AaHsc-3b	1.191	(574474-576459)	662	73.4	*Hsc70*-3	n/a

Amino acid (AA), Molecular weight (MW), not available (n/a). [a] Best match in *D. melanogaster* after BLASTp search of Flybase (FB2008_10). [b] not available due to major gaps in the sequence assembly. [c] two distinct mutations (nonsense and 1 bp deletion) interrupt the coding sequence of this gene, the data are presented as if the ORF were intact.

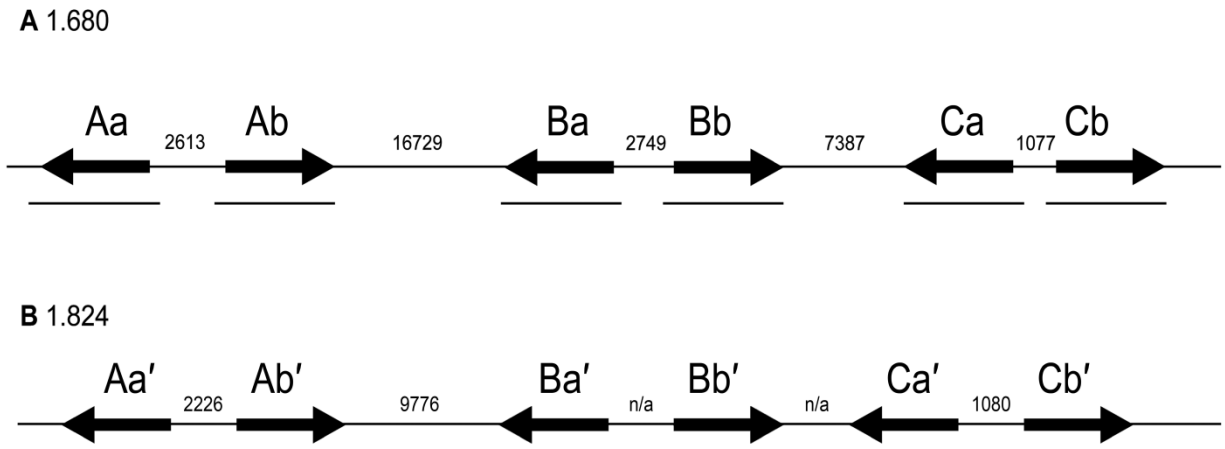


Figure 2.1 Genome organization and relationship of *AaHsp70* genes. Arrangement of *AaHsp70* genes in *Ae. aegypti*. *AaHsp70* genes are indicated by solid arrows. Lines under each gene indicate areas re-amplified, cloned, and sequenced in this project. Distances between genes are labeled in base pairs.

sequences as a query against the *Ae. aegypti* genomic supercontigs (Nene *et al.* 2007). Both DQ453756 (Isoe *et al.* 2007) and AI658418 (Fulton *et al.* 2001) were found to map to genomic supercontig 1.116 (positions 1031943-1032601 and 1032458-1035831, respectively). An open reading frame was detected in this region and the predicted protein sequence was used as a query to search the *An. gambiae* and *D. melanogaster* genomes using tBLASTn. As shown in Table 2.2 and Fig. 2.2, the heat shock-like gene identified on supercontig 1.116 is most closely related to *AgHsc70B* and *DmHsc70-4,* suggesting that the most appropriate annotation for this gene is *AaHsc70-4*.

As the two clusters of six *AaHsp70* genes were near identical to each other, we focused the rest of our analysis on the cluster located on scaffold 1.680, where the sequence assembly was of higher quality with fewer gaps. In order to document the exact start and stop of transcription of *AaHsp70* transcripts, we performed rapid amplification of cDNA ends (RACE). Ninety-five percent of 5' RACE clones (35/37 clones) supported a consistent start of transcription, which was found to be 182 bases upstream of the start of translation for *AaHsp70Aa* and *AaHsp70Cb*, 181 bases upstream for *AaHsp70Ba*, *AaHsp70Bb* and *AaHsp70Ca*, and 180 bases upstream for *AaHsp70Ab*. Multiple 3' RACE products were sequenced for each *AaHsp70* paralog, with the longest, most abundant clone assumed to represent the end of transcription. The 3' untranslated regions were found to be far more variable in length than the 5' region, with *AaHsp70Ab* (14/21 clones) being the shortest (135 nt 3'UTR), followed by *AaHsp70Ca* (4/7 clones, 145 nt), *AaHsp70Aa* (5/8 clones, 150 nt), *AaHsp70Bb* (6/11 clones, 150 nt), *AaHsp70Ba* (3/4 clones, 208 nt), and *AaHsp70Cb* (9/9 clones, 282 nt). Each of the six putative open reading frames were nearly identical in length, with *AaHsp70Aa*, *-Ab*, *-Ba*, and *-Bb* at 1914 nt; *AaHsp70Ca* at 1911 nt; and *AaHsp70Cb* at 1908 nt. In order to better resolve the

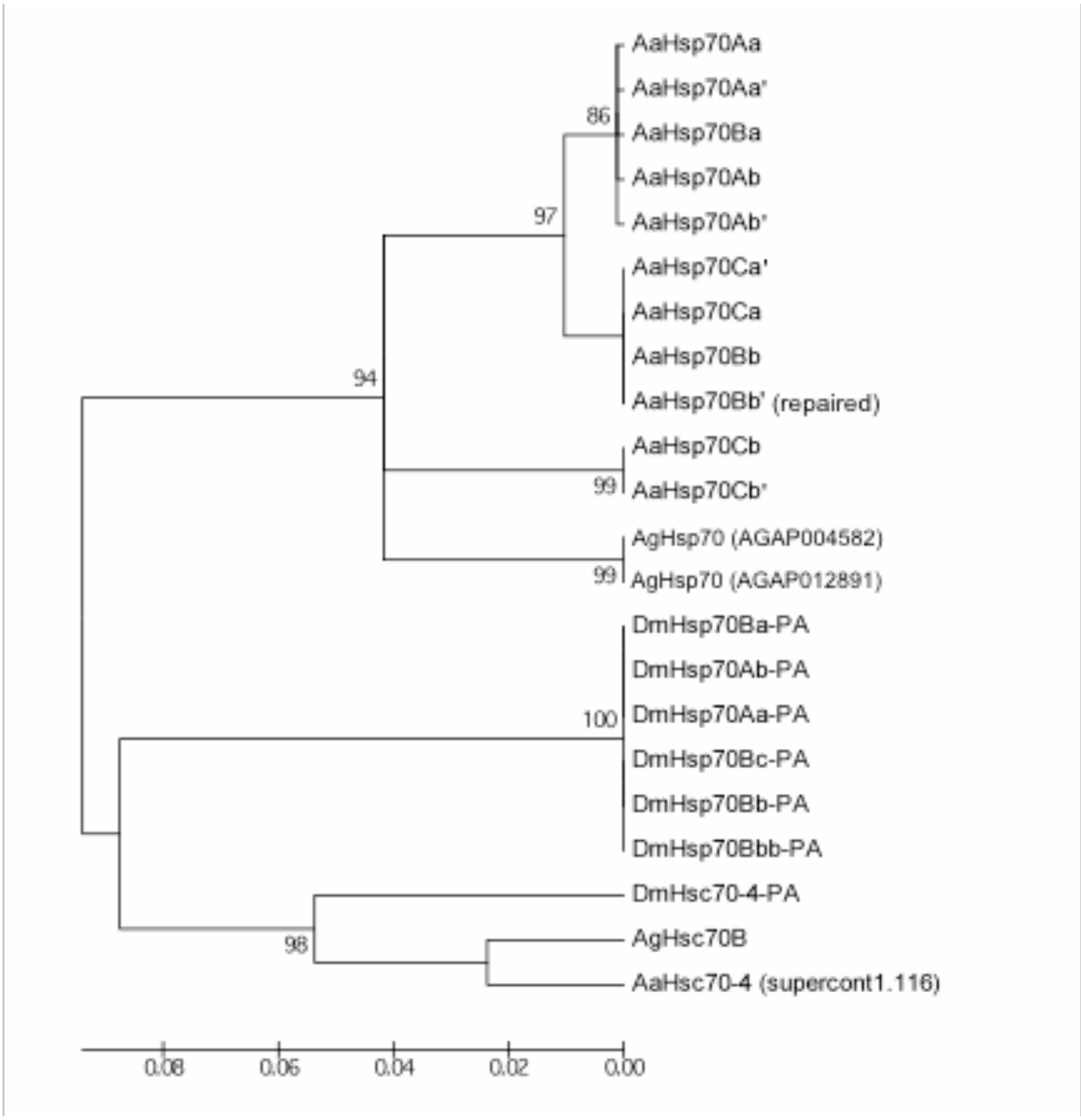


Figure 2.2 Alignment *of Ae. aegypti, An. gambiae and D. melanogaster hsp70* predicted proteins. Gene sequences listed in Materials and Methods were aligned using MEGA 4.1 and bootstrap analysis was performed using the neighbor-joining method with 1000 replications. Bootstrap support (>80) is listed on the node of each branch, where applicable. AaHsp70Bb' (repaired) indicates the predicted protein after removing two deleterious frameshifts.

within-cluster phylogenetic relationship of *AaHsp70* genes, Geneious software (Biomatters, Aukland, New Zealand) was used to produce a ClustalW alignment of putative *AaHsp70* gene transcripts (Fig. 2.3). Open reading frames of *AaHsp70* genes were found to be 77-96% identical at the nucleotide level with *AaHsp70Cb* being the most divergent. The 5' untranslated regions of putative *AaHsp70* genes were found to share 88% nucleotide identity, while the 3' untranslated regions share only 38% identity. Pairwise comparisons between putative *AaHsp70* genes revealed that *AaHsp70Bb* and *AaHsp70Ca* were most similar, with greater than 95% identity at the nucleotide level (Table 2.3). No other strong pairwise relationship was evident, as *AaHsp70Aa*, *AaHsp70Ab* and *AaHsp70Ba* were essentially equally similar to each other (93.5%), while *AaHsp70Cb* was equivalently divergent from the other five genes (Table 2.3). This is in agreement with the protein alignment presented in Fig. 2.1, where three *AaHsp70* clades were also identified (Aa, Ab and Ba; Bb and Ca; and Cb).

Expression of AaHsp70 genes

In order to confirm our bioinformatic annotation of this cluster as true heat shock genes, we next sought to determine whether mRNA expression could be induced through exposure to heat shock. Female *Ae. aegypti* were exposed to heat shock regimens with temperatures ranging from 35°C to 41°C in 2 degree increments for 1 hour. Total RNA was harvested at various times following exposure to heat shock and was subject to Northern analysis using a probe capable of hybridizing with putative *AaHsp70* genes, but not the closely related heat shock cognates (Fig. 2.4). Expression of *AaHsp70* mRNAs was induced to various extents in all heat shock regimens, while no expression was detected in control mosquitoes not subjected to heat shock (Fig. 2.4). The highest levels of expression were observed following exposure to 39°C, with only modest

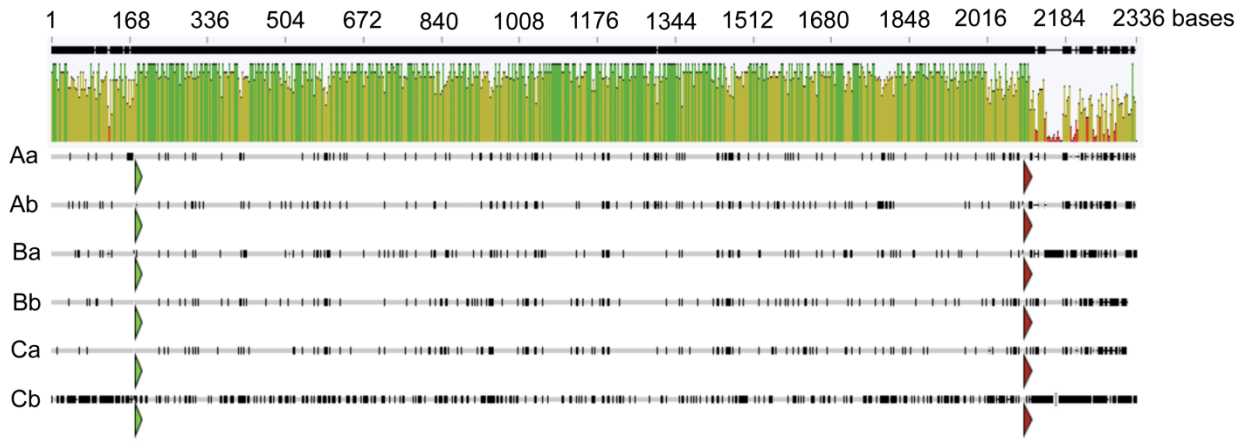


Figure 2.3 Sequence comparison of *AaHsp70* genes. Complete transcripts for six *AaHsp70* genes were aligned using ClustalW (Geneious,Biomatters, Aukland, New Zealand) to produce an identity graph. The start of each open reading frame is marked with a green arrow, and the end with a red arrow. Dark marks on lines representing each *AaHsp70* gene indicate base changes from the consensus *AaHsp70* sequence.

Table 2.3. Nucleotide identity matrix of *AaHsp70* open reading frames on supercontig 1.680

	Aa	*Ab*	*Ba*	*Bb*	*Ca*	*Cb*
AaHsp70Aa	1.000	0.937	0.935	0.910	0.914	0.774
AaHsp70Ab		1.000	0.935	0.899	0.904	0.775
AaHsp70Ba			1.000	0.908	0.909	0.783
AaHsp70Bb				1.000	0.956	0.782
AaHsp70Ca					1.000	0.781
AaHsp70Cb						1.000

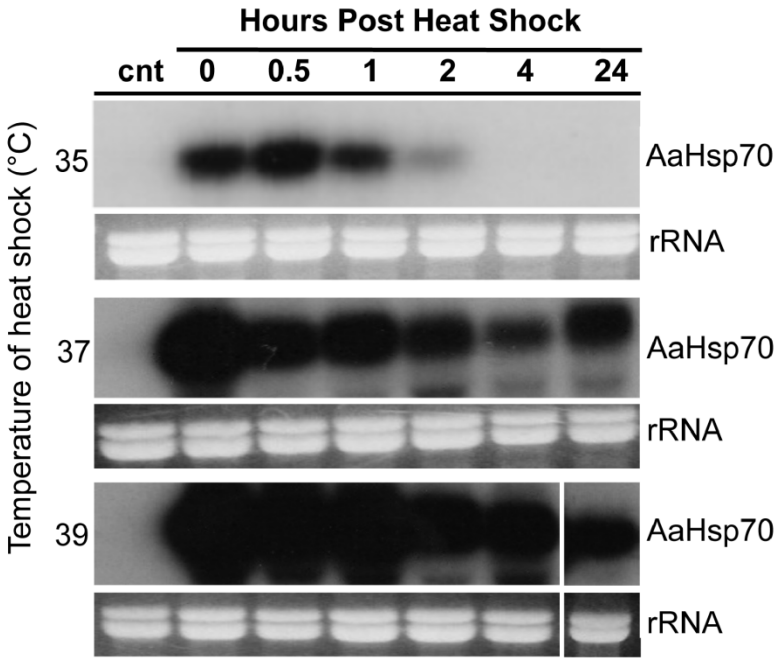


Figure 2.4 Expression levels of *AaHsp70* genes increase with heat shock temperature. Female *Ae. aegypti* were heat shocked at temperatures from 35-39ºC and total RNA was hybridized to a ^{32}P-labeled probe common to all *AaHsp70* genes. All blots were hybridized simultaneously to ensure equivalent exposure and probe normalization (specific activity = 5.4×10^7 CPM). Control mosquitoes (cnt) were not heat shocked. Ethidium bromide-stained rRNA loading controls are shown below each blot.

induction occurring at 35°C and heat shock transcripts detected only up to 2 hours after heat shock. After heat shock at 37°C, *AaHsp70* transcripts were detected up to 24 hours, though expression did not match that seen at 39°C. *AaHsp70* expression at 41°C could not be documented because all mosquitoes died during the 1 hour heat shock regimen.

Multiple copies of genes with the same function can indicate inactive evolutionary remnants (Ingolia *et al.* 1980). Thus it was possible that not all of the *AaHsp70* genes we identified were still active, or responded to heat shock identically. Indeed, at least one of the *AaHsp70* genes (*Bb'*) contained deleterious frameshifts. In order to document the expression levels of each putative *AaHsp70* gene, we repeated our heat shock regimen with female *Ae. aegypti* heat shocked at 39°C for 1 hour. Following a rest period of 0-4 hours, total RNA was extracted and Northern analysis was performed using six paralog-specific probes derived from the unique 3'UTR of each gene. Of the six putative *AaHsp70* genes we examined, at least five showed detectable expression by this method, with only *AaHsp70Ab* failing to produce a detectable hybridization signal (Fig. 2.5). The highest levels of expression were observed for *AaHsp70Aa*, *AaHsp70Ba*, and *AaHsp70Ca* transcripts, with weak expression seen for *AaHsp70Cb* (Fig. 2.5).

In *D. melanogaster*, *hsp70* genes are expressed ubiquitously throughout the body of the fly following heat shock. In order to document any tissue specificity of putative *AaHsp70* genes, RNA from heat shocked *Ae. aegypti* heads, salivary glands, thoraxes lacking salivary glands, ovaries, midguts, as well as from heat shocked larvae, pupae, and males was subject to Northern analysis using a probe capable of hybridizing with *AaHsp70* genes, but not the closely related cognates. As expected, robust expression was observed following heat shock in all tissues tested (Fig. 2.6A), and in all life stages and male mosquitoes (Fig. 2.6B).

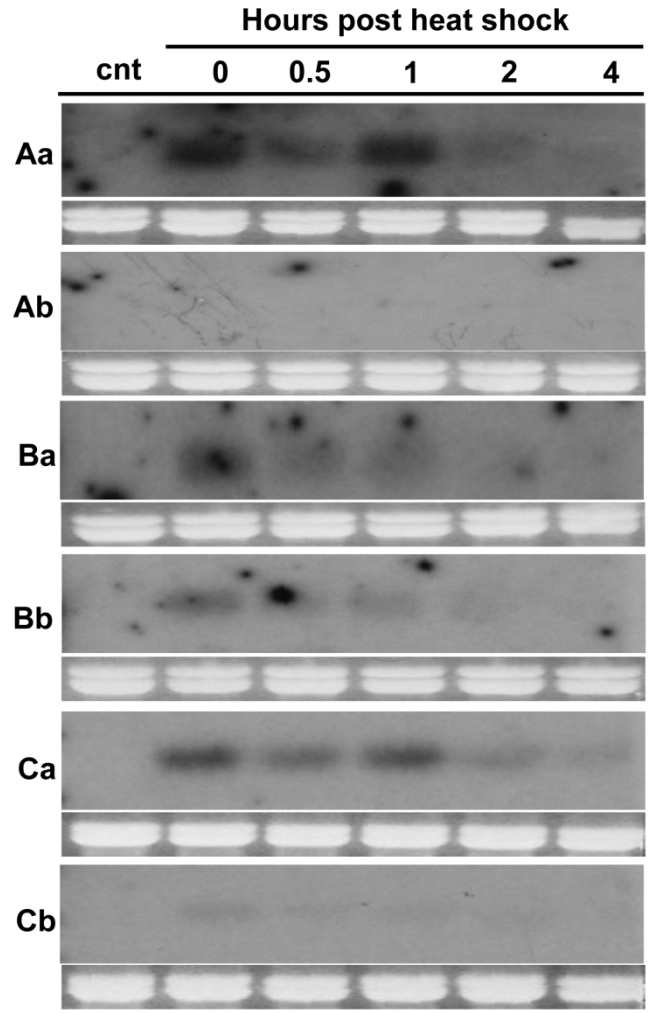


Figure 2.5 Northern analysis of individual *AaHsp70* paralogs. Northern analysis was performed using a ^{32}P-labeled probe derived from the 3' untranslated regions of *AaHsp70* paralogs. Exposure times were standardized based on specific activity of each probe ($7.3x10^7$-$3.0x10^8$CPM). Mosquitoes were heat shocked at 39ºC and control mosquitoes (cnt) were not heat shocked. Ethidium bromide-stained rRNA loading controls are shown below each blot.

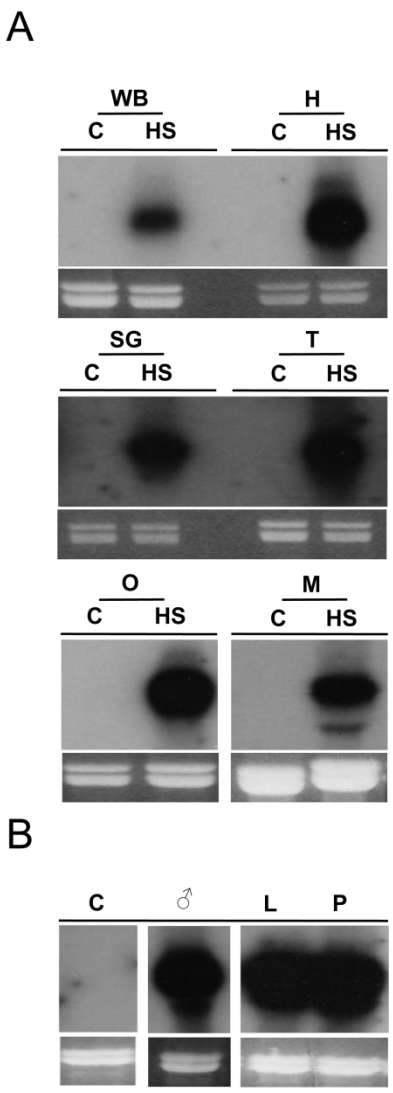


Figure 2.6 Tissue and life stage expression of *AaHsp70* genes. Expression in specific tissues (**A**) and life stages (**B**) was analyzed by Northern analysis using a ^{32}P-labeled probe designed to hybridize to all *AaHsp70* genes. Tissues were dissections from female *Ae. aegypti* that had been heat shocked at 39°C for 1 hour, or kept at 28°C as controls (C). Tissues include whole body (WB), head (H), salivary glands (SG), thorax without salivary glands (T), ovaries (O), and midguts (M). RNA from males (♂), 3rd and 4th instar larvae (L), and pupae (P) heat shocked at 39°C for 1 hour was used for the life stage study. Ethidium bromide-stained rRNA loading controls are shown below each blot.

DISCUSSION

The basic arrangement of *hsp70* genes in *Ae. aegypti* closely resembles that of *D. melanogaster* (Gong and Golic 2004) and *An. albimanus* (Benedict *et al.* 1993), with a pair of nearly identical open reading frames organized as an inverted pair. We have successfully identified 2 clusters of three such pairs in close proximity in the *Ae. aegypti* genome. While the presence of these two clusters of six *AaHsp70* genes would indicate that this mosquito has up to 12 *hsp70* paralogs, the fragmented nature of the genomic assembly does not allow us to rule out the possibility that these two clusters are allelic variants. Phylogenetic analysis of *AaHsp70* genes compared to *hsp70* genes from *D. melanogaster* and *An. gambiae* reveals a pattern of divergence in which both *Ae. aegypti* and *An. gambiae* genes diverged from *D. melanogaster* before duplication of the ancestral inverted pair within each species, as predicted by Benedict *et al.* (1993). *DmHsp70* genes are grouped at two loci on the right arm of chromosome 3 with the first inverted pair separated by approximately 1.7 Kb, followed by 3 or 4 genes at the second locus organized with one gene proximal to the centromere separated by approximately 40 Kb from 2 or 3 tandem genes of inverted polarity (Craig 1985, Gong and Golic 2004). It is believed that a duplication of the evolutionarily ancient, highly stable two gene cluster lead to the current organization of *DmHsp70* genes, with tandem duplications leading to the 5^{th} and 6^{th} genes allowing *D. melanogaster* greater thermotolerance and niche expansion (Bettencourt and Feder 2001). It is possible that multiple copies of *AaHsp70* may also contribute to the distribution and heat tolerance of *Ae. aegypti*. *DmHsp70* genes are separated by large amounts of repetitive DNA resulting from transposable S-elements, and these are thought to have played a role in the duplication of heat shock genes from 2 to 4 in Drosophila (Bettencourt and Feder 2001, Evgen'ev *et al.* 2004, Gong and Golic 2004). The *Ae. aegypti* genome is highly repetitive, with

approximately 70% of the genome being transposable elements/repetitive DNA (Waterhouse *et al.* 2008). At least two duplication events of the ancestral *hsp70* gene two gene pair appear to have occurred in *Ae. aegypti* after divergence from both *D. melanogaster* and *An. gambiae*, and these may have occurred by similar mechanisms and for similar reasons. Unlike Drosophila however, whose two pairs of heat shock genes are separated by over 500 kb (Evgen'ev *et al.* 2004), and *An. albimanus*, whose two pairs are separated by ~20 cM (Benedict *et al.* 1993), all three pairs of *AaHsp70* genes were spaced within a span of 42 kb. As the scaffold 1.824 is unmapped, the spacing between the two clusters of *AaHsp70* genes remains unknown. In addition, unlike *hsp70* genes described in *An. albimanus*, *Ae. aegypti hsp70* genes were not found to be more closely related within each inverted pair compared to between pairs (Benedict *et al.* 1993). It is possible that the differences we observe between the *AaHsp70* genes are the result of gene inactivation followed by drift rather than being a strict footprint of inheritance. Ultimately, we cannot draw firm conclusions at this point as to how the ancestral two-gene pair evolved into the six gene clusters we observed. Future studies of *hsp70* genes from other members of the genus *Aedes* would likely provide additional insight.

The putative *Ae. aegypti* heat shock cognate identified as *AaHsp70* by Isoe *et al.* (2007) was mapped to supercontig 1.116 on chromosome 3 (Nene *et al.* 2007). However, when we compared this sequence to the *D. melanogaster* genome, the best match is actually a heat shock cognate, a closely related gene that is constitutively expressed and not heat inducible. As shown in Figure 2.2, the cognate identified by Isoe *et al.* (2007) forms a separate clade with cognates from both *D. melanogaster* and *An. gambiae*, further supporting the idea that this gene may be a heat shock cognate. Gene annotations are often the result of predictions based on homology prior

to complete genome sequencing. However, examples such as this underscore the importance of confirming such predictions prior to experimentation.

In order to balance the benefits of *hsp70* gene expression under stress conditions with detrimental effects of expression under normal conditions, *DmHsp70* genes have been found to be self-regulating (Craig 1985, Lindquist 1986). *DmHsp70* genes are targeted for degradation by signals in the 3' untranslated regions, and under normal conditions *hsp70* transcripts are rapidly degraded (Petersen and Lindquist 1989, Feder *et al.* 1992). In *D. melanogaster* cultured cells, induced expression of *DmHsp70* genes under normal conditions was deleterious to cells and resulted in a decreased growth rate (Solomon *et al.* 1991, Feder *et al.* 1992). Extreme temperatures induce long-lasting synthesis and very stable *DmHsp70* mRNA, while at lower temperatures expression proceeds until heat shock proteins have accumulated to a level proportional to the severity of heat shock before further transcription is repressed (Lindquist 1986, Nover 1991). *AaHsp70* genes were found to exhibit a similar expression pattern, as under extreme heat shock conditions (39°C), high levels of expression are initiated rapidly and transcript was detected up to 24 hours after heat shock, while expression of *hsp70* transcripts was less robust and short-lived under a more modest heat shock of 35^{o}C (Fig. 2.4).

Induced mRNA expression levels were found to vary among the *AaHsp70* paralogs we examined. Interestingly, the first gene of each inverted pair seemed to be expressed preferentially over the second (see Fig. 2.1, 2.5), with *AaHsp70Aa* > *AaHsp70Ab*, *AaHsp70Ba* > *AaHsp70Bb* and *AaHsp70Ca* > *AaHsp70Cb*. What's more, this expression seemed to be proportional, so that the highest/lowest level of expression was observed from the *AaHsp70Aa/Ab* pair, the next highest/lowest expression from *AaHsp70Ca/Cb*, followed by *AaHsp70Ba/Bb* which had almost equivalent expression between the two genes. The reasons for this are currently unknown, but

may be related to the positioning of replication complexes during transcriptional pausing. In *D. melanogaster*, RNA polymerase II is constantly bound upstream of the start of transcription to maintain an open chromatin configuration (Karpov *et al.* 1984, Craig 1985) as heat shock genes must be available for near immediate action under stress conditions. This configuration is likely to occur in *Ae. aegypti* as well, and is consistent with the observation of high levels of *AaHsp70* expression detected immediately after 37°C and 39°C heat shocks. Though we could not detect *AaHsp70*Ab expression by Northern analysis, RACE transcripts were recovered for this gene, indicating some level of transcription.

AaHsp70 genes are expressed in all life stages and in all tissues tested in response to heat shock. Therefore, isolating heat inducible *AaHsp70* promoter elements would be valuable for transgenesis and gene function studies, particularly when it is important to minimize the presence of exogenous sequences. Further studies of *AaHsp70* regulatory regions would make such studies possible in *Ae. aegypti*. *Hsp70* gene expression also serves as a marker for stress and monitoring levels of *AaHsp70* gene expression in wild populations could serve as a physiological indicator at the population level.

Chapter 3

Characterization of endogenous Hsp70-based inducible promoters in *Aedes aegypti* cells and embryos

ABSTRACT

Although inducible promoters capable of controlling transgene expression in specific tissues have been characterized for *Aedes aegypti*, no whole body, whole life stage inducible promoter has been described for this mosquito. Previously I characterized the gene structure and expression of heat shock 70 genes in *Aedes aegypti* (Gross *et al.* 2009). Preliminary experiments using *hsp70*-derived genomic fragments to drive transgene expression demonstrated high levels of transcription under stress conditions, as well as repression under control conditions for constructs containing the entire intergenic region between the *AaHsp70Aa*/*AaHsp70Ab*, and *AaHsp70Ba*/*AaHsp70Bb* genes. In order to further define an optimal *Aedes aegypti* Hsp70 promoter, regions between *AaHsp70Aa*/*AaHsp70Ab* and *AaHsp70Ba*/*AaHsp70Bb* of varying length were cloned into luciferase reporter constructs. Constructs were assayed in heat shocked *Aedes aegypti* cells and embryos. Putative promoter constructs possessing the ideal balance of manageable construct size while retaining heat inducibility were identified. Isolating inducible *AaHsp70* promoter elements would be valuable for transgenesis and gene function studies, particularly when it is important to minimize the presence of exogenous sequences.

INTRODUCTION

Aedes aegypti is a significant vector of disease agents, capable of transmitting the viruses that cause yellow fever, dengue fever, and the more severe dengue hemorrhagic fever (Tatem *et al.* 2006). According to the WHO (2008), 2.5 billion people are at risk from dengue, with 50

million dengue infections worldwide each year. Current efforts to control mosquito populations including insecticides and habitat removal have not been not sufficient to reduce the spread of mosquito borne disease. As such, genetic control strategies are being developed to reduce disease transmission (Wise de Valdez *et al.* 2011).

Comparative genomics studies between *Drosophila melanogaster* and mosquitoes including *Aedes aegypti* and *Anopheles gambiae* have resulted in identification of useful endogenous promoter sequences in *Aedes aegypti*. For example, *nanos* transcripts, initially found to be responsible for embryogenesis and abdomen formation in *D. melanogaster* (Nusslein-Volhard et al. 1987, Lehmann and Nusslein-Volhard 1991), were found to accumulate in the posterior end of *Aedes aegypti* embryos and in oocytes (Calvo *et al.* 2005). The endogenous *nanos* promoter has since been used to drive transgene expression targeted to ovaries in *Aedes aegypti* (Adelman *et al.* 2007).

Inducible promoters based on Hsp70 genes have been well characterized in *Drosophila melanogaster*. Such promoters have been used to drive the expression of exogenous gene products, to induce transposition events, and also for gene function studies in *D. melanogaster* and other species (Golic and Lindquist 1989, Wimmer 2003). An ortholog of this gene has been characterized in *Anopheles albimanus* (Zhao and Eggleston 1999). However, the DmHsp70 promoter lacks heat inducibility and maintains high levels of basal activity in some species, including *Aedes aegypti* (Morris *et al.* 1991, Zhao and Eggleston 1999, Kalosaka *et al.* 2006). An inducible promoter such as the DmHsp70 promoter would be invaluable as a tool to further characterize *Aedes aegypti* genes in an effort to better understand vector competence.

Recently we characterized six members of the Hsp70 family in *Aedes aegypti* (Gross *et al.* 2009). In an effort to identify an endogenous inducible promoter with both high levels of

induced expression and low levels of basal expression, constructs containing varying amounts of sequence between *AaHsp70Aa/AaHsp70Ab* and *AaHsp70Ba/AaHsp70Bb* were designed. The objective of these studies was to determine whether regions upstream of AaHsp70 genes can act as inducible promoters in *Aedes aegypti* cells and embryos. AaHsp70 promoter constructs were found to drive heat inducible expression of a marker gene in both cells and embryos, with heat induction levels between 10 and 20 fold.

MATERIALS AND METHODS

Mosquito rearing

The Liverpool strain of *Aedes aegypti* was reared at 28°C and approximately 60% relative humidity with a 14:10 daylight cycle. Larvae were fed pulverized fish food and reared ≈ 400 per pan in 4 liters of reverse osmosis (RO) purified water until pupation. Adult mosquitoes were maintained on sucrose and were blood fed using artificial membrane feeders and defibrinated sheep blood (Colorado Serum Company, Denver, CO).

Cell culture and transfection

Cultures of C6/36 (*Aedes albopictus*) cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. Cells were maintained in 28°C and 5% CO_2. Twelve-well plates were seeded with C6/36 cells to be transfected at approximately 80% confluency with EGFP promoter plasmid constructs. Plasmids were prepared and transfected using the Qiagen Effectene Reagent protocol (Qiagen, Valencia, CA) with 0.5 μg of DNA and 5 μL Effectene. Transfected cells

were maintained at 28°C in CO_2 incubators. Approximately 17 hours after transfection, cells were heat shocked by placing them in a 37°C, 5% CO_2 incubator for one hour. After heat shock, cells were maintained at 28°C, 5% CO_2 for 24 hours. Photographs of cells were taken at 24 and 48 hours post heat shock using a Zeiss Axiovert 200 microscope with a Cannon Powershot A260 camera. Photographs were taken at both 10X and 20X magnifications. Cells were counted and averages calculated by counting cells in 20X photos with 3 photos for each construct tested.

Aedes aegypti Aag2 cells were maintained at 28°C and 5% CO_2 in Schneider's Drosophila medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Twelve-well plates were seeded with Aag2 cells to be transfected at approximately 80% confluency with luciferase promoter plasmid constructs. Experimental plasmids were transfected in a 1:1 ratio with a Renilla luciferase control plasmid driven by the Cytomegalovirus (CMV) enhancer and immediate/early promoter elements (Promega). A total of 0.5 µg of DNA was transfected with 12.5 µl of Effectene. After 24 hours, experimental cells were heat shocked at 37°C for one hour. All cells were harvested at 26 hours post transfection and luciferase assays were performed. Dual luciferase reporter assays (Promega) were performed on cell lysates after active cell lysis using rubber policemen according to the manufacturer's protocol for 12-well plates. Luciferase readings were measured using a Hidex Chameleon plate reader (Turku, Finland).

Embryo assays

Aedes aegypti embryos were collected from bloodfed females and injected 30 to 90 minutes after being laid. Injected embryos were then placed under normal rearing conditions. Injection mixes consisted of 300 ng/µl experimental firefly luciferase plasmid, 15 ng/µl Renilla

luciferase control plasmid, and 1X injection buffer (5 mM KCl, 0.1 mM NaH_2PO_4, pH 6.8) (Jasinskiene *et al.* 1998). Twenty-four hours after injection, experimental groups were heat shocked for one hour at 39°C. Embryos were harvested at 26 hours post injection and snap frozen in liquid nitrogen, then crushed in a total of 100 µl of passive lysis buffer (Promega). Dual luciferase assays were performed on embryo lysates according to the Promega protocol for cell lysates.

Identification of putative transcription factor binding sites

Putative transcription factor binding sites were predicted using the TransFac function TFSearch (Heinemeyer *et al.* 1998). The regions between *AaHsp70Aa/AaHsp70Ab* and *AaHsp70Ba/AaHsp70Bb* gene sets were used as query using the arthropod classification with the default threshold level of 85 percent identity.

Construction of short putative promoter reporter plasmids

AaHsp70 putative promoter regions were amplified from *Aedes aegypti* Liverpool genomic DNA using Platinum *Pfx* polymerase chain reaction (PCR; Invitrogen, Carlsbad, CA) (2X *Pfx* amplification buffer, 0.3 mM dNTPs, 1 mM $MgSO_4$, 0.3 µM primers, 1 U Platinum *Pfx* DNA polymerase). Genomic DNA was digested with either *Sac*II or *Bgl*II to separate inverted *hsp70* gene pairs. Putative promoter regions from *AaHsp70Ba,* and -*Ca* were amplified using *Sac*II digested DNA from male *Aedes aegypti* (94°C, 5 min; 94°C, 15 sec, 56°C, 30 sec, 68°C, 1 min, 10 cycles; 94°C, 15 sec, 66°C, 30 sec, 68°C, 1 min, 25 cycles; 68°C, 10 min). The putative promoter region from *AaHsp70Aa* was amplified from genomic DNA digested with *Bgl*II (94°C, 5 min; 94°C, 15 sec, 59°C, 30 sec, 68°C, 1 min, 10 cycles; 94°C, 15 sec, 65°C, 30 sec, 68°C, 1

min, 25 cycles; 68°C, 10 min). PCR products were visualized on an agarose gel, and bands were excised and purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Oligonucleotides used to amplify putative promoter fragments are listed in table 3.1. Both *AaHsp70Aa* and -*Ca* were re-amplified from the purified PCR product to increase yield (94°C, 5 min; 94°C, 15 sec, 65°C, 30 sec, 68°C, 1 min, 35 cycles; 68°C 10 min). Putative promoter regions for *AaHsp70Ma* (partial) and -*Mb* were synthesized and cloned into reporter vectors. Putative promoter regions were cloned into a green fluorescent protein expressing reporter vector and pGL3Basic (Promega, Fitchburg, WI) using *Mlu*I and *Nco*I. *AaHsp70Ba* was cloned in two pieces, moving the amplified portion first into the vector using *Mlu*I and *Nco*I, and the synthesized piece next using *Mlu*I and *Bgl*II. The *AaHsp70Aa* putative promoter fragment was extended by adding a fragment of synthesized sequence in the same manner as *AaHsp70Ba*. Clones were sequenced for verification using the primers listed in table 3.1. Reporter plasmids were prepared for transfection using either Qiagen Plasmid Mini kit or Plasmid Midi kit.

Construction of large putative promoter reporter constructs

AaHsp70A was assembled by adding sequence to the previously cloned *AaHsp70Aa*-2 luciferase reporter construct. A second fragment of synthesized DNA was added consisting of the sequence corresponding to the genomic region from 380341 to 383225 on supercontig 1.680, due to difficulties amplifying upstream regions of *AaHsp70A*. *AaHsp70Aa*-2-Luc was digested with *Mlu*I and *Tth111*I. The plasmid containing the synthesized upstream sequence was first digested with *Mlu*I, then partially digested with *Tth111*I and a 2331 base pair product was ligated

into *AaHsp70Aa*-2-Luc. The resulting plasmid was sequenced for confirmation using primers listed in Table 3.2.

The *AaHsp70Ab* 3' untranslated region (UTR) was amplified using Platinum *Pfx* (94°C, 5 min; 94°C, 15 sec, 61°C, 15 sec, 68°C, 30 sec, 35 cycles; 68°C 10 min), and oligonucleotides containing *Xba*I sites which are represented by lowercase letters (5'-tttttctagaTAAATTGAGTTGAGATACGAGACTGAATGAG-3', 5'-tttttctagaATCAAGATTGTCGACCAACTAGAAGGACC-3'). Amplicons were ligated into *Xba*I sites in the previously cloned AaHsp70A construct. Clones were sequenced using a single primer (5'-ATCAGAGAGATCCTCATAAAGGCCAAGAAGG-3').

AaHsp70B was assembled in pieces beginning with the previously cloned *AaHsp70Bb*-Luc construct. The region between *AaHsp70Ba* and *AaHsp70Bb* was amplified using Phusion polymerase (Thermo Scientific, Lafayette, CO) and genomic DNA digested with *Bgl*II (98°C,

Table 3.1 Oligonucleotides to amplify and sequence short putative promoter fragments

Primer Name	Sequence (5'-3')*
Aa Mlu F	ttttacgcgtAACTCGAACTTTCTAGATCTGCTCGTTTCTACTATATAAG
Aa Nco R	ttttccatggACATTTTTGCTTTCGTACTGATGTTCAACAC
Ba Mlu F	ttttacgcgtCTCACACAAAGCAATGAAATATGCGAC
Ba Nco R	ttttccatggACATTTTTGCTTTCGTACTGATGTTTAACAC
Ca Mlu F	ttttacgcgtTGGGCGCTTATATAGTGGAAACG
Ca Nco R	ttttccatggACATTTTTGCTTCTGTACTGATGTTTAACAC
ZA41	TTGTAAAACGACGGCCAGTG
ZA57	AACTCCAGCAGGACCATGTGATCGC
ZA559	TTTCATAGCTTCTGCCAACCGAACGG

*Lowercase letters indicate restriction sites

Table 3.2 AaHsp70A sequencing oligonucleotides

Primer Name	Sequence (5'-3')
ZA353	CCGCAGGCAAAGTGTGTGCAGGATA
ZA355	CGAGAGTAGTCGAGTACCTCCACGAATCTG
ZA362	ATCAAACTTTCTGCTCTCGATACATGTTTGC
ZA 446	TGTTCGAGTACTTTCGTATGAGTATGGGC
ZA448	TTGATCTAACCAGCAAACATGTATCGAGAGC
ZA450	ATCTAAACTCCTCACCTGCATATTTTGCG
ZA451	TTATTGCTCTTGCTTGTGATTCGTTCTCTTGC

30 sec; 98°C, 1 min 30 sec, 55°C, 1 min, 72°C, 2 min, 35 cycles; 72°C, 10 min) using the following oligonucleotides (5'-AGGTGTCTTATAAAATCTCGAATTCCACT-3', 5'-TGGAAACAGGCTACCATTAACTGATGAGTC-3'). The resulting amplicon was digested, along with *AaHsp70Bb*-Luc, with *Eco*RI and *Apa*I and ligated into *AaHsp70Bb*-Luc. The *AaHsp70Ba* putative promoter fragment was amplified using Phusion (98°C, 30 sec; 98°C, 10 sec, 60°C, 30 sec, 72°C, 30 sec, 35 cycles; 72°C, 10 min) with the following oligonucleotides (5'-ttttctcgagATGCATTTTGCTTTCGTACTGATGTTTAACAC-3', 5'-GAGAATACTCTGGATTGATACATCTAGCC-3'). The resulting amplicon was digested with *Xho*I and *Eco*RI and ligated into the *Xho*I and *Eco*RI digested *AaHsp70Ba-Bb*-Luc intermediate plasmid produced in the last cloning step. After the digestion with *Eco*RI, an unexpected 500 base pair restriction fragment was noted. This fragment resulted from a previously unidentified *Eco*RI site in the fragment between the *AaHsp70Ba* and *AaHsp70Bb* genes. This fragment was digested from the original amplicon of the region between the *AaHsp70Ba* and *AaHsp70Bb* genes using *Eco*RI and ligated into a partially digested plasmid resulting from the previous cloning step. The resulting AaHsp70B putative promoter-reporter construct was sequenced using the oligonucleotides listed in Table 3.3.

The *AaHsp70Bb* 3'UTR was amplified from genomic DNA digested with *Sac*II using Platinum *Pfx* polymerase (94°C, 5 min; 94°C, 15 sec, 61°C, 15 sec, 68°C, 30 sec, 35 cycles; 68°C 10 min) and oligonucleotides containing *Xba*I sites (5'-tttttctagaTTGAGGAAGTCGACTAAAGCGAATGGAGAGG-3', 5'-tttttctagaGGCAATTTCGTAGCTCTATTGACATGTCCAG-3'). The resulting amplicon was digested with *Xba*I and the resulting fragment was ligated into *Xba*I-digested pGL3Basic (Promega). Clones were sequenced with a single oligonucleotide to confirm

Table 3.3 AaHsp70B sequencing oligonucleotides

Primer Name	Sequence (5'-3')
ZA403	TGGAAACAGGCTACCATTAACTGATGAGTC
ZA459	GTCGCATATTTCATTGCTTTGTGTG
ZA460	CATCGGTGAATCACTTCGAAGC
ZA461	TTACAGTTGAGGTCGACATACGGGTATCTG
ZA558	ATAGGCTGTCCCCAGTGCAAGT
ZA559	TTTCATAGCTTCTGCCAACCGAACGG

the correct orientation of the 3'UTR (5'-ATCAGAGAGATCCTCATAAAGGCCAAGAAGG-3'). AaHsp70B was then digested *Xho*I and *Nco*I along with pGL3Basic containing the *AaHsp70Bb* 3'UTR. The resulting AaHsp70B putative promoter fragment was ligated into pGL3Basic containing the *AaHsp70Bb* plasmid.

Construction of additional AaHsp70 putative promoter constructs of varying lengths

Additional AaHsp70A putative promoter constructs were created based on the initial 2589 base pair construct. To produce AaHsp70A-1734, AaHsp70A was digested with *Nco*I and *Hind*III, and the resulting 1916 base pair fragment was ligated into pGL3Basic that had been digested with *Nco*I and *Hind*III. To produce AaHsp70A-1383, AaHsp70A was linearized with *Mlu*I in a 100 µl reaction. The reaction was then split into 5 smaller reactions and *Ase*I was added and the reaction was allowed to proceed at room temperature for 5 – 25 minutes in 5-minute intervals. The digests were run together in a gel and the desired fragment was excised and purified using the Qiaquick gel extraction kit (Qiagen). Fragments containing the desired putative promoter fragment as well as luciferase and the remaining pGL3 backbone were treated with Klenow to produce blunt ends and were self ligated.

Additional AaHsp70B putative promoter constructs were also created based on the initial large construct. To produce both AaHsp70B-1696 and -767, AaHsp70B was linearized with *Mlu*I as for AaHsp70A. Partial digests were performed using *EcoR*I in the same way as *Ase*I for AaHsp70A. Two desired fragments resulted from these digests, and were treated with Klenow and self-ligated. AaHsp70B-1456 resulted from an effort to move the AaHsp70B-1696 fragment into a new vector for the purpose of making a transgenic donor construct, when a small fragment was lost due to an unpredicted restriction site. The completed AaHsp70A-1373 and AaHsp70B-

1456 constructs correspond to genomic regions 380841 – 382390 and 405057 – 406759 respectively on supercontig 1.680.

RESULTS

AaHsp70 putative promoter regions

In order to test whether regions upstream of *AaHsp70* genes can act as inducible promoters, short putative promoter regions (250 to 525 bases in length) were cloned into enhanced green fluorescent protein (EGFP) reporter constructs. *AaHsp70Ab* and *AaHsp70Cb* did not drive expression levels that were detectable by Northern analysis, therefore they were not pursued (Gross *et al.* 2009). *AaHsp70Aa* and *AaHsp70Bb* constructs exhibited higher levels of EGFP expression both under heat shock and control conditions when compared to the other constructs tested (Figure 3.1). Other *AaHsp70* putative promoters drove low levels of EGFP expression and were not pursued as candidates for further study. In order to amplify AaHsp70 putative promoter regions, it was necessary to digest *Aedes aegypti* genomic DNA to separate the divergently transcribed pairs. As a result of the required digests the *AaHsp70Aa* putative promoter construct was significantly shorter than the other putative promoter constructs. Therefore this promoter was extended by another 386 base pairs to test whether regions necessary for repression under control conditions were present. The *AaHsp70Aa* putative promoter containing 344 bases exhibited very similar core promoter activity as compared to the initial construct (Figure 3.1).

Based on the results from initial putative promoter testing, new putative promoter constructs were designed including either the entire genomic region between *AaHsp70Aa* and *AaHsp70Ab* (genomic position 380841-383453 on supercontig 1.680), or *AaHsp70Ba* and *AaHsp70Bb* (genomic position 404011-406759 on supercontig 1.680).

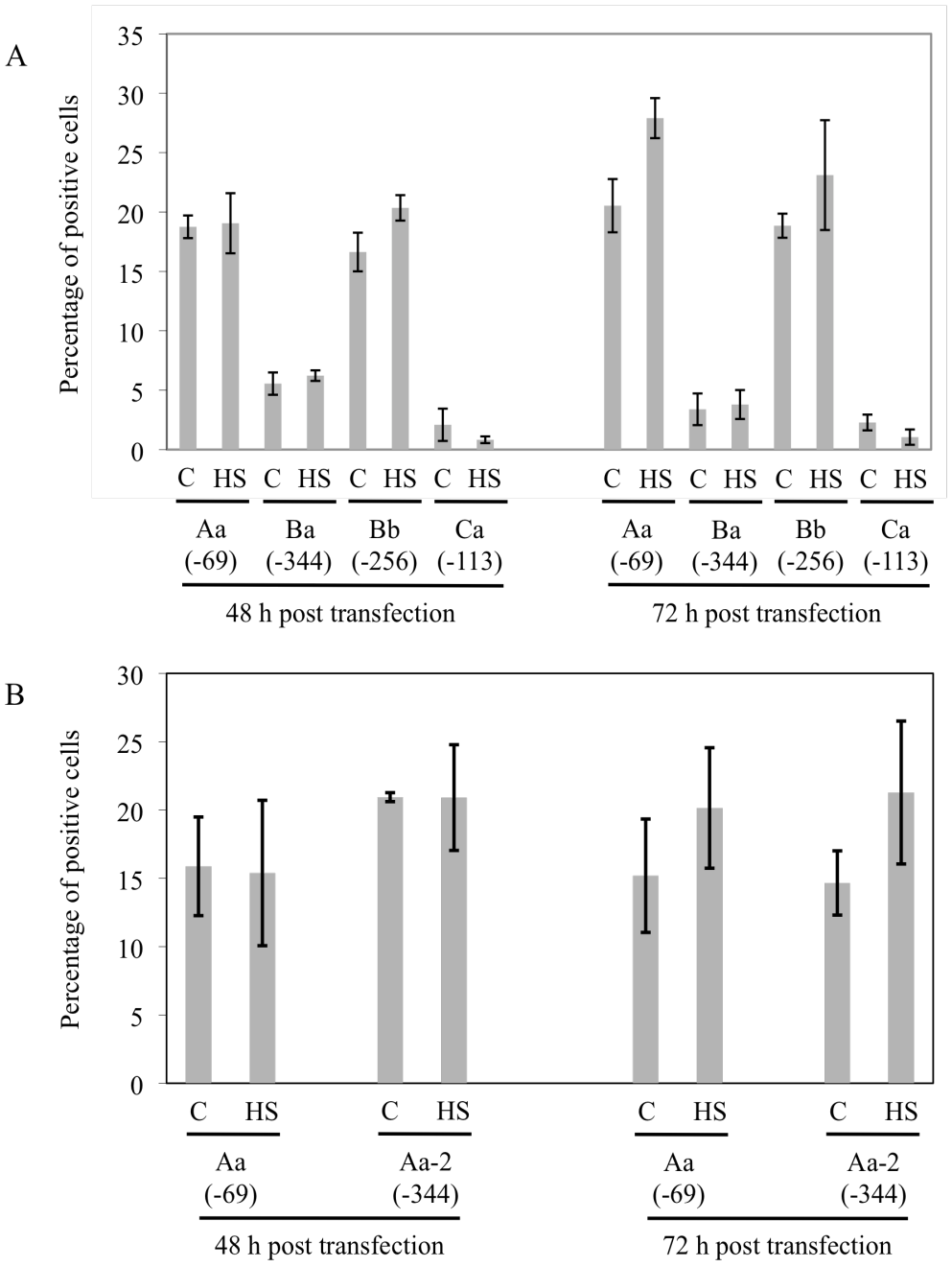


Figure 3.1 Activity of short *AaHsp70* putative promoter constructs. (A) C6/36 cells were transfected with short (250-530 bp) putative promoter constructs driving GFP expression. Promoter sequences were derived from genomic sequences upstream from indicated genes. Each construct includes the 181-182 bp 5'UTR of the downstream gene. Cells were heat shocked (HS) at 37° C for one hour 24 hours after transfection or maintained at standard rearing conditions (C). Photos were taken 48 and 72 hours post tranfection and the number of cells expressing GFP within a series of photos were counted and expressed as the percentage of positive cells out of all the cells counted. (B) An additional 275 bases of upstream sequence was added to the Aa putative promoter and assayed as in (A).

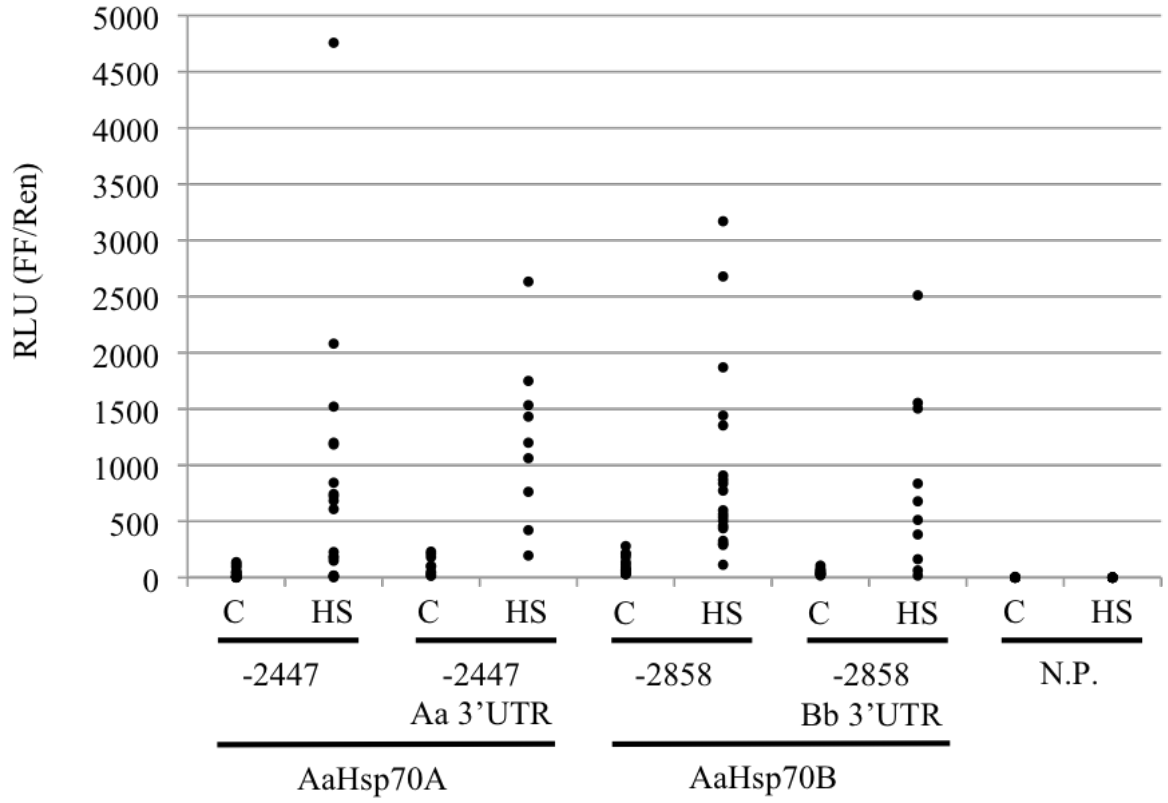


Figure 3.2 Activity of putative *AaHsp70* promoter constructs in *Aedes aegypti* embryos. *Aedes aegypti* embryos were injected with putative *AaHsp70* promoter constructs and a promoterless (N.P.) construct 30-90 minutes after being laid. Each data point represents 80-120 embryos harvested as a group. Control (C) and heat shocked (HS) embryos were assayed.

These constructs were injected into *Aedes aegypti* embryos and after 24 hours experimental groups were heat shocked at 39°C. Luciferase assay results indicate robust expression driven by each putative promoter construct in embryos with approximately 10-20 fold induction under heat shock (Figure 3.2). The level of expression between control and heat shocked embryos was significantly different according to Tukey's Honestly Significant Difference (HSD) test performed on data transformed into ranks (Figure 3.3).

In *Drosophila DmHsp70* genes, the 3' untranslated region (UTR) serves to target transcripts for degradation (Petersen and Lindquist 1989). In order to test whether the presence of the endogenous 3'UTR in our putative promoter constructs would affect the expression of luciferase, the SV40 3'UTR present in putative promoter constructs was replaced by either *AaHsp70Aa* or *AaHsp70Bb* 3'UTR in their respective constructs. Expression levels between each of the different *AaHsp70* putative promoter constructs were not found to be significantly differently one hour post heat shock according to Tukey's HSD performed on ranks (Figure 3.3). Therefore, constructs with SV40 3'UTRs were used for further study.

Putative Transcription Factor Binding Sites

The number of putative HSTF binding sites has been positively correlated with the level of expression driven by heat shock promoters (Fernandes *et al.* 1995). In order to predict sequences which might be important to the regulation of expression driven by *AaHsp70* promoters, both AaHsp70A and AaHsp70B constructs were analyzed using the TransFac function TFsearch (Heinemeyer *et al.* 1998). Putative HSTF binding sites were identified in both promoter sequences, with more overall sequences identified in AaHsp70A as compared to AaHsp70B (Fig. 3.4, 3.5).

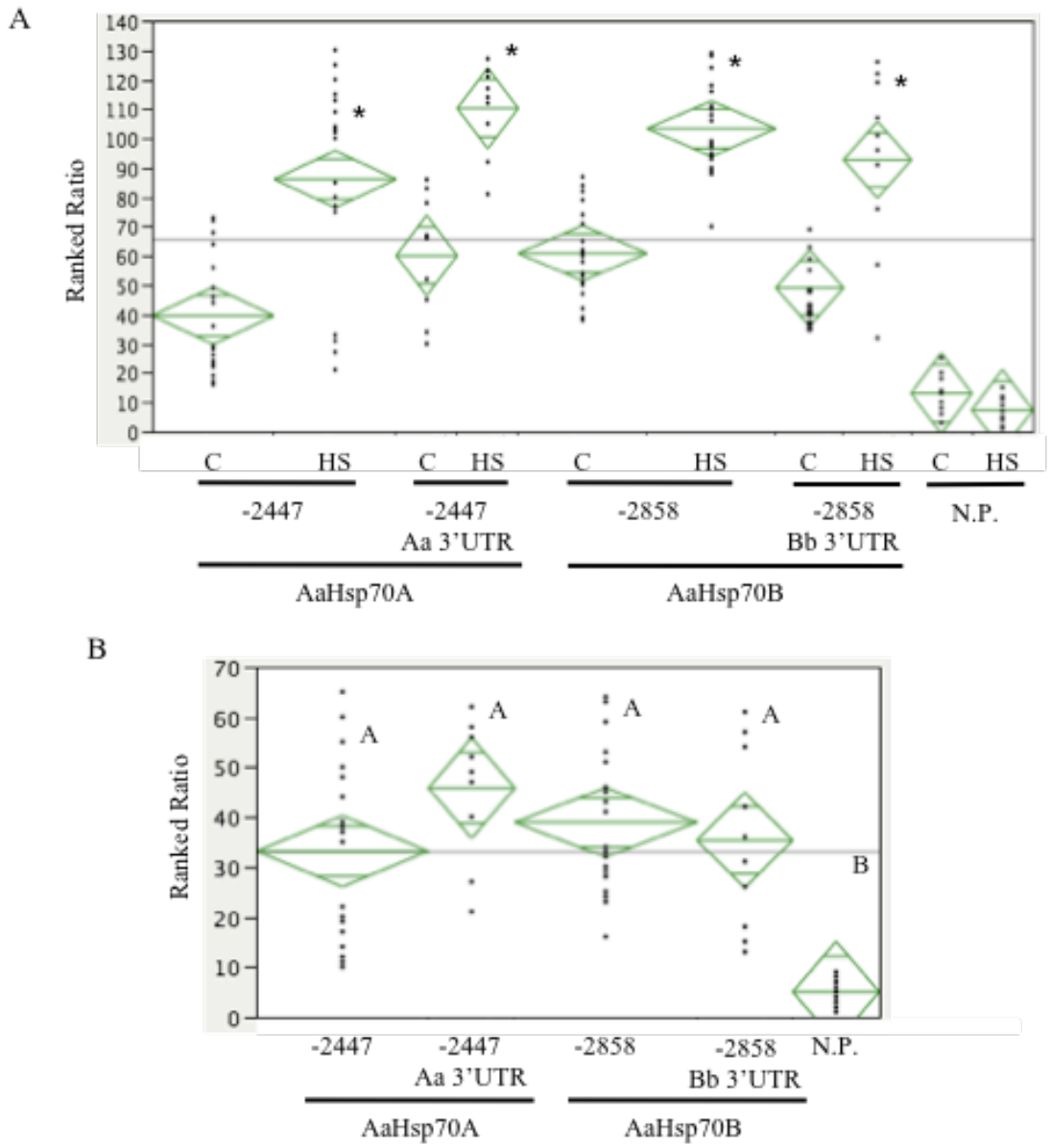
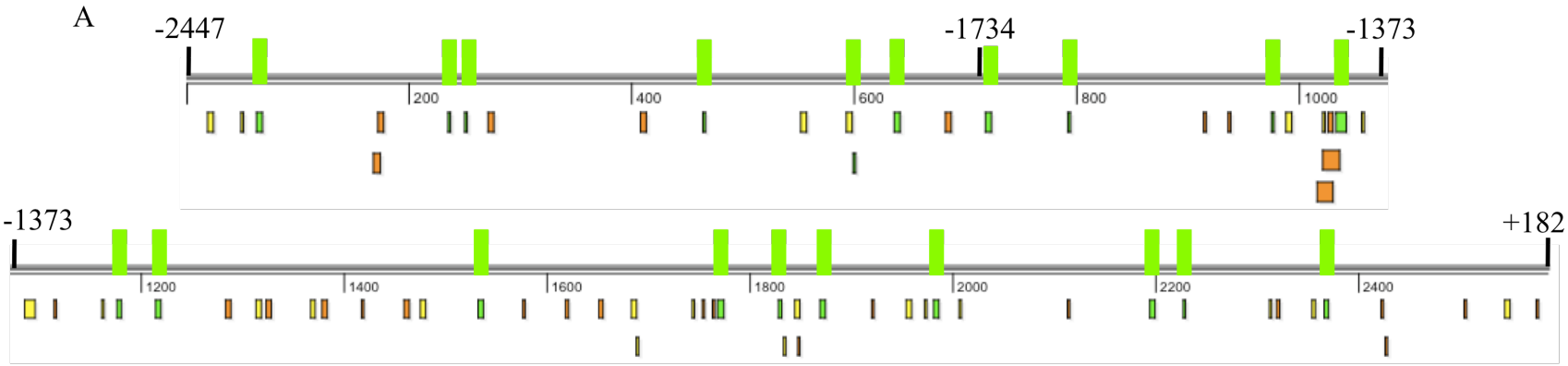


Figure 3.3 Analysis of putative *AaHsp70* promoter construct activity in *Aedes aegypti* embryos. (A) Luciferase assay readings were transformed into ranks and ANOVA was perfomed. Diamonds represent the 95% confidence interval of the mean, indicated by the middle line of the diamond. Means were compared using Tukey's Honestly Significant Difference test (HSD). Heat shock columns marked with a star are significantly different from control values for that construct. (B) Heat shocked constructs were compared separately to one another to test for significant differences. Constructs not connected by the same letter are significantly different according to Tukey's HSD.

A

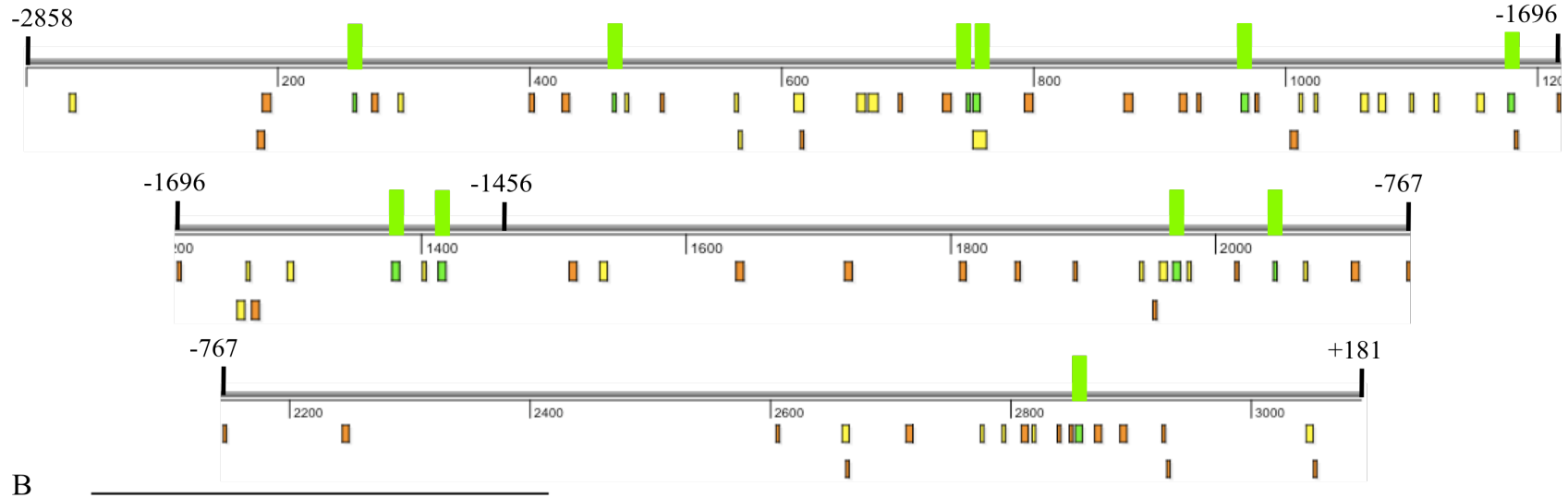


B

AaHsp70 A Construct	Identity of binding site	Number
-2447	100%	20
	90 - 99%	23
	85 - 90%	29
-1734	100%	14
	90 - 99%	21
	85 - 90%	24
-1373	100%	10
	90 - 99%	16
	85 – 90%	19

Figure 3.4 Putative Heat Shock Transcription Factor Binding Sites in AaHsp70A. (A) Putative heat shock transcription factor (HSTF) binding sites were identified using the TFsearch function of TransFac (Heinemeyer et al. 1998) using the region between *AaHsp70Aa* and *AaHsp70Ab* genes as query. Green boxes indicate sites with 100 percent identity, yellow boxes indicate 90-99 percent identity, and orange boxes indicate 85-90 percent identity. (B) The number of putative binding sites in each construct.

A



B

AaHsp70 B Construct	Identity of binding site	Number
-2858	100%	11
	90 - 99%	30
	85 - 90%	40
-1696	100%	5
	90 - 99%	14
	85 - 90%	23
-1456	100%	3
	90 - 99%	10
	85 – 90%	22
-767	100%	1
	90 - 99%	5
	85 – 90%	12

Figure 3.5 Putative Heat Shock Transcription Factor Binding Sites in AaHsp70B. (A) Putative heat shock transcription factor (HSTF) binding sites were identified using the TFsearch function of TransFac (Heinemeyer et al. 1998) using the region between *AaHsp70Ba* and *AaHsp70Bb* genes as query. Green boxes indicate sites with 100 percent identity, yellow boxes indicate 90-99 percent identity, and orange boxes indicate 85-90 percent identity. (B) The number of putative binding sites in each construct.

Further Characterization of AaHsp70 Promoter Sequences

Additional constructs based on AaHsp70A and AaHsp70B were designed to determine whether the amount of upstream sequence could be reduced without losing the pattern of induction and repression exhibited by the larger constructs already tested. AaHsp70A-based and AaHsp70B-based constructs were first tested in *Aedes aegypti* Aag2 cells, and then *Aedes aegypti* embryos.

Two additional constructs based on AaHsp70A were designed and initially tested in *Aedes aegypti* Aag2 cells. Expression levels under heat shock were found to be significantly upregulated from control expression levels for the original AaHsp70A-2447 construct, as well as for the AaHsp70A1734. The third construct, AaHsp70A1373, was not found to have significantly different expression under heat shock according to Tukey's HSD (Fig. 3.6).

AaHsp70A based constructs were further characterized in *Aedes aegypti* embryos. All three AaHsp70A constructs drove significantly higher levels of heat induced expression when compared to control conditions (Fig. 3.7, 3.8). When heat shocked expression was compared between the three constructs, no loss of heat inducible expression was noted as a result of decreased construct size (Figure 3.8B). As a result, AaHsp70A-1373 was chosen as the best candidate for further experimentation.

Simultaneously, three additional AaHsp70B based constructs were characterized in both Aag2 cells and *Aedes aegypti* embryos. Initial characterization in cells demonstrated significant

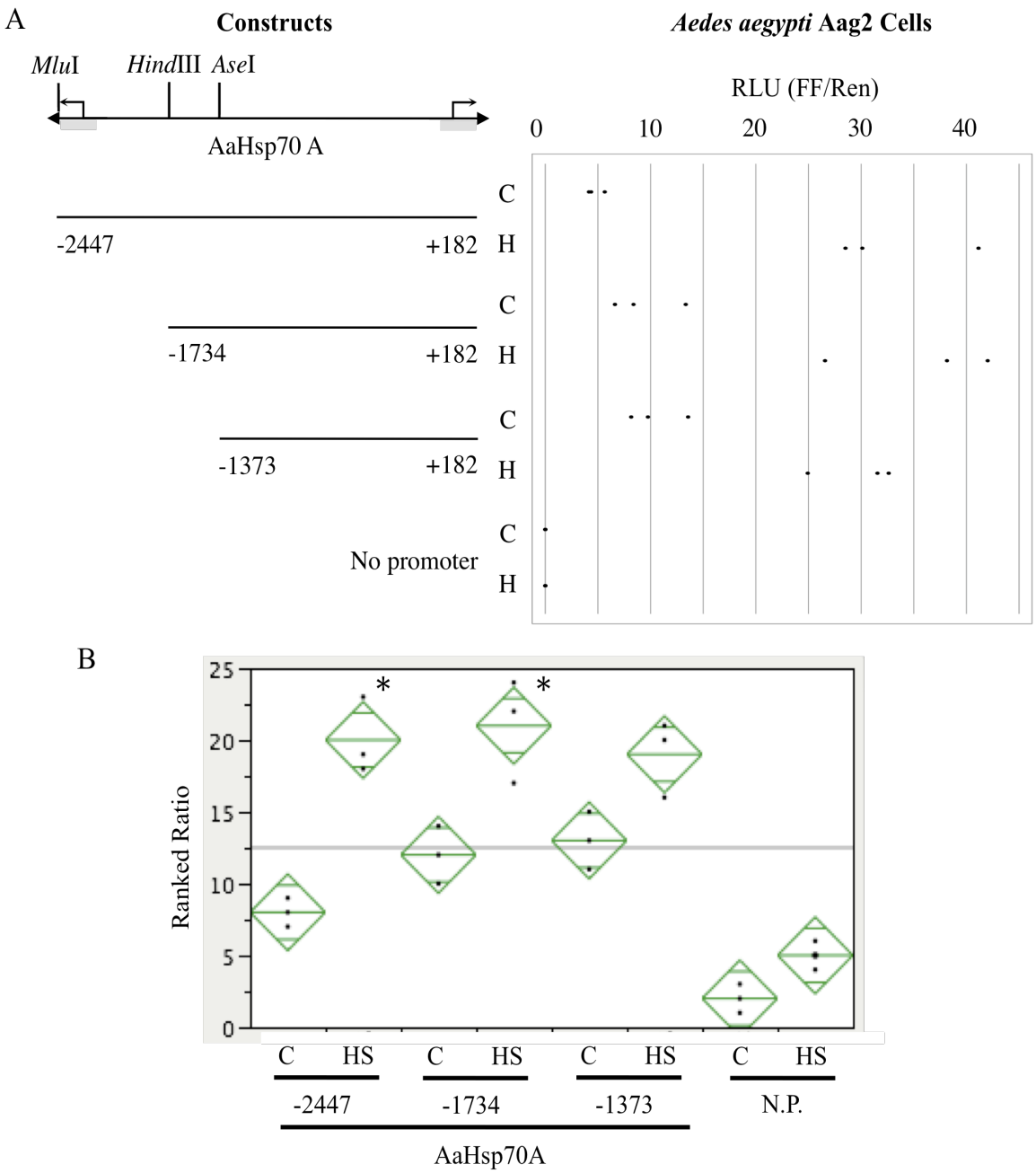


Figure 3.6 Activity of AaHsp70A putative promoter constructs in *Aedes aegypti* Aag2 cells. (A) Aag2 cells were transfected with AaHsp70A putative promoter constructs driving luciferase expression. Cells were maintained at 28° C and 5% CO_2. Heat shocked cells (H) were incubated at 39° C for one hour approximately 24 hours post transfection. Heat shocked and control (C) cells were harvested approximately 26 hours post transfection and luciferase assays were performed on lysates. (B) Luciferase assay readings were transformed into ranks and ANOVA analysis was performed. Tukey's HSD was used to compare means, and heat shocked values that are significantly different from control values within each construct are marked with a star.

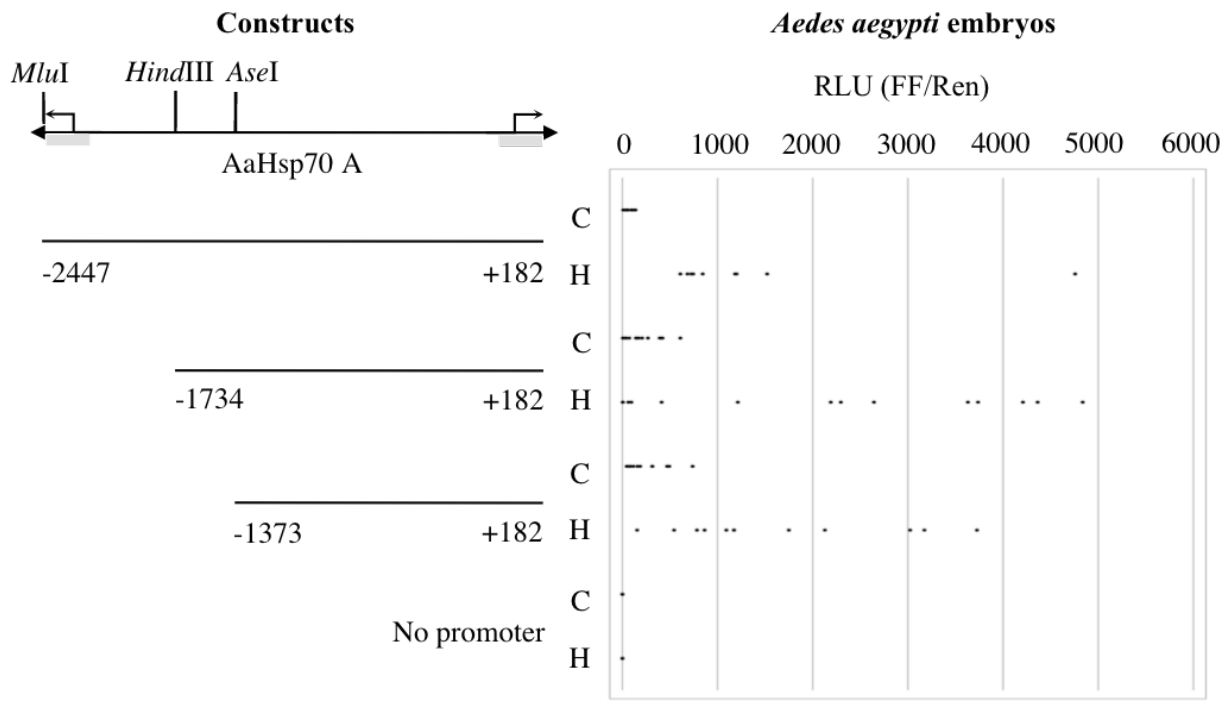


Figure 3.7 Activity of AaHsp70A putative promoter constructs in *Aedes aegypti* embryos. Groups of *Aedes aegypti* embryos were injected with AaHsp70A putative promoter constructs 30-90 minutes after being laid. Embryos were maintained at 28°C with 60-70% relative humidity. Heat shocked embryos (H) were heat shocked at 39°C for one hour approximately 24 hours post injection. All embryos were harvested approximately 26 hours post injection. Each data point represents a group of 80-100 embryos.

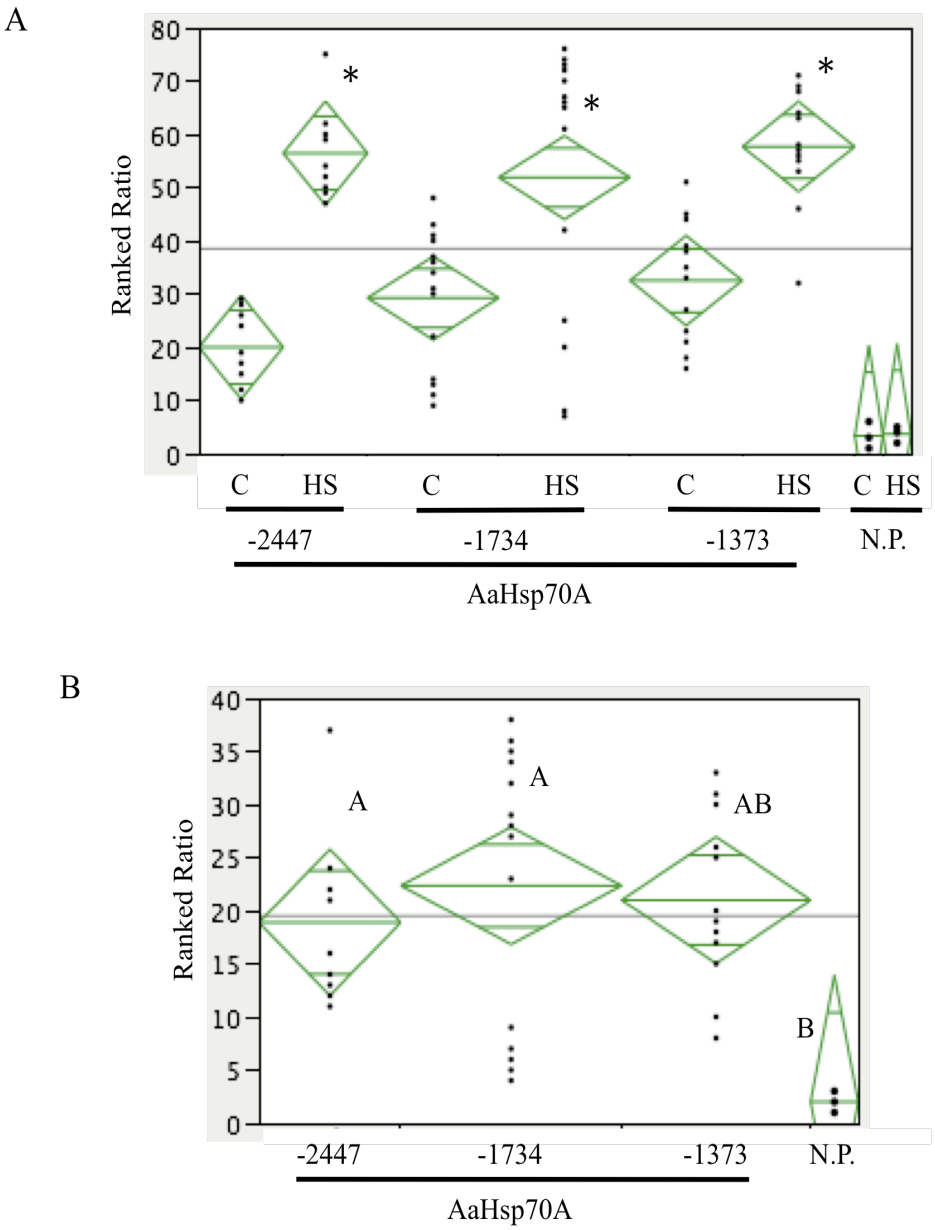


Figure 3.8 Analysis of putative *AaHsp70* promoter construct activity in *Aedes aegypti* embryos. (A) Luciferase assay readings were transformed into ranks and ANOVA was performed. Diamonds represent the 95% confidence interval of the mean, indicated by the middle line of the diamond. Means were compared using Tukey's Honestly Significant Difference test (HSD). Heat shock columns marked with a star are significantly different from control values for that construct. (B) Heat shocked constructs were compared separately to one another to test for significant differences. Constructs not connected by the same letter are significantly different according to Tukey's HSD.

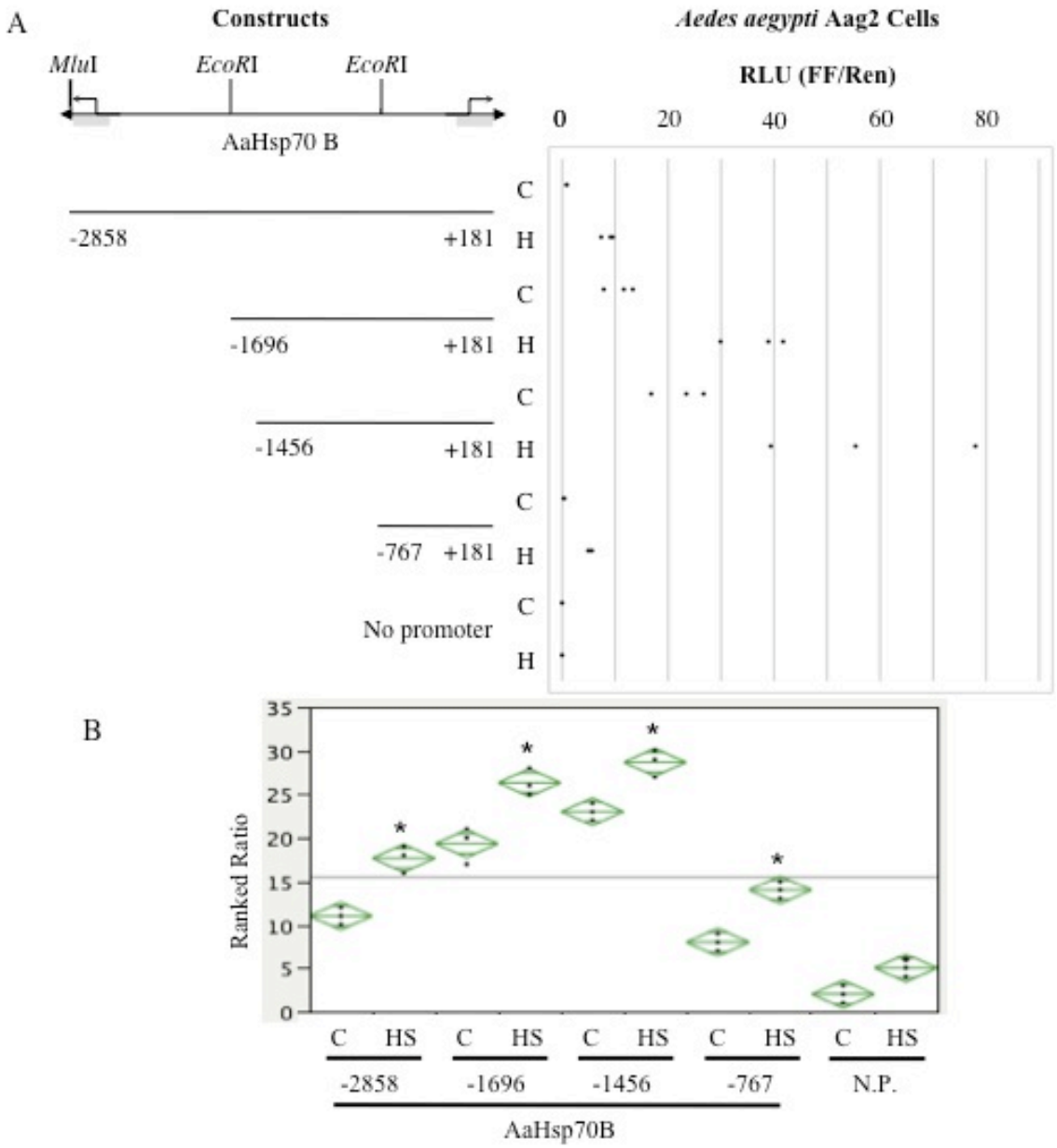


Figure 3.9 Activity of AaHsp70B putative promoter constructs in *Aedes aegypti* Aag2 cells. (A) Aag2 cells were transfected with AaHsp70A putative promoter constructs driving luciferase expression. Heat shocked cells (H) were incubated at 39°C for one hour approximately 24 hours post transfection. Heat shocked and control (C) cells were harvested approximately 26 hours post transfection and luciferase assays were performed on lysates. (B) Luciferase assay readings were transformed into ranks and ANOVA analysis was performed. Tukey's HSD was used to compare means, and heat shocked values that are significantly different from control values within each construct are marked with a star.

differences between control and heat shock expression for all constructs (Fig. 3.9). However, significantly less expression was observed for the AaHsp70B-767 construct. Characterization in embryos showed statistically significant levels of heat induced expression for three of the four constructs, with the least amount of expression driven by AaHsp70B-767 (Fig. 3.10, 3.11). Significant differences between control and heat shock expression levels were again observed for all four constructs. When heat shock expression levels were compared between each construct, AaHsp70B, AaHsp70B-1696, and AaHsp70B-1456 were not found to be statistically different (Fig. 3.11). However, AaHsp70B-767 heat shock expression was significantly less than the other AaHps70B based constructs. As a result, AaHsp70B-1456 was chosen as the best candidate for further experimentation.

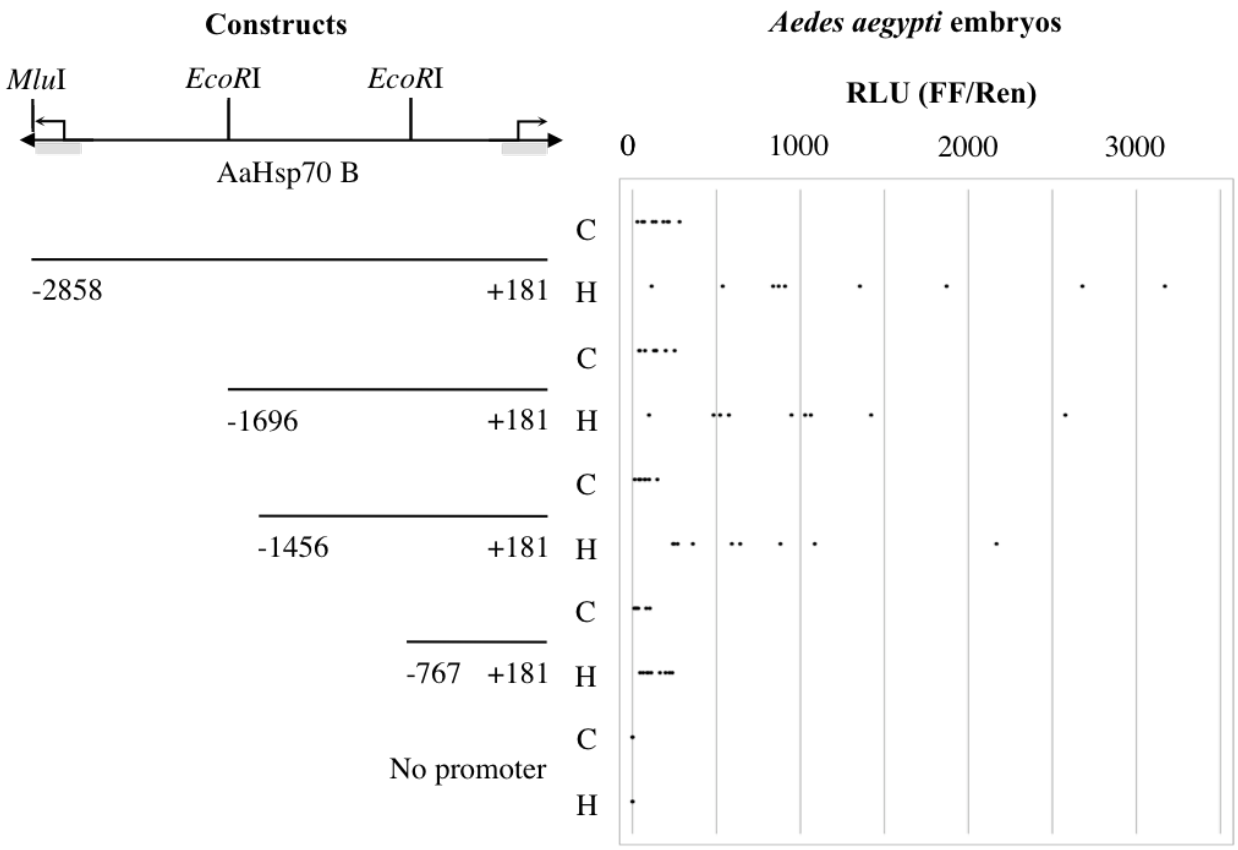


Figure 3.10 Activity of AaHsp70B putative promoter constructs in *Aedes aegypti* embryos. Groups of *Aedes aegypti* embryos were injected with AaHsp70A putative promoter constructs 30-90 minutes after being laid. Embryos were maintained at 28°C with 60-70% relative humidity. Heat shocked embryos (H) were heat shocked at 39°C for one hour approximately 24 hours post injection. All embryos were harvested approximately 26 hours post injection. Each data point represents a group of 80-100 embryos.

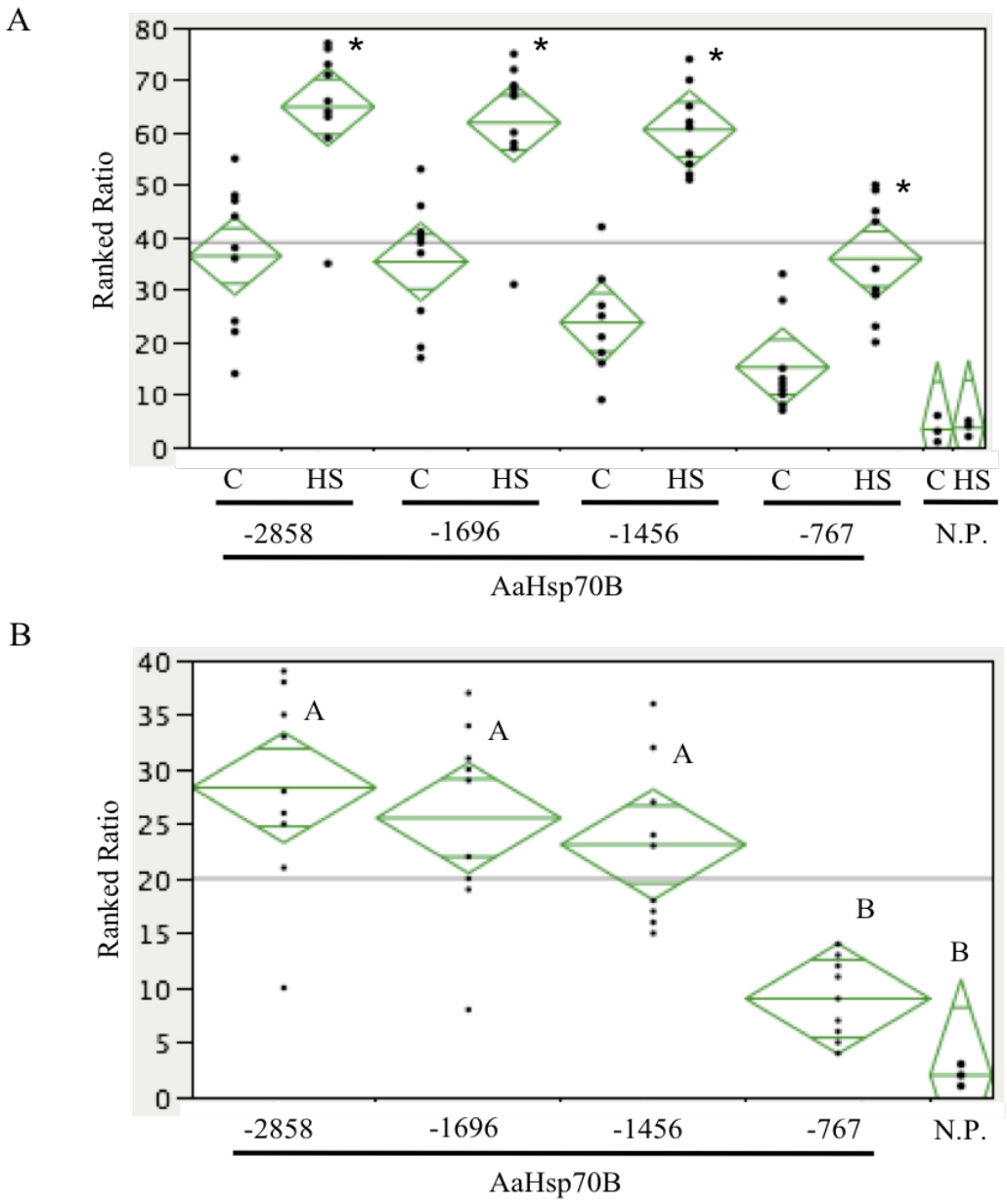


Figure 3.11 Analysis of putative *AaHsp70* promoter construct activity in *Aedes aegypti* embryos. (A) Luciferase assay readings were transformed into ranks and ANOVA was performed. Diamonds represent the 95% confidence interval of the mean, indicated by the middle line of the diamond. Means were compared using Tukey's Honestly Significant Difference test (HSD). Heat shock columns marked with a star are significantly different from control values for that construct. (B) Heat shocked constructs were compared separately to one another to test for significant differences. Constructs not connected by the same letter are significantly different according to Tukey's HSD.

DISCUSSION

Hsp70 genes require specific upstream elements in order to maintain heat inducibility as well as tightly controlled repression under normal conditions (Pelham 1982, Tang *et al.* 2000). The regions upstream of *D. melanogaster* hsp70 genes containing the elements required to drive robust heat inducible expression while maintaining repression under normal conditions have been well described (Amin *et al.* 1987). In an effort to determine whether regions upstream of *AaHsp70* genes could act as inducible promoters, short 250 to 525 base pair sequences found upstream of *AaHsp70* genes were tested in cells. These sequences were found to drive varying degrees of expression of a green fluorescent protein (EGFP) marker gene. Sequences were sufficient to possess varying levels of core promoter activity. Two promoter sequences, Aa and Bb, were found to possess the greatest core promoter activity, and were therefore chosen for further characterization.

The genomic regions between *AaHsp70Aa/AaHsp70Ab* and *AaHsp70Ba/AaHsp70Bb* were analyzed for the presence of putative heat shock transcription factor binding sites. Previous studies in *Drosophila melanogaster* have shown that more HSTF binding sites correlate with higher levels of inducible expression, with upstream elements leading to cooperative binding of downstream elements (Topol *et al.* 1985, Perisic *et al.* 1989). Overall, the AaHsp70A region contained more putative binding sites when compared to the AaHsp70B region. Constructs were designed including these regions and tested using luciferase assays to quantify the amount of expression driven by each putative promoter. Surprisingly, changes in expression levels due to heat shock for each putative promoter were statistically similar with 10 to 20 fold induction. With the AaHsp70A construct containing 20 putative HSTF binding sites and AaHsp70B

containing only 11, a significant difference in the level of heat induced expression between the two constructs was expected. It may be possible that while more putative HSTF binding sites drive higher absolute levels of expression, the level of induction may not be as dependent upon the increased binding of transcriptional units.

Additional putative promoter constructs were designed to determine whether promoters could be reduced in size without the loss of heat induction and repression characteristic of endogenous *AaHsp70* genes. Constructs were tested in both *Aedes aegypti* cells and embryos. For both constructs, the fold changes in expression due to heat shock were different in cells than embryos. For AaHsp70A, the fold changes in embryos were consistently higher (6.4-19X) than cells (2.8-7X). However, for AaHsp70B the pattern was not consistent with fold changes ranging from 3.6-10.7X in embryos and 2.6-13.9X in cells. Some of the variation is likely due to small sample sizes in cell assays, with only three total samples for each construct. Additionally, both cell transfection and embryo injection introduce additional sources of stress that cannot be controlled.

As expected, some loss of heat inducible expression was noted with the reduction in construct size along with a loss of repression under control conditions. The constructs that retained the highest levels of heat induction with the shortest sequence, AaHsp70A-1373 and AaHsp70B-1456, were chosen as the best candidates for further experimentation. These constructs retained enough sequence to remain heat inducible, while the reduction in size was favorable for ease of cloning and success of transformation events.

Overall, we found that regions upstream of *AaHsp70* genes are capable of driving heat inducible expression of marker genes in *Aedes aegypti* cells and embryos. Further characterization of putative *AaHsp70* promoter regions was necessary to determine whether

these promoters would behave similarly to endogenous genes after integration into *Aedes aegypti* chromosomes.

Chapter 4

Activity of *AaHsp70* promoter constructs in transgenic *Aedes aegypti*

INTRODUCTION

Successful genetic transformation protocols exist for multiple insect species, including *Drosophila melanogaster, Bombyx mori, Tribolium castaneum,* and *Aedes aegypti*. As reviewed by Wimmer (2003), genetic transformation has allowed for gene characterization studies as well as the development of vector species refractory to pathogen transmission. Genetic manipulation of vector/pathogen interactions requires well characterized promoter elements to drive transgene expression (Zhao and Eggleston 1999).

Ectopic expression and gene knockdown are common methods of deducing the function of uncharacterized genes. The knockdown of developmentally important genes must be precisely timed in order to be able to study the phenotypic result of knockdown without interrupting development. A wide range of promoter elements have been characterized and utilized to drive transgene expression and for gene knockdown in *Aedes aegypti*. Such elements include the salivary gland specific *apyrase* (Coates *et al.* 1999) and *30K* (Mathur *et al.* 2010) promoters, midgut expressed *carboxypeptidase* promoter (Moreira *et al.* 2000), ovary and fat body specific *vitellogenin* promoters (Kokoza *et al.* 2001, Cho *et al.* 2006) and testes specific *β2 tubulin* promoter (Smith *et al.* 2007) as well as the full body *polyubiquitin* promoter (Anderson *et al.* 2010). Targeted transgene expression has been proposed to drive expression of anti-pathogen genes to tissues of significance such as the midgut to inhibit parasite development (Moreira *et al.* 2000, Kokoza *et al.* 2001). While many of the promoters available for use in *Aedes aegypti* are tissue, life stage, or sex specific, there is no full body inducible promoter yet characterized for

this species. Inducible, whole body expression may be desirable for driving expression of anti-pathogen genes in multiple tissues under specific conditions, as well as for the precise timing of transgene expression.

The *Drosophila melanogaster hsp70* promoter element has been used extensively as a ubiquitous inducible promoter both in *D. melanogaster* and other species (Berger *et al.* 1985, Zhao and Eggleston 1999, Uhlirova *et al.* 2002, Kalosaka *et al.* 2006). The *DmHsp70* promoter was used in *Bombyx mori* to drive a heat inducible RNAi system for the investigation of endogenous eclosion hormone gene function (Dai *et al.* 2007). However, the *DmHsp70* promoter exhibits high levels of constitutive expression in addition to being heat inducible in *Aedes aegypti* and is not useful for studies requiring a full body inducible promoter repressed under control conditions (Morris *et al.* 1991). Therefore, AaHsp70 promoter regions were chosen for characterization as possible inducible promoters that may be active in all tissues.

Having determined through cell culture and embryo assays that portions of AaHsp70 promoter elements act as inducible promoters under heat stress, the activity of AaHsp70 promoters was studied in a chromosomal context using genetically transformed *Aedes aegypti*. Both cell culture transfection assays and embryo injection provide potential sources of stress that may induce expression of luciferase driven by AaHsp70 promoters. In order to characterize AaHsp70 promoter activity in unstressed conditions, transgenic lines containing AaHsp70A-1373-Luc and AaHsp70B-1456-Luc cassettes were produced. Luciferase activity in these lines was characterized in various life stages and in multiple tissues. Both promoter constructs were found to drive heat inducible expression in larvae, pupae and adults as well as heads, midguts, salivary glands and ovaries.

MATERIALS AND METHODS

Mosquito strains and rearing

The Liverpool strain of *Aedes aegypti* were reared at 28°C and approximately 60% relative humidity with a 14:10h daylight cycle. Larvae were fed pulverized fish food and reared ≈400 per pan with 4 liters of RO purified water until pupation. Adult mosquitoes were maintained on sucrose and were blood fed using artificial membrane feeders and defibrinated sheep blood (Colorado Serum Company).

Construction of donor plasmids

Donor transgenesis constructs were designed based on AaHsp70A-1373 and AaHsp70B-1456 promoter constructs characterized in Chapter 3. pMos-pUbDsRed-5HE-MCS-5HE, a plasmid containing *Mos*1 inverted terminal repeats as well as DsRed driven by the *polyubiquitin* promoter as a marker for transgenesis (Anderson *et al.* 2010), was digested with *Bgl*II in order to linearize the plasmid and then digested with either *Asc*I or *Pac*I. AaHsp70A-1373 was digested sequentially with *Mlu*I and then *BamH*I. The resulting promoter/reporter fragment was ligated into the *Asc*I and *Bgl*II sites of the digested *Mos*1 plasmid. AaHsp70B-1456 was digested sequentially first with *Pvu*I and then *BamH*I and ligated into the *Pac*I and *Bgl*II sites of the digested *Mos*1 plasmid. Resulting clones were sequenced using primers listed in Tables 4.1 and 4.2.

Mos1-mediated transformation of Aedes aegypti

Transgenic *Aedes aegypti* were produced by injecting Liverpool strain embryos with 0.5 µg/µl donor plasmid (pMos-pUbDsRed-AaHsp70A-1373 or pMos-pUbDsRed-AaHsp70B-1456) and 0.3 µg/µl pGL3-*PUb*Mos1 in 1X injection buffer [5mM KCl, 0.1mM NaH_2PO_4, pH 6.8 (Coates *et al.* 1998)]. Microinjections were performed using a Leica micromanipulator and a FemtoJet microinjector (Eppendorf, Westbury, NY). Needles were produced using a Sutter Instruments (Novato, CA) Model P-2000 needle puller (Heat = 270, Fil = 3, Vel = 37, Del = 250, Pul = 140). Surviving G_0 females were pooled into groups of 20-25 individuals and mated to Liverpool males. G_0 males were mated individually to 5 Liverpool females and then pooled into groups of 24 males and 120 females, as previously described (Anderson *et al.* 2010). Pools were bloodfed and eggs were collected. G_1 progeny were screened for $DsRed^+$ bodies using a Leica MZ-16FL microscope. Photos were taken using a Cannon Powershot S3 IS digital camera with a 0.8 second exposure time.

Heat shock regimens and luciferase assays

Transgenic *Aedes aegypti* were heat shocked as larvae, pupae, and adults. Adults were heat shocked at 39°C as described previously (Gross *et al.* 2009). Larvae and pupae were heat shocked in pre-warmed water for one hour and were returned to control conditions for one hour before being harvested. Larvae and pupae were pooled into groups of 5 and snap frozen in liquid nitrogen. Adult females were frozen individually for luciferase assays, or in groups of 6 for RNA extraction. Larvae, pupae and adults were lysed in 100 µl of 1X passive lysis buffer and luciferase assays were performed according to the manufacturer's protocol (Promega, Dual Luciferase Assay, firefly reading only).

For tissue assays, adult female transgenic mosquitoes were heat shocked at 3-6 days post eclosion for one hour at 39°C. Tissues were dissected 1-4 hours post heat shock and snap frozen in pools of 10 heads, 10 pairs of salivary glands, 10 midguts, and 10 pairs of ovaries. Tissues were lysed in 50 µl of 1X passive lysis buffer and luciferase assays were performed according to the manufacturer's protocol (Promega, Dual Luciferase Assay). Firefly luciferase readings were measured using a Hidex Chameleon plate reader (Turku, Finland).

Timecourse experiments

Adult female AaHsp70A-1373 transformed mosquitoes from P10 were heat shocked at 39°C and were collected at 0, 1, 2, 4, and 24 hours post heat shock. Individuals were snap frozen for luciferase assays, and pools of 6 females were frozen for RNA extraction. Luciferase assays were performed on individual adults crushed in 100 µL 1X passive lysis buffer according to the manufacturer's protocol (Promega, Dual Luciferase Assay, firefly reading only).

Northern Analysis

Total RNA was extracted from pools of 6 females using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA (5 µg) from each experimental group was electrophoresed in a 1.2 percent agarose, 1X MOPS (0.023 M MOPS, 0.3 mM NaOAc, 0.2 mM EDTA), 2% formaldehyde gel at 90 V. RNA was blotted onto a positively charged nylon membrane (Immobilon-NY$^+$, Milllipore, Concord, MA). Blots were prehybridized at 65°C in pre-warmed Church's buffer (0.25 M sodium phosphate buffer, 1mM EDTA, 7% SDS) using a Fisher Scientific Isotemp hybridization oven. Random primed probes were labeled with [α-^{32}P]dATP, specific activity 3000 Ci/mmol using the Amersham Megaprime DNA Labeling System (GE

Healthcare, Buckinghamshire, UK). The specific activity of the probes was determined using a Beckman-Coulter LS6500 Multi-purpose Scintillation Counter. Probes were purified using illustra NICK columns (GE Healthcare) and added to pre-warmed Church's buffer to hybridize overnight at 65°C. Blots were washed twice with 2X SSC, 0.1% SDS for 20 minutes each at 65°C and twice with 0.2X SSC (0.03 M sodium chloride, 0.003 M sodium citrate), 0.1% SDS for 20 minutes each at 65°C. Following washes, the blots were exposed to Kodak BioMax maximum sensitivity film at -80°C. A probe specific to the luciferase open reading frame was generated by digesting pGL3Basic (Promega) with *Xba*I and *Nco*I, resulting in an approximately 1.6 kb fragment (Moreira *et al.* 2000). Separate blots were hybridized with a probe common to AaHsp70 open reading frames. Expression levels were quantified using a Storm 820 phosphoimager and ImageQuantTL software (GE Healthcare).

Table 4.1 AaHsp70A-1373 donor construct sequencing oligonucleotides

Primer Name	Sequence (5'-3')
ZA34	CGCCACCACCTGTTCCTGTA
ZA66	CTCTACAAATGTGGTATGGC
ZA68	CTTACTTCCAACTGCTCTGCGA
ZA343	TCGTTTCTACTATATAAGCGCCCGGTTCG
ZA353	CCGCAGGCAAAGTGTGTGCAGGATA
ZA450	ATCTAAACTCCTCACCTGCATATTTTGCG
ZA499	TTCAGCAGAATATTCGAGGACATTCGC
ZA509	CGAATAAAGAGCAAACGCGGACAC
ZA542	TTTGTCGTCGAATTCGAGCTCGC
ZA549	TGATACCTGGCAGATGGAACCTCTTG
ZA565	TAGTAAATGACACCGCTCTGATCCCACCAA
ZA1266	AATCGAATACGTTTCCTAGTGGAGTGAAC
ZA1341	ACGCGTTCATAATCAGCCATACCACATTTGTAGAG

Table 4.2 AaHsp70B-1456 donor construct sequencing oligonucleotides

Primer Name	Sequence (5'-3')
ZA25	CGAAACGGTGAATACGGCACGCTA
ZA34	CGCCACCACCTGTTCCTGTA
ZA59	ATCAGAGAGATCCTCATAAAGGCCAAGAAGG
ZA66	CTCTACAAATGTGGTATGGC
ZA68	CTTACTTCCAACTGCTCTGCGA
ZA112	CTTGCCGTATGTGATGGAGCGTTGTCATGG
ZA334	ttttgaattcTATCCGCTGGAAGATGGAACC
ZA403	TGGAAACAGGCTACCATTAACTGATGAGTC
ZA460	CATCGGTGAATCACTTCGAAGC
ZA461	TTACAGTTGAGGTCGACATACGGGTATCTG
ZA509	CGAATAAAGAGCAAACGCGGACAC
ZA558	ATAGGCTGTCCCCAGTGCAAGT
ZA559	TTTCATAGCTTCTGCCAACCGAACGG
ZA1266	AATCGAATACGTTTCCTAGTGGAGTGAAC

*Lowercase letters indicate restriction sites

Southern Analysis

AaHsp70A-1373 transgenic mosquitoes from all three lines were snap frozen and genomic DNA was extracted from pools of six females by grinding in Bender buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris, pH 9.0, 0.05 M EDTA, 0.5 M SDS). The resulting homogenates were incubated overnight at 50°C with Proteinase K followed by phenol-chloroform extraction and ethanol precipitation. DNA was resuspended in 200 µl of DEPC treated water. Genomic DNA was digested with either *EcoR*I or *Hind*III overnight, followed by gel electrophoresis and capillary transfer to a nylon membrane, as described for Northern Analysis. Radiolabeled probes were produced corresponding to *Hind*III restriction fragments of *Mos1* construct, as described previously (Anderson *et al.* 2010). Blots were hybridized, washed and exposed to film in the same manner as Northern analysis.

RESULTS

Mos1 transposition of AaHsp70 putative promoter constructs in Aedes aegypti

Both AaHsp70A-1373 and AaHspB-1456 promoters were shown in Chapter 3 to drive heat inducible expression in *Aedes aegypti* cells and embryos. In order to determine the activity of these promoters within the mosquito chromosome, *Mos1* transformation constructs were designed to include each promoter region and were successfully integrated in the mosquito genome (Figure 4.1). G_1 progeny resulting from injection survivors crossed with wild type were screened as larvae for full body expression of DsRed with putative transgenic individuals identified in three

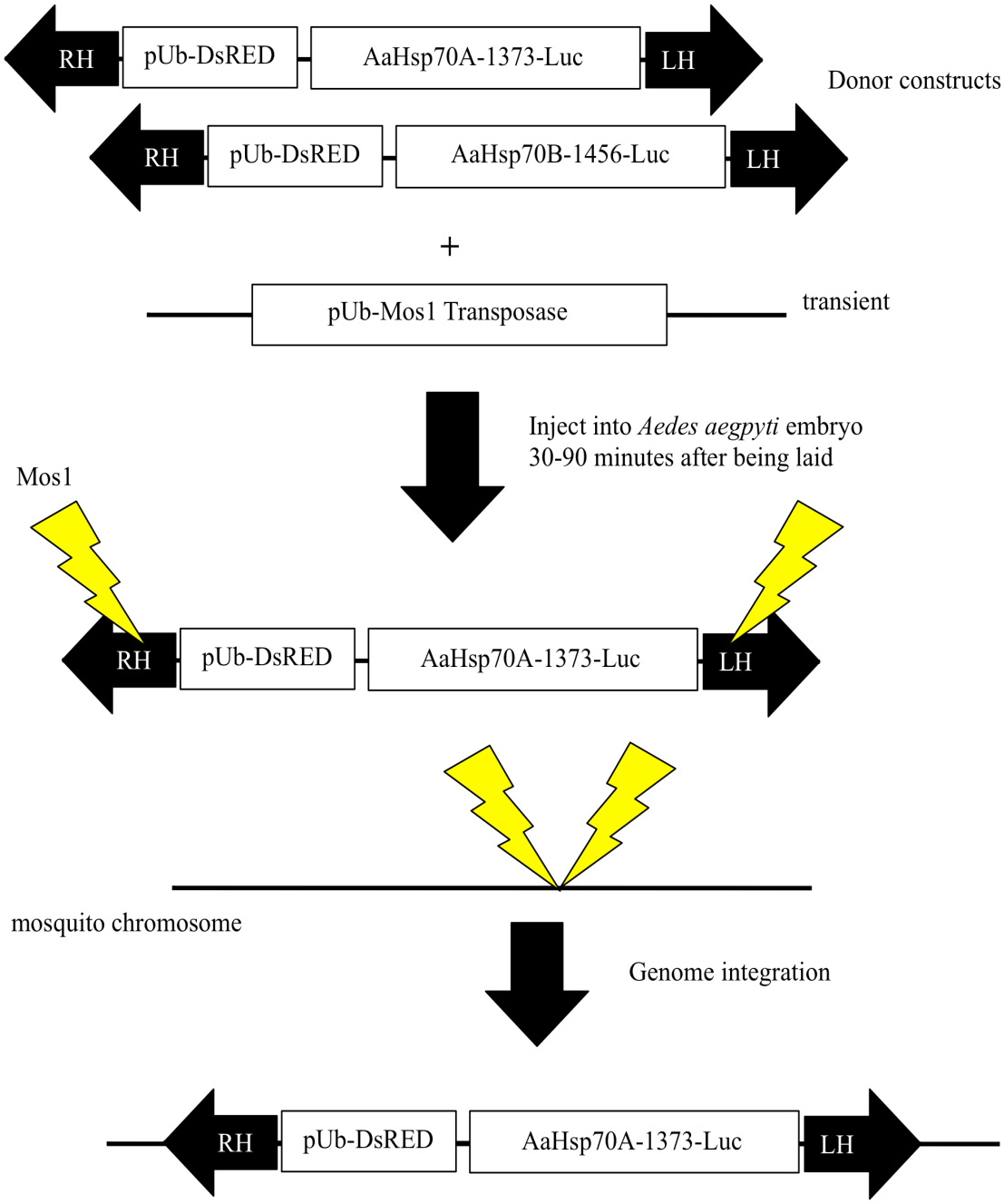


Figure 4.1 Genome integration of transgenic constructs. Donor DNA constructs were designed with *Mos1* terminal inverted repeats and co-injected with a transient *Mos1* helper plasmid. Once injected, *Mos1* transposase is transcribed and translated, and the resulting protein recognizes the terminal inverted repeats in the donor DNA construct. Donor DNA is integrated into the mosquito chromosome in a cut-and-paste fashion.

AaHsp70A-1373 pools and four AaHsp70B-1456 pools (Table 4.3). Injections and establishment of AaHsp70B-1456 lines were performed by Azadeh Aryan. Transformed individuals exhibited robust expression of DsRed in larvae, pupae, and adults (Figure 4.2).

In order to confirm genomic integration in AaHsp70A-1373 lines, Southern analysis was performed (Figure 4.3). Genomic DNA from each of the three $DsRed^{+}$ pools was digested with *EcoR*I, which had no recognition site within the transgenic insertion. DNA was also digested with *Hind*III, which should result in a band of 1621 bp as well as a second band of variable size. All three AaHsp70A-1373 lines, P2, P5 and P10, displayed banding patterns that likely indicate single insertions (Figure 4.3).

Promoter activity in transgenic Aedes aegypti

AaHsp70A-1373 and AaHsp70B-1456 activity was characterized in larvae, pupae and adult transformed individuals. Heat inducible expression was demonstrated in all three AaHsp70A-1373 lines in both larvae and pupae (Figure 4.4). Induction ranged from 1.7 to 9 fold under heat shock conditions, with significant differences between control values and heat shock values for all lines. Significance was determined using Tukey's Honestly Significant Difference (HSD) test on luciferase values transformed into ranks. Heat shock induction in adults ranged from 5.6-fold in P2, to 12-fold in P5, and 13-fold in P10 adults. Statistically significant heat induced changes in expression compared to control values were noted in each line according to Tukey's HSD (Figure 4.5). These results indicated the AaHsp70A-1373 was capable of driving robust heat induced expression in adults for all three lines.

The same experiments were performed on AaHsp70B-1456 transformed larvae, pupae and adults. Changes in expression upon heat induction were not as strong in AaHsp70B-1456

Table 4.3 *Mos1*-mediated transformation of *Aedes aegypti* with *AaHsp70* promoter cassettes

Mos1 Donor	# embryos injected	# G_0 survivors (%)	# Pools	# G_1 progeny screened	Pools with $DsRed^+$ progeny (+/total)	$G2^+$/Total (%+)
AaHsp70 A-1373-Luc	1508	556 (36.87%)	11	23 438	P2 (31+/1774)	102/249 (41%)
					P5 (29+/2851)	128/220 (58%)
					P10 (6+/2008)	127/294 (43%)
AaHsp70 B-1456-Luc*	2524	459 (18.2%)	17	~13 300	P1 (5+)	180/290 (62%)
					P3 (19+)	215/450 (48%)
					P6 (17+)	160/312 (51%)
					P7 (7+)	92/200 (46%)

* Lines created by Azadeh Aryan

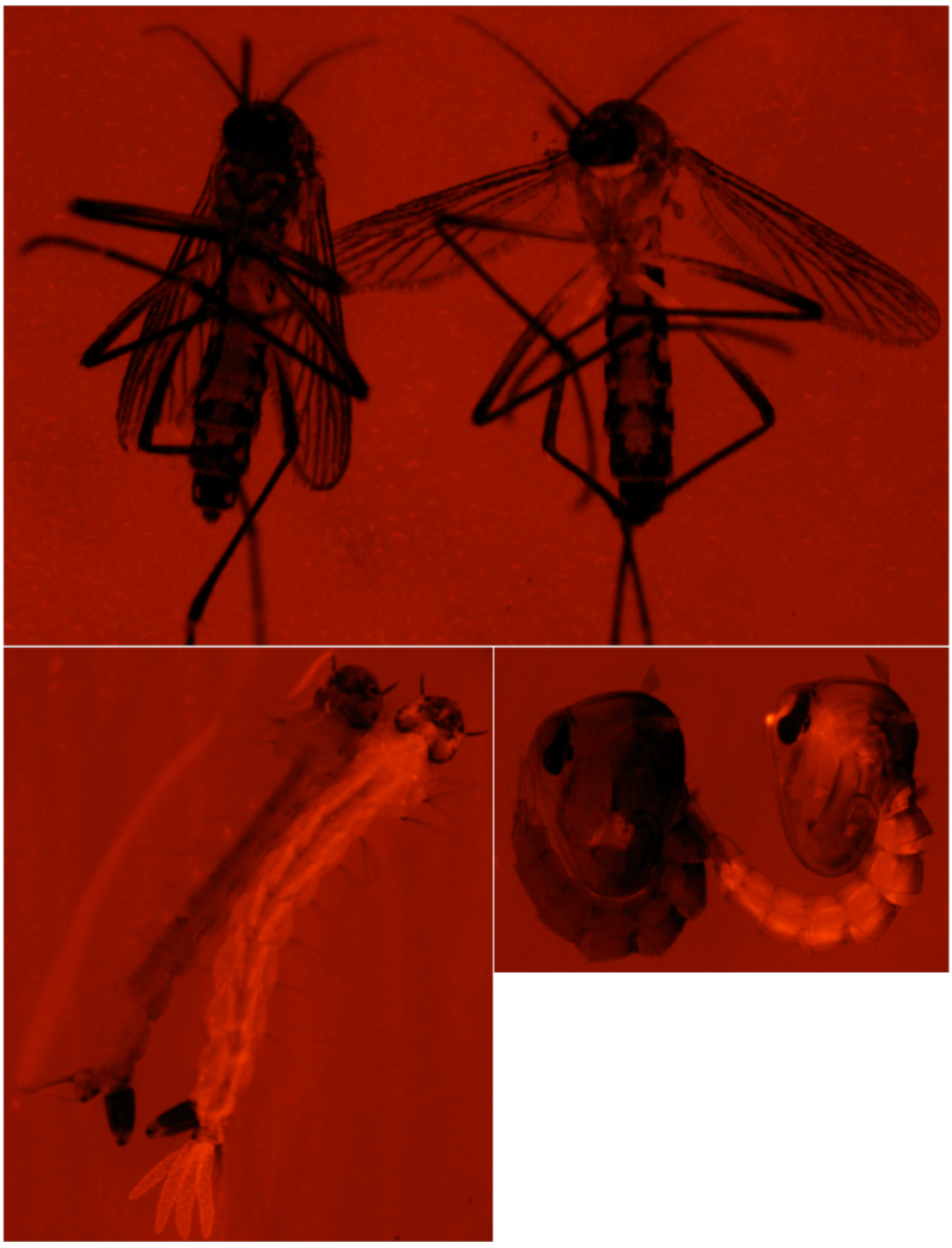


Figure 4.2 pMos-PUbDsRed-AaHsp70A-1373-Luc transformed *Aedes aegypti*. L4 larvae, pupae, and adults exhibit robust, full-body expression of the DsRed marker gene. In order to identify transformants, larvae or pupae are screened for DsRed expression.

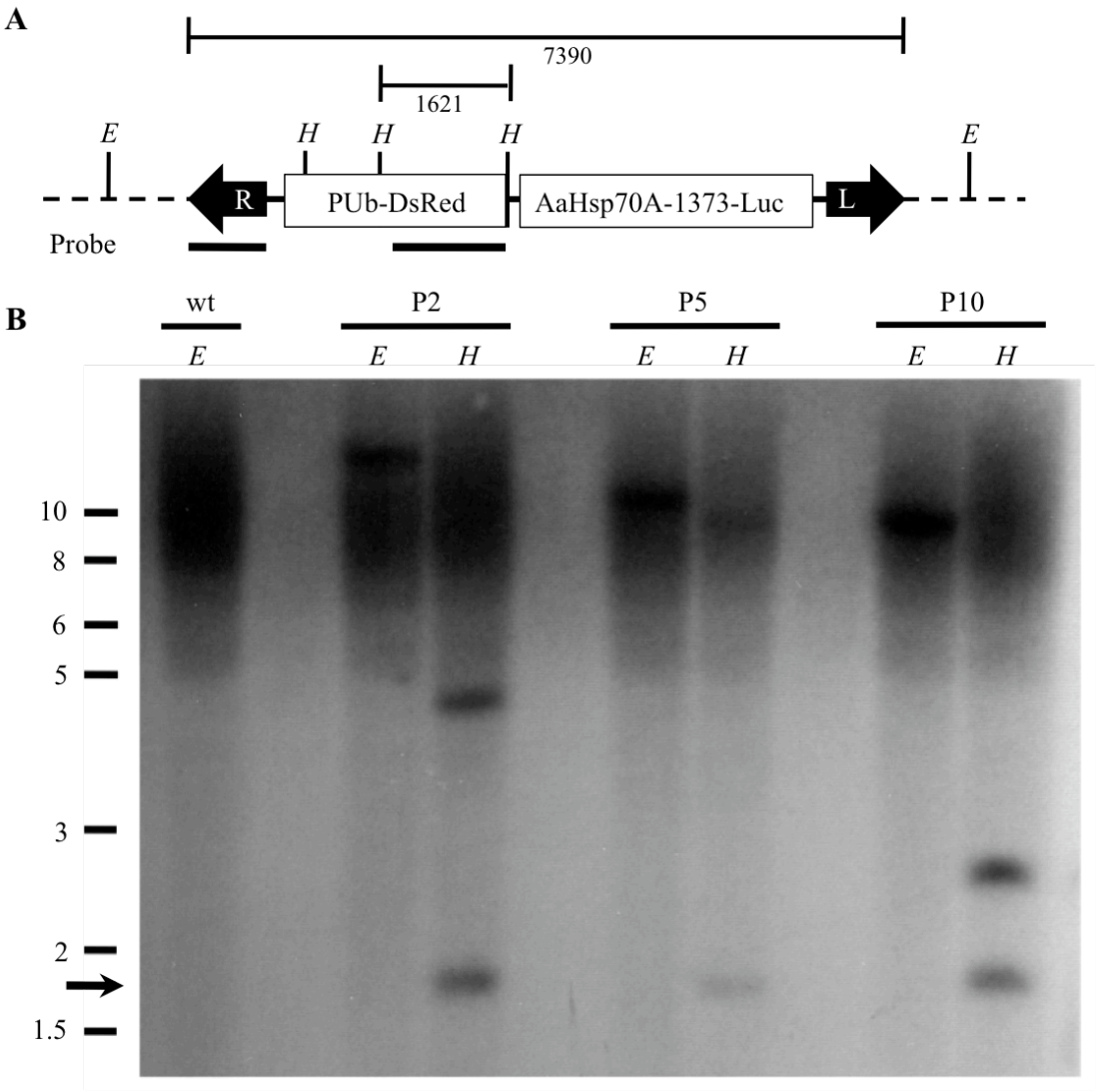


Figure 4.3 Southern analysis of AaHsp70A-1373 in *Mos1* transformed *Aedes aegypti*. (A) Hypothetical representation of a transgene insertion. Block arrows represent right (R) and left (L) arms of *Mos1* transposon. The dashed line indicates *Aedes aegypti* genomic DNA. Predicted restriction sites for *Eco*RI (*E*) and *Hind*III (*H*) are indicated above the construct. The bars above the construct indicate the size of the entire insertion as well as the expected size of one of the bands resulting from *Hind*III digestion. (B) Genomic DNA from wild type (wt), P2, P5 and P10 transgenic mosquitoes was hybridized with a probe corresponding to the *Mos*1 right arm and DsRed cassette, as indicated by the bars below the construct in A. Molecular weight markers are indicated to the left of the image as well as an arrow indicating the size of the expected hybridization signal.

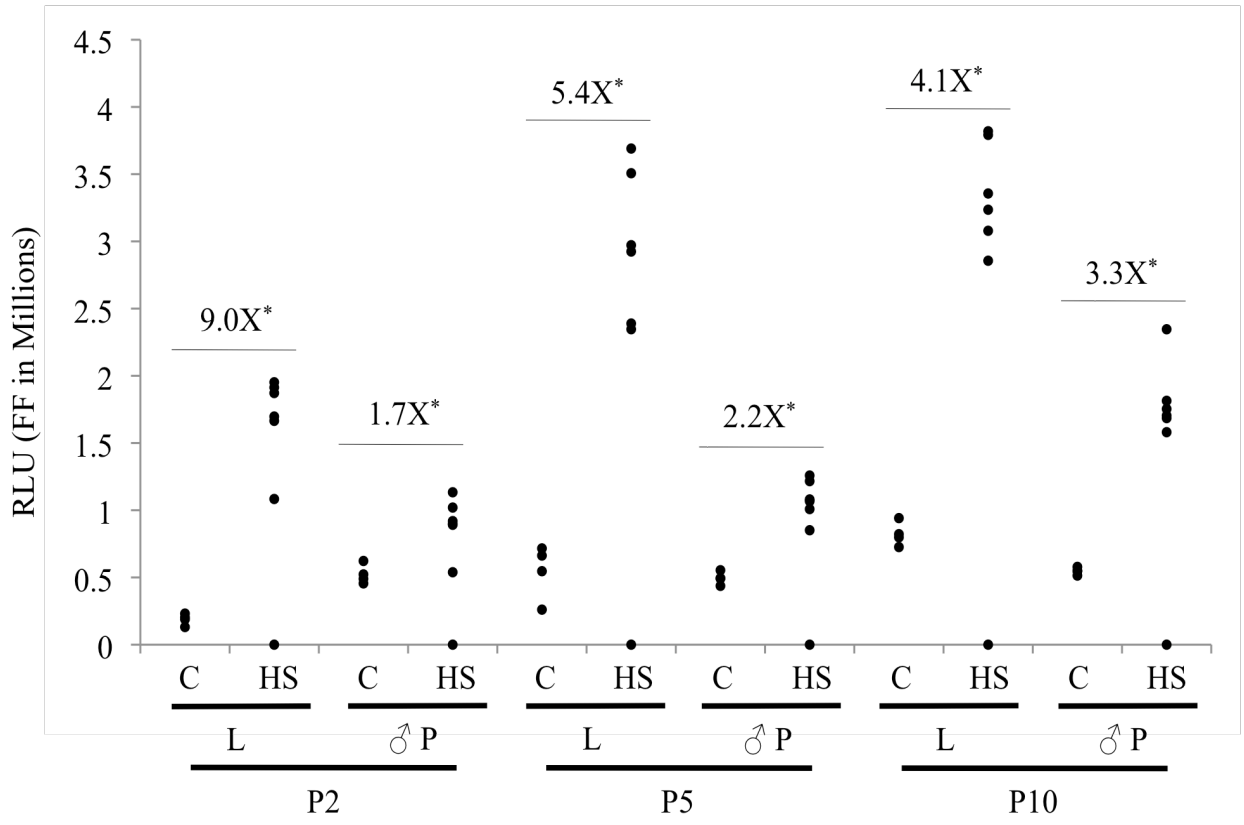


Figure 4.4 Analysis of AaHsp70A-1373 promoter activity in transgenic *Aedes aegypti* larvae and pupae. pMos-PUb-AaHsp70A-1373 transformed pools of 5 larvae (L) and 5 male pupae (P) were heat shocked at 39°C for 1 hour, then allowed to recover for 1 hour Before luciferase assays were performed. Fold changes between control and heat shock values are marked, and values marked with a star are significantly different from control values within the experimental group according to Tukey's HSD performed on ranked values.

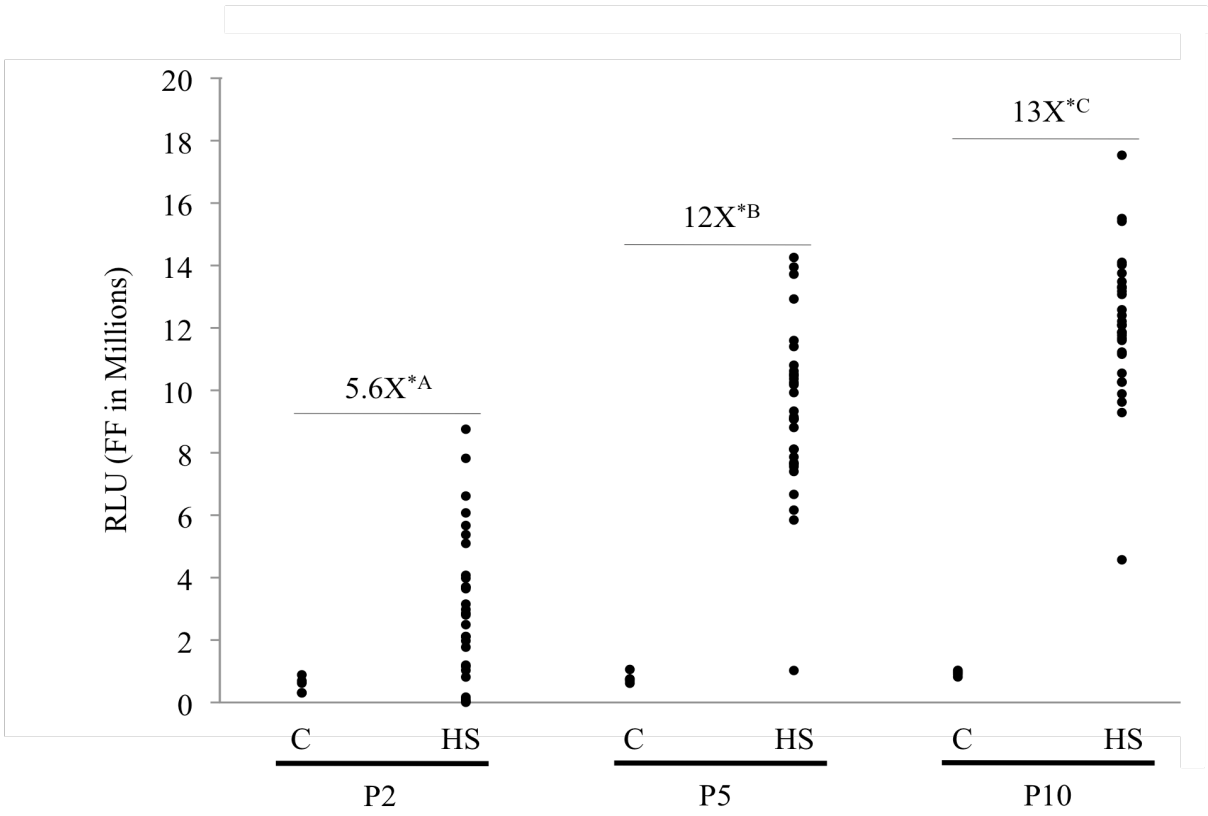


Figure 4.5 Analysis of AaHsp70A-1373 promoter activity in transgenic adult female *Aedes aegypti*. pMos-PUb-AaHsp70A-1373 transformed adult females were heat shocked at 39°C for 1 hour, then allowed to recover for 1 hour. AaHsp70A-1373 activity was measured via luciferase assays, and fold changes in luciferase activity are indicated. Values marked with a star are significantly different than control values within the experimental group, and values not connected by the same letter are significantly different according to Tukey's HSD performed on ranked values.

transformed larvae and pupae as compared to AaHsp70A-1373 (Figure 4.6). Heat shock values were significantly different from controls only in P1 larvae, which exhibited approximately 13.6 fold induction under heat shock. Fold changes under heat shock for the remaining larvae and pupae ranged from 0.8 to 5. In contrast, AaHsp70B-1456 transformed adults exhibited significant changes under heat shock for all lines (Figure 4.7). Fold changes ranged from 10.2X for P1 to 13.4X for P7, with P3 and P6 around 12X.

AaHsp70A-1373 promoter activity in specific tissues

In order to determine the pattern of AaHsp70A-1373-driven expression, luciferase activity was assayed in specific tissues in each of the three separately transformed lines of mosquitoes (Figure 4.8). Statistically significant levels of heat induction were documented in heads in all three lines, and expression levels between each line were similar. The fold change in luciferase levels between control and heat shock was similar for P2 and P10, and slightly lower for P5 (Figure 4.8A). The level of heat induced luciferase activity in salivary glands was statistically similar between all three lines, but was not significantly higher than control values for any line (Figure 4.8B). In midguts, heat induced luciferase activity levels were significantly different from control values in all three lines, and were also statistically different between each line, with the highest levels of expression in P10 and the lowest in P5 (Figure 4.8C). However, the level of induction for P5 was notably higher (166.1X)

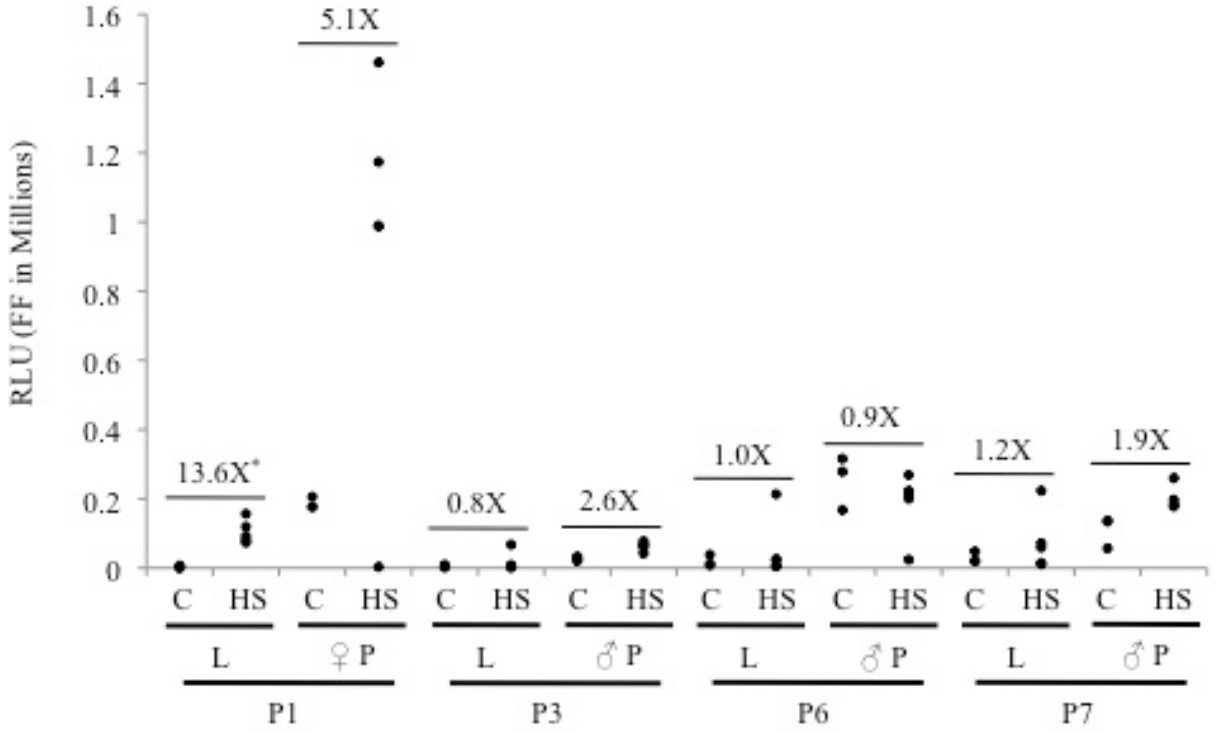


Figure 4.6 Analysis of AaHsp70B-1456 promoter activity in transgenic *Aedes aegypti larvae and pupae*. pMos-PUb-AaHsp70B-1456 transformed pools of 5 larvae (L) and 5 male or female pupae (P) were heat shocked at 39°C for 1 hour, then allowed to recover for 1 hour. AaHsp70B-1456 activity was measured via luciferase assays, and fold changes in expression are marked above each group. Values marked with a star are significantly different than control values within the experimental group according to Tukey's HSD performed on ranks.

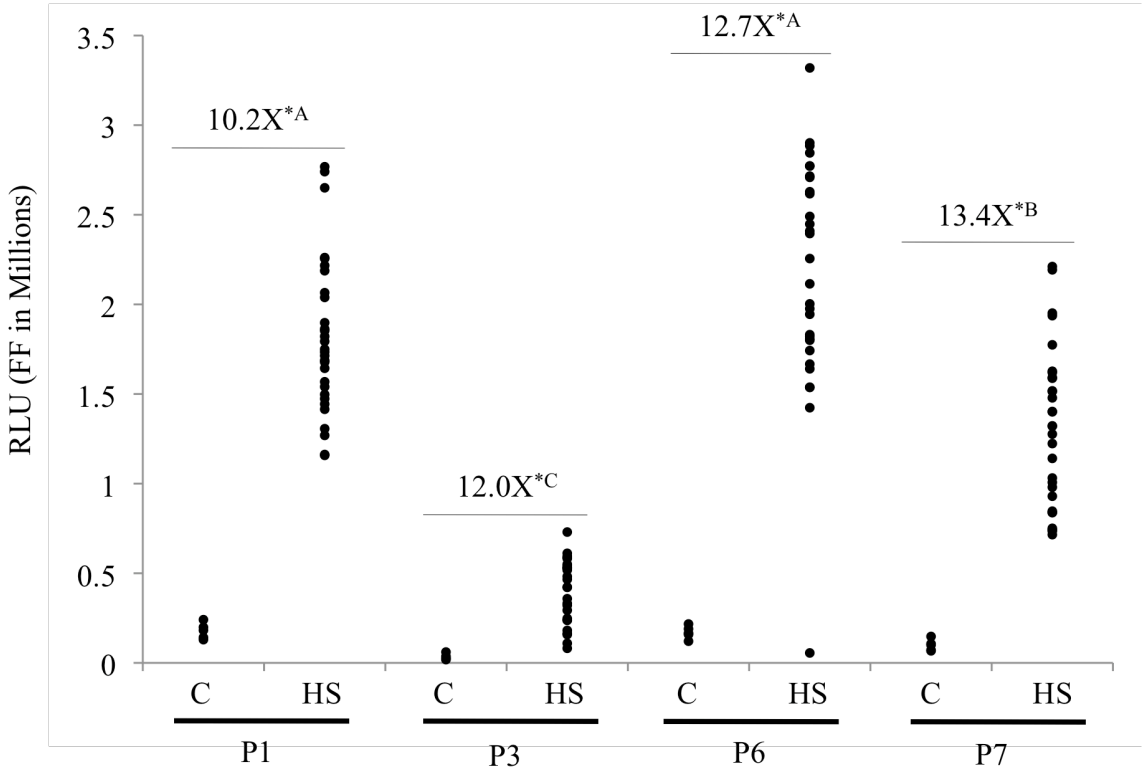


Figure 4.7 Analysis of AaHsp70B-1456 promoter activity in transgenic adult female *Aedes aegypti*. pMos-PUb-AaHsp70B-1456 transformed adult females were heat shocked at 39°C for 1 hour, then allowed to recover for 1 hour. AaHsp70B-1456 activity was measured via luciferase assays. Heat shock values marked with a star are significantly different than control values within the experimental group. Heat shock values not connected by the same letter are significantly different according to Tukey's HSD performed on ranked values.

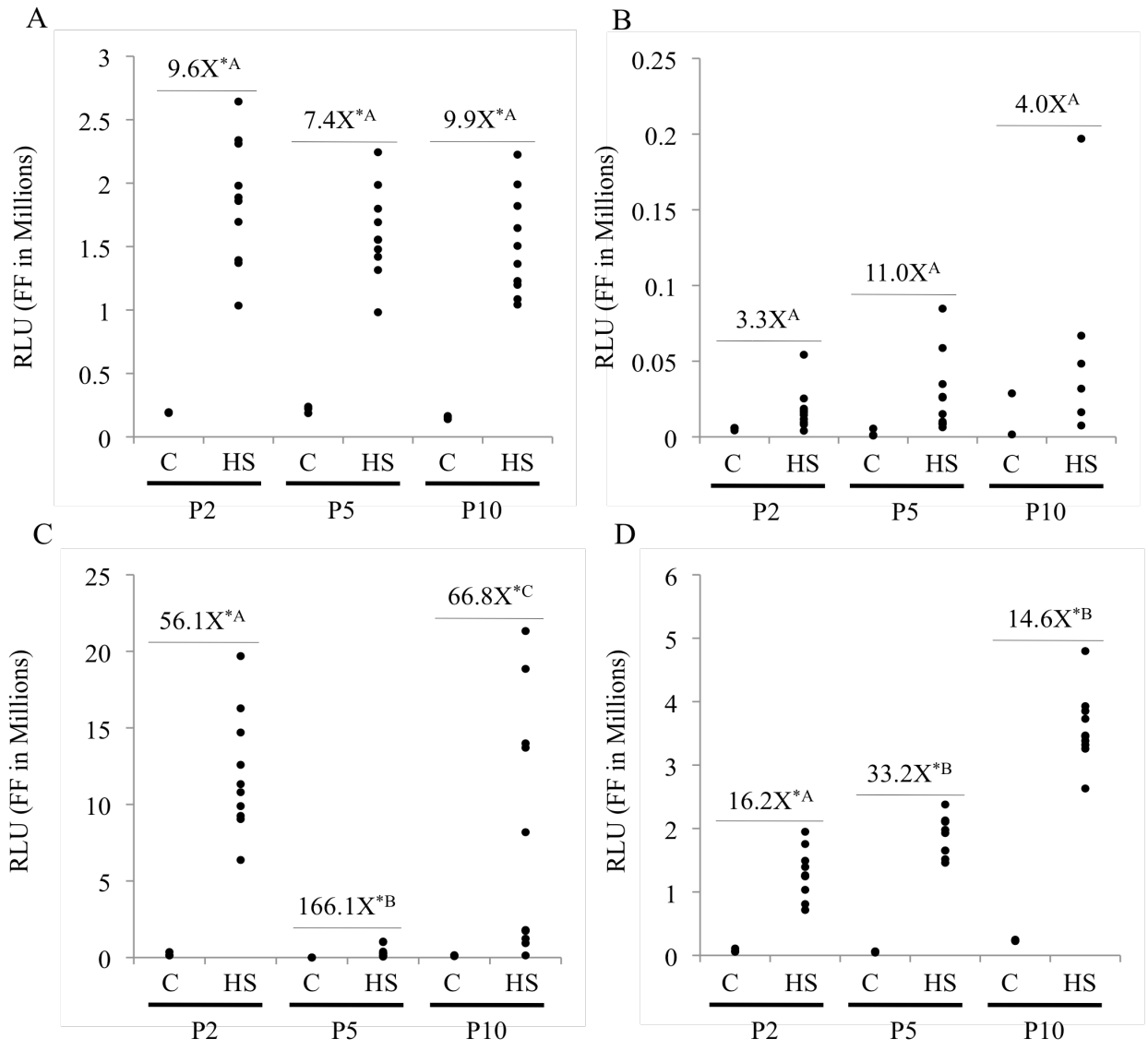


Figure 4.8 Analysis of AaHsp70A-1373 promoter activity in transgenic *Aedes aegypti* tissues. pMos-PUb-AaHsp70A-1373 transformed adult females were heat shocked at 39°C for 1 hour, then allowed to recover for 1 hour before being dissected 2-4 hours post heat shock. AaHsp70A-1373 activity was measured in heads (A), salivary glands (B), midguts (C), and ovaries (D) via luciferase assays. Fold changes due to heat shock are marked above each group, and those marked with have heat shock values that are significantly different from control values. Groups not connected by the same letter have heat shock values that are significantly different between lines according to Tukey's HSD performed on ranked values.

than both P2 (56.1X) and P10 (66.8X). Overall, induction levels were highest in midguts. Significant heat induced expression was also observed in all lines in ovaries, with expression levels for P2 that were significantly different from P5 and P10 (Figure 4.8D). Induction levels exhibited a pattern similar to those in salivary glands, with the highest level of induction in P5 and similar levels of induction in P2 and P10.

AaHsp70A-1373 heat shock time course

In an effort to measure the accumulation of transcripts and protein at specific times, AaHsp70A-1373 P10 adult females were heat shocked and transcript abundance was measured by Northern analysis while luciferase activity was measured in individuals. Northern analysis of heat shocked AaHsp70A-1373 P10 RNA revealed a pattern of luciferase mRNA levels similar to native AaHsp70 genes described previously (Gross *et al.* 2009), with abundant transcripts initially that taper off and are almost undetectable 24 hours post heat shock (Figure 4.9). When the level of luciferase transcripts was compared with the level of *AaHsp70* transcripts, luciferase transcripts were less abundant overall. It should be noted that the probe used to detect *AaHsp70* transcripts recognized all 12 AaHsp70 genes, while the luciferase probe recognized only transcripts resulting from AaHsp70A-1373-Luc expression. When compared to *AaHsp70* transcript abundance at 4 hours which exhibited the highest transcript abundance, luciferase transcripts were 20, 18, 13, and 6 percent of the expression at 0, 1, 2, and 4 hours respectively. Compared to transcript abundance at 4 hours, *AaHsp70* transcripts were 75, 65, and 81 percent as abundant at 0, 1, and 2 hours.

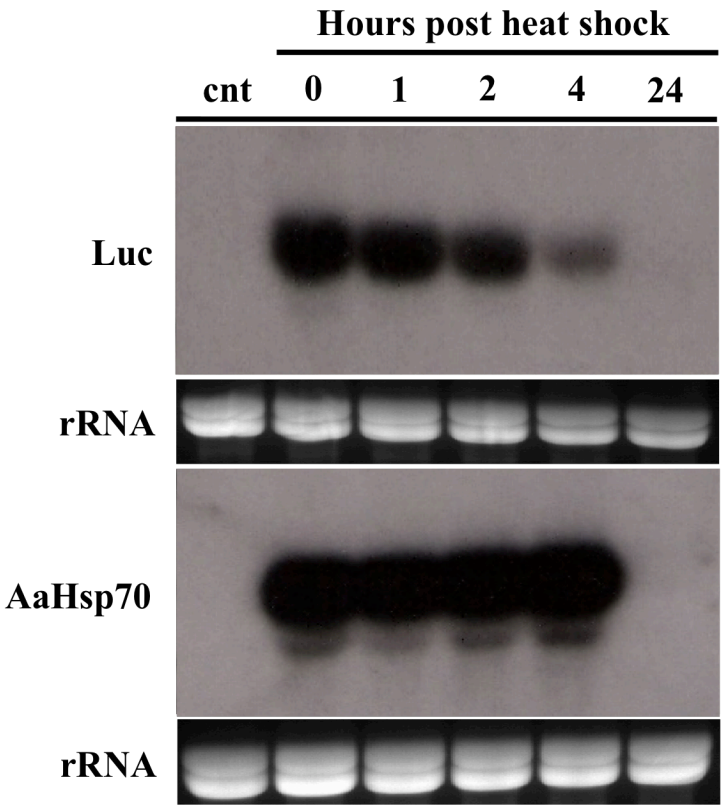


Figure 4.9 Luciferase and *AaHsp70* transcript abundance in transgenic *Aedes aegypti* following heat shock. AaHsp70A-1373 P10 adult females were heat shocked at 39°C for one hour. Pools of 6 females were collected and snap frozen 0, 1, 2, 4, and 24 hours post heat shock. Total RNA was hybridized either with a probe recognizing luciferase (specific activity = $1.2x10^8$) or AaHsp70 transcripts (specific activity = $1.5x10^8$). Blots were given equal exposure time within the same cassette. Control mosquitoes (cnt) were not heat shocked. Ethidium bromide stained rRNA loading controls are shown below each blot.

The number of light units produced in luciferase assays has been found to be proportional to the number of luciferase molecules in a given sample (de Wet *et al.* 1987, Nguyen *et al.* 1988) and therefore provide a good indicator for the amount of functional protein. As such, luciferase assays implied a pattern of protein abundance similar to that of transcript abundance (Figure 4.10). Luciferase activity increased from low levels under control conditions (6.84×10^5 RLU) to peak levels at 4 hours post heat shock (1.76×10^7 RLU) followed by declining levels 24 hours post heat shock (1.3×10^6 RLU). However, luciferase activity was not as strong immediately after heat shock and also did not dissipate as quickly as transcripts after heat shock.

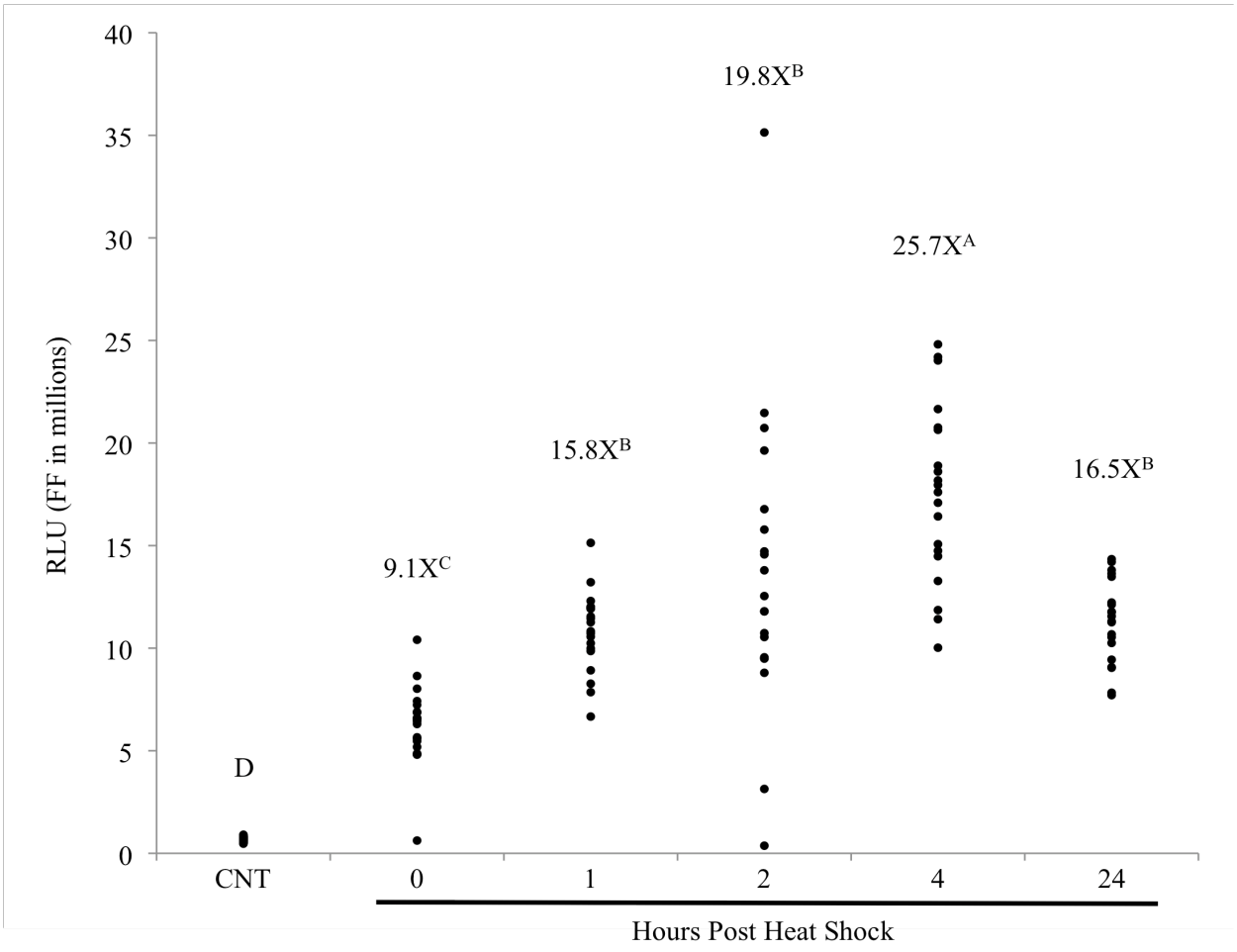


Figure 4.10 Luciferase activity in transgenic *Aedes aegypti* following heat shock. pMos-PUbDsRed-AaHsp70A-1373-Luc transformed adults from P10 were heat shocked for 1 hour at 39°C. Individual adults were collected immediately after heat shock, 1, 2, 4, and 24 hours post heat shock and luciferase activity was measured. Fold changes in heat shock values compared to control values are indicated, and those not connected by the same letter are significantly different according to Tukey's HSD performed on ranks.

DISCUSSION

Aedes aegypti were successfully transformed with both AaHsp70A-1373-Luc and AaHsp70B-1456-Luc gene cassettes. Both promoter elements were found to drive heat inducible expression in multiple life stages, and in multiple tissues, verifying that both elements behave as inducible promoters.

Each putative promoter was found to be active in larvae, pupae and adults to varying degrees. Expression in various life stages was expected, as previous studies in *D. melanogaster* have confirmed heat induced Hsp70 expression in larvae and pupae (Feder *et al.* 1996) as well as adults (Dahlgaard *et al.* 1998), and *AaHsp70* genes have been shown to be expressed under heat shock conditions in these life stages (Gross *et al.* 2009). Expression levels under heat shock conditions for AaHsp70A-1373 were significantly higher than control levels in larvae, pupae, and adults, however expression levels driven by AaHsp70B-1456 were found to be significantly higher than control levels only in adults and P1 larvae. Variation between siblings was also noted in the various life stages with some individuals exhibiting significantly higher or lower expression levels than others, especially in P1 pupae.

Overall, AaHsp70A-1373 was found to drive higher expression levels than AaHspB-1456. Lower expression levels driven by AaHsp70B-1456 may be due to the presence of fewer heat shock transcription factor (HSTF) binding sites, as previous studies in *D. melanogaster* have shown that expression levels are positively correlated with more copies of HSTF binding sites (Amin *et al.* 1987). Upstream HSTF binding sites allow for cooperative binding of transcriptional machinery to downstream elements, thus increasing transcriptional activity. As described in Chapter 3, AaHsp70B-1456 contains 3 putative HSTF binding sites with 100 percent identity (Figure 3.5), whereas AaHsp70A-1456 contains 10 such sites.

Robust expression of AaHsp70A-1373-Luc was observed in all tissues tested, consisting of heads, salivary glands, midguts, and ovaries. Heat induced expression of native Hsp70 has been previously confirmed in *D. melangaster* in all larval tissues assayed, including brain, salivary glands, midgut and female gonadal disks (Krebs and Feder 1997). All tissues dissected from transgenic *Aedes aegypti* exhibited significantly higher levels of expression under heat shock conditions compared to control conditions with the exception of salivary glands. Expression levels between AaHsp70A-1373 lines in both midguts and ovaries were found to be significantly different from each other. This difference in expression patterns could be a result of chromosomal location of the transgene insertion, with regulatory elements capable of acting over long distances (Chambeyron and Bickmore 2004). It is possible that regulatory elements from other genes around the inserted constructs could act in a repressive manner. Additionally, studies in *D. melanogaster* larval tissues suggest a difference in the expression kinetics of Hsp70 in different tissues (Krebs and Feder 1997). The brain, salivary glands, imaginal disks, and hindgut were found to express Hsp70 more quickly in response to heat shock than the fat body, caeca, midgut and Malphigian tubules. While tissue specific kinetics of adult Hsp70 expression have not been established, it may be possible that the timing of Hsp70 expression varies in *Aedes aegypti*.

The steady state level of luciferase transcripts and protein was measured using Northern analysis and luciferase assays at specific times after heat shock. Luciferase transcripts were initially very abundant after heat shock, which is similar to the pattern exhibited by AaHsp70 genes (Gross *et al.* 2009). However, luciferase transcripts levels decreased quickly when compared to *AaHsp70* transcripts. This is notable because transformation constructs did not include the native AaHsp70 3' untranslated region (UTR). This region is important for heat

shock transcript regulation in *Drosophila melanogaster* as it targets messages for degradation once stress conditions have passed (Petersen and Lindquist 1989, Feder *et al.* 1992, Dellavalle *et al.* 1994). Without the native AaHsp70 3'UTRs, the stability of luciferase transcripts was expected to increase. However, transcripts were degraded in a pattern similar to native AaHsp70 transcripts resulting from lower levels of heat shock, as described in Chapter 2. Protein stability was measured indirectly through luciferase assays. Luciferase has a half-life of approximately 3 hours at 37°C, making it a more suitable reporter protein compared to other more stable proteins (Thompson *et al.* 1991). Enzyme activity increased from 1 to 4 hours post heat shock before returning to approximately the same level as 1 hour post heat shock by 24 hours.

AaHsp70 transcripts could not be detected by Northern analysis in non-heat shocked *Aedes aegypti*, however, some luciferase activity is present in control samples. Basal levels of Hsp70 protein abundance have not been established in *Aedes aegypti*, so it may be possible that there is some level of basal protein expression under normal rearing conditions that cannot be detected with this assay. High throughput sequencing techniques would allow for the detection of rare transcripts and could establish whether native *AaHsp70* transcripts are present under normal conditions.

In conclusion, AaHsp70 promoter regions have been shown to act as inducible promoters in multiple life stages and tissues. These promoters could be useful for whole-body gene knockdown studies, especially of genes which would require precise timing of knockdown such as those important to development. Also, AaHsp70 promoters can be used to study the effect of a range of treatments on *Aedes aegypti* as indicators of stress conditions. While the stress response of *D. melanogaster* has been studied extensively (as reviewed in Craig 1985, Lindquist 1986), there is only limited understanding of the conditions which induce stress response in

Aedes aegypti. The transgenic lines created for this study could be useful in establishing a range of conditions that induce heat shock gene expression. Also, high throughput sequencing techniques could be used to compare the expression of endogenous *AaHsp70* genes under different conditions. This technique is highly sensitive and could not only detect rare transcripts, but it could also differentiate between *AaHsp70* genes due to polymorphisms in the open reading frames. This would also contribute to the knowledge of the heat shock response in *Aedes aegypti*.

Chapter 5

Characterization of the effect of heat shock on the endogenous *Aedes aegypti* RNAi pathway

INTRODUCTION

RNA interference (RNAi) is an innate immune defense pathway against viral infection that has been shown to act in *Aedes aegypti* and other species, including *C. elegans* and *D. melanogaster* (Ding 2010). RNAi is triggered by double stranded RNA, which is processed into short interfering RNAs (siRNAs). One strand of the siRNA is loaded into the RNA-induced silencing complex (RISC), which targets homologous transcripts.

Heat shock 70 proteins may also play a role in the response to viral infection, however the role that Hsp70 proteins in modulation of viral infection has not been well characterized. Some studies suggest that heat shock proteins accumulate as a result of viral infection and act to facilitate viral proliferation (Phillips *et al.* 1991, Glotzer *et al.* 2000, Serva and Nagy 2006). However, the mechanism of this induction of heat shock protein synthesis is not well understood. Other studies suggest that Hsp70 is associated with viral entry into cells (Reyes-Del Valle *et al.* 2005). Cytopathic effects resulting from viral infection may also cause increased levels of Hsp70 expression, where Hsp70 is acting to protect the cell rather than facilitate viral replication (Sim *et al.* 2005). Interestingly, the *Anopheles gambiae* heat shock 70 cognate protein was found to impede o'nyong-nyong virus replication (Sim *et al.* 2007). Still other studies suggest that heat shock 70 proteins have both positive and negative effects on viral replication (Nover 1991, Weeks *et al.* 2010). Without a proper understanding of how Hsp70 modulates viral infection, further investigation is necessary.

It is not currently known whether heat shock has an effect on the RNAi pathway. Increased seasonal temperatures in areas with endemic arboviral diseases have been associated with more frequent outbreaks of disease, however the interplay of temperature and disease transmission is complicated (consult Weaver and Reisen 2010). Increased temperatures are known to decrease the extrinsic incubation period of arboviruses, however other contributing factors are not well understood.

In order to test whether heat shock has a direct effect on the RNAi pathway, "Sensor" mosquitoes (Adelman *et al.* 2008), which express a phenotypic indicator based on the status of the RNAi pathway, were examined for a response to heat shock. *Aedes aegypti* Sensor mosquitoes have been transformed with a construct driving DsRed expressed in the eyes as a marker for transgenesis, followed by EGFP also expressed in the eyes and an inverted repeat sequence derived from a portion of the EGFP open reading frame (Adelman *et al.* 2008). When the RNAi pathway is functioning, the eyes of the mosquito should exhibit DsRed expression but not EGFP expression. However, when the RNAi pathway is compromised, EGFP expression is visible along with DsRed expression. Changes in the levels of EGFP transcripts were noted in heat shocked samples, with some samples containing increased levels of EGFP transcripts. However, changes between heat shocked and control levels of EGFP were not consistent between samples and could not be replicated.

MATERIALS AND METHODS

Heat shock regimen

Transgenic *Aedes aegypti*, Sensor #2, 3xP3 R-G #P11A, and 3xP3 R-G #P11A-Cre, were heat shocked at 39°C for one hour 3-5 days post eclosion, heads were dissected and snap frozen in liquid nitrogen at 12, 24, 48, and 72 hours post heat shock. Control heads were snap frozen at 24, 48 and 72 hours after experimental groups were heat shocked.

For constant rearing assays, Sensor #2 mosquitoes were vacuum hatched and reared at either 28°C, 32°C, or 35°C with approximately 400 larvae per pan. Heads were dissected and snap frozen 1, 3, 5, 7, and 10 days post eclosion.

RNA extraction and cDNA preparation

Total RNA was extracted from pools of 10-15 heads using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was then DNase treated, phenol-chloroform extracted, and ethanol precipitated. Alternatively, RNA was extracted using TRI Reagent RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. First strand cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Real time PCR

Power SYBR® Green PCR Master Mix (Appied Biosystems) was used in PCR reactions with primer concentrations of 250 nM, and 0.2 µl of cDNA. Reactions were run on a Step One Real Time PCR System (Applied Biosystems) as follows: 95°C, 10 min; 40 cycles of 95°C, 15

sec, 51°C, 30 sec, 72°C, 30 sec; followed by a dissociation curve of 95°C, 15 sec, 60°C, 60 sec, with a slow ramp of 0.1°C per second to 95°C taking constant fluorescence measurements (hold at 95°C for 15 sec). Real time PCR was performed in triplicate for each cDNA using primers for EGFP and a reference gene (AaElav) listed in Table 5.1. Analysis was performed using the Step One software (Applied Biosystems) using the ddC_T method. AaElav values were used as the endogenous control and non-heat shocked controls as the calibrator. Age appropriate controls were used in each analysis. The cycle threshold (C_T) and baseline were automatically set.

Bloodfeed Study

Aedes aegypti Liverpool strain mosquitoes were fed either: 28°C sugar, 28°C blood, or 37°C blood. Midguts and thoraxes were dissected from engorged mosquitoes immediately after feeding, 1, 2, and 4 hours post feed. Total RNA was extracted from pools of midguts and thoraxes using TRIzol reagent (Invitrogen) and 5 μg was electrophoresed in a 1.2% agarose, 1X MOPS (0.023 M 3-morpholinopropane sulfonic acid, 0.3 mM NaOAc, 0.2 mM EDTA), and 2% formaldehyde get at 90 V. RNA was blotted onto a positively charged nylon membrane (Immobilon-NY^+; Millipore, Concord, MA). The resulting blot was prehybridized at 65°C in pre-warmed Church's buffer (0.25 M sodium phosphate buffer, 1 mM EDTA, 7% SDS) using a Fisher Isotemp hybridization oven (Fisher, Pittsburgh, PA). A random primed probe was labeled with [α-^{32}P] dATP (specific activity 3000 Ci/mmol) using the Amersham Megaprime DNA labeling system (GE Healthcare, Buckinghamshire, UK). The specific activity of the probe

Table 5.1 Oligonucleotide primers for transgenic real time assays

Primer Name	Sequence (5'-3')
EGFP F	AGCTGGACGGCGACGTAAA
EGFP R	TCGCCGATGGGGGTGTTCTGC
AaElav F	AAAGAAGCTGAACGTGCCATTG
AaElav R	TCTCCTCCCATCGGTGAAAAG

was determined using a Beckman-Coulter LS6500 Multi-purpose Scintillation Counter. The probe was purified using illustra NICK columns (GE Healthcare) and added to pre-warmed Church's buffer to hybridize overnight at 65°C. The blot was washed twice with 2X saline-sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) for 20 min each at 65°C and twice with 0.2X SSC (0.03 M sodium chloride, 0.003 M sodium citrate) and 0.1% SDS for 20 min each at 65°C. The DNA template to be radiolabeled was digested using KpnI from a clone containing a sequence common to all identified AaHsp70 genes (Gross *et al.* 2009).

RESULTS

Real time analysis of EGFP expression in transgenic Aedes aegypti

In order to determine whether EGFP expression was effected by heat shock, transgenic *Aedes aegypti* Sensor mosquitoes were heat shocked and assayed for EGFP using quantitative real time PCR. As EGFP and DsRed expression are both driven by the eye-specific synthetic 3xP3 promoter (Horn *et al.* 2000), a normalizing gene also expressed in the head was necessary. *Aedes aegypti Elav,* whose *Drosophila* ortholog is expressed in neurons (Campos *et al.* 1985), was used to normalize the data (Adelman *et al.* 2008). Three lines were assayed including Sensor mosquitoes, 3xP3 R-G #P11A, and 3xP3 R-G #P11A-Cre. The 3xP3 R-G #P11-A line contains the same DsRed-EGFP construct as the Sensor line but lacks the inverted repeat portion of EGFP. The 3xP3 R-G #P11A-Cre contains EGFP in the same chromosomal location as 3xP3 R-G #P11A with the DsRed excised.

For the Sensor line of mosquitoes, experiments were performed with 12 biological replicates in an effort to achieve consistent results. In the first round of the heat shock experiments, the results seemed to indicate a large increase in the number of EGFP transcripts 12 hours after heat shock at 35°C (Figure 5.1 A). Increases were also noted 12 and 24 hours after

heat shock at 37°C, and 12 hours after heat shock at 39°C. However, this pattern was not seen in any of the other three replicates. The third replicate also indicated potentially significant differences in EGFP transcript abundance after heat shock at 39°C, but the pattern was not demonstrated in any other replicates. For the final set of replicates, the method of RNA extraction was also modified to reduce the amount of sample handling in an effort to avoid degrading RNA samples. However, the modified extraction method did not affect the consistency of the results.

To determine whether changes in EGFP expression were a result of actual changes in the status of the RNAi pathway or a nuance of the assay technique itself, experiments were performed on both 3xP3 R-G #P11A and 3xP3 R-G #P11A-Cre transgenic lines, also with variable results (Figure 5.2, 5.3). In the first replicate of heat shock experiments with 3xP3 R-G #P11A, a significant change in the abundance of EGFP transcripts was noted after heat shock at 39°C. An 18-fold increase was documented 12 hours after heat shock for one of the triplicates in the experiment, as well as increased abundance through the rest of the time course (Figure 5.2). However, this pattern did not hold true within the triplicates of the experiment, or in the second replicate. No significant changes were noted in the second replicate of the heat shock experiment. In experiments with 3xP3 R-G #P11A-Cre, changes of nearly 5-fold were observed in some samples after heat shock at all three temperatures. Again, however, changes in transcript

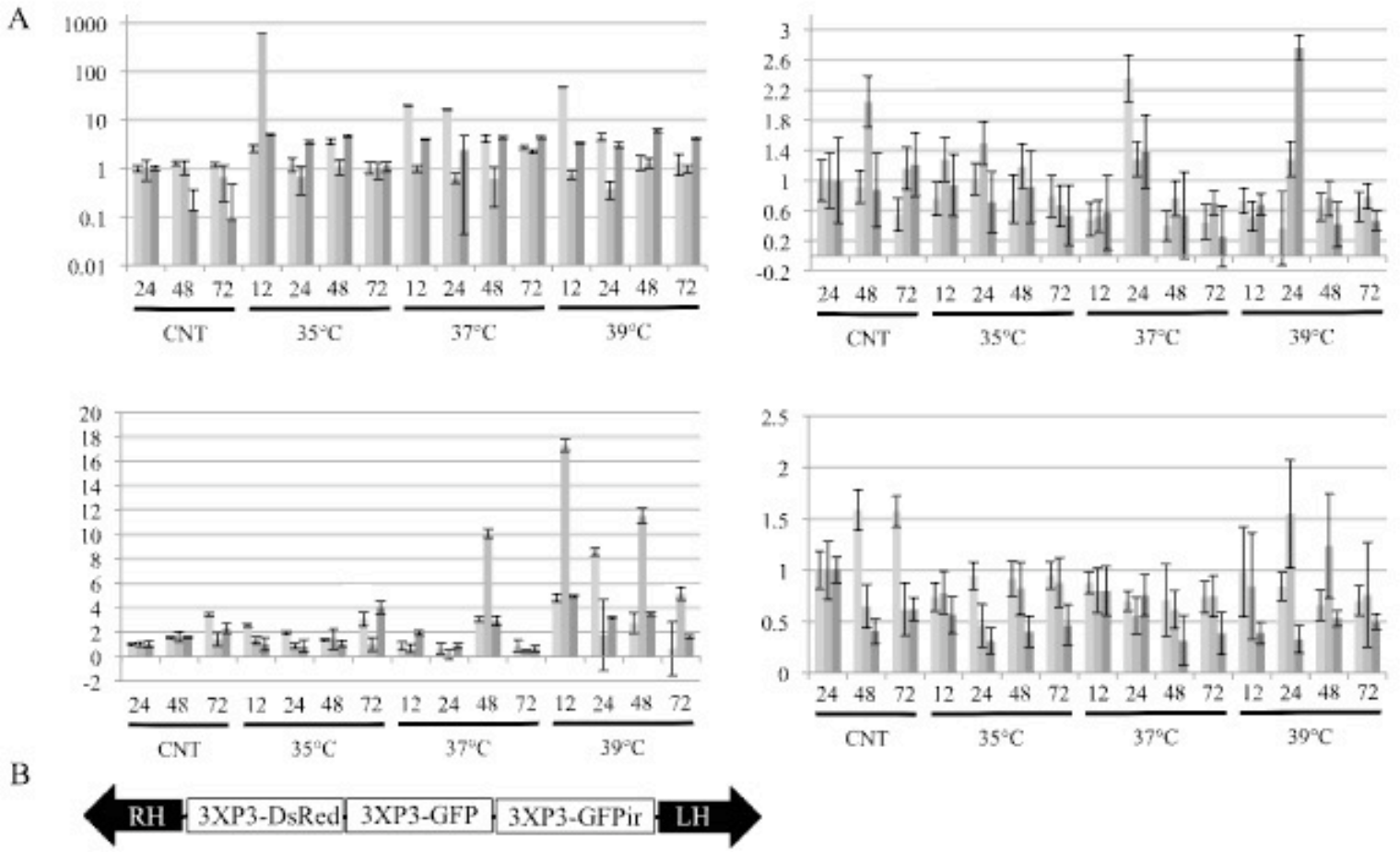


Figure 5.1 Real time analysis of EGFP expression in transgenic Sensor #2 mosquitoes. (A) Sensor #2 mosquitoes were heat shocked at varying temperatures for 1 hour and were harvested over a time course beginning 12 hours after heat shock. The levels of EGFP expression were measured using quantitative real time PCR to determine whether heat shock has a detrimental effect on the endogenous RNAi pathway. (B) A graphical representation of the Sensor construct.

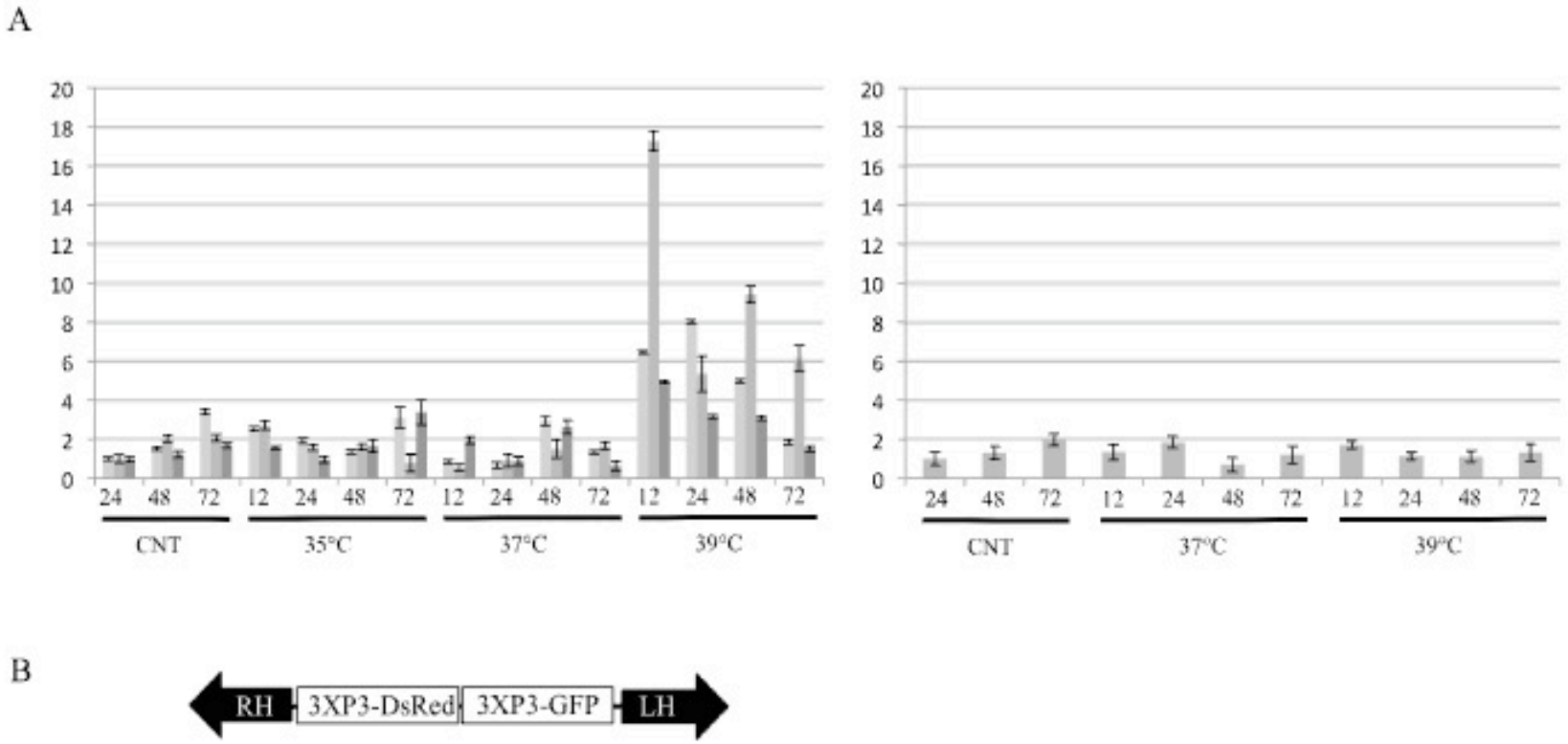
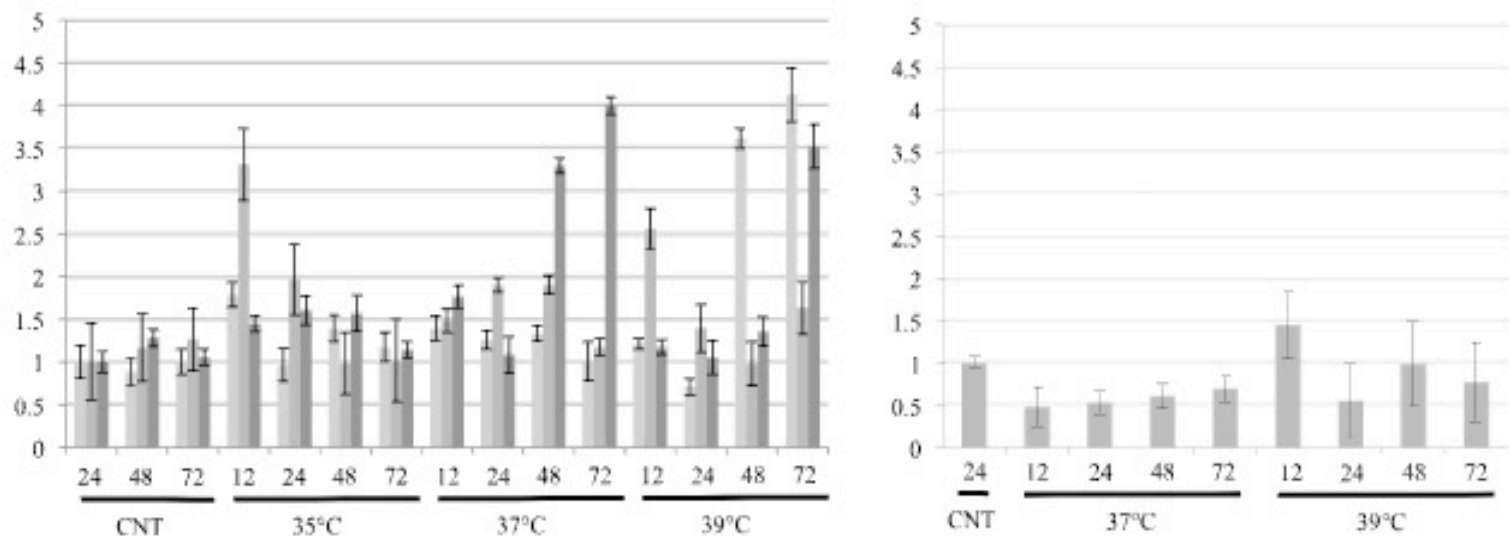


Figure 5.2 Real time analysis of EGFP expression in transgenic 3XP3 R-G #P11A mosquitoes. (A) Transgenic *Aedes aegypti* were heat shocked at varying temperatures for 1 hour and were harvested over a time course beginning 12 hours after heat shock. The levels of EGFP expression were measured using quantitative real time PCR. (B) A graphical representation of the donor construct, which is similar to the Sensor construct but lacking the EGFP inverted repeat.

A



B

Figure 5.3 Real time analysis of EGFP expression in transgenic 3XP3 R-G #P11A-Cre mosquitoes. (A) Transgenic *Aedes aegypti* were heat shocked at varying temperatures for 1 hour and were harvested over a time course beginning 12 hours after heat shock. The levels of EGFP expression were measured using quantitative real time PCR. (B) A graphical representation of the donor construct, which is in the same chromosomal location as the construct in Figure 5.2, but has had the DsRed excised.

abundance were not uniform between samples in the first replicate, and the second replicate did not seem to indicate any significant changes between control and heat shocked samples.

Real time analysis of EGFP expression as a result of varied rearing temperatures

Experiments varying the rearing conditions of transgenic Sensor mosquitoes were also performed. Sensor mosquitoes were reared at either 28°C, 32°C, or 35°C and harvested at 1, 3, 5, 7, and 10 days post eclosion. Triplicate experiments were not possible for mosquitoes reared at 35°C because the majority died before reaching 10 days post eclosion. In the first experimental replicate, one of the triplicates indicated no change in EGFP levels based on rearing conditions, while the other two exhibited variable changes. Results between triplicates were again variable, with no change exhibited when mosquitoes were reared at the highest temperature, and some samples indicating increased levels of EGFP expression even under 28°C control conditions (Figure 5.4). Changes in EGFP transcript abundance remained below 4-fold in these experiments. Overall, it was not possible to accurately determine whether heat shock had an effect on the RNAi pathway using real time PCR to measure EGFP transcripts due to inconsistent results.

Expression of AaHsp70 genes in response to a blood meal

As a whole body heat shock of 35°C is sufficient to induce expression of AaHsp70 genes (Gross *et al.* 2009), the effect of ingesting 37°C blood was investigated to determine whether blood feeding induces a stress response. *Aedes aegypti* mosquitoes were fed 28°C sugar as a control, 28°C blood, or 37°C blood. Northern analysis was performed on RNA extracted from midguts and thoraxes of engorged mosquitoes that had been extracted 0, 1, 2, and 4 hours post blood meal. RNA from midguts dissected from heat shocked adult female mosquitoes was used

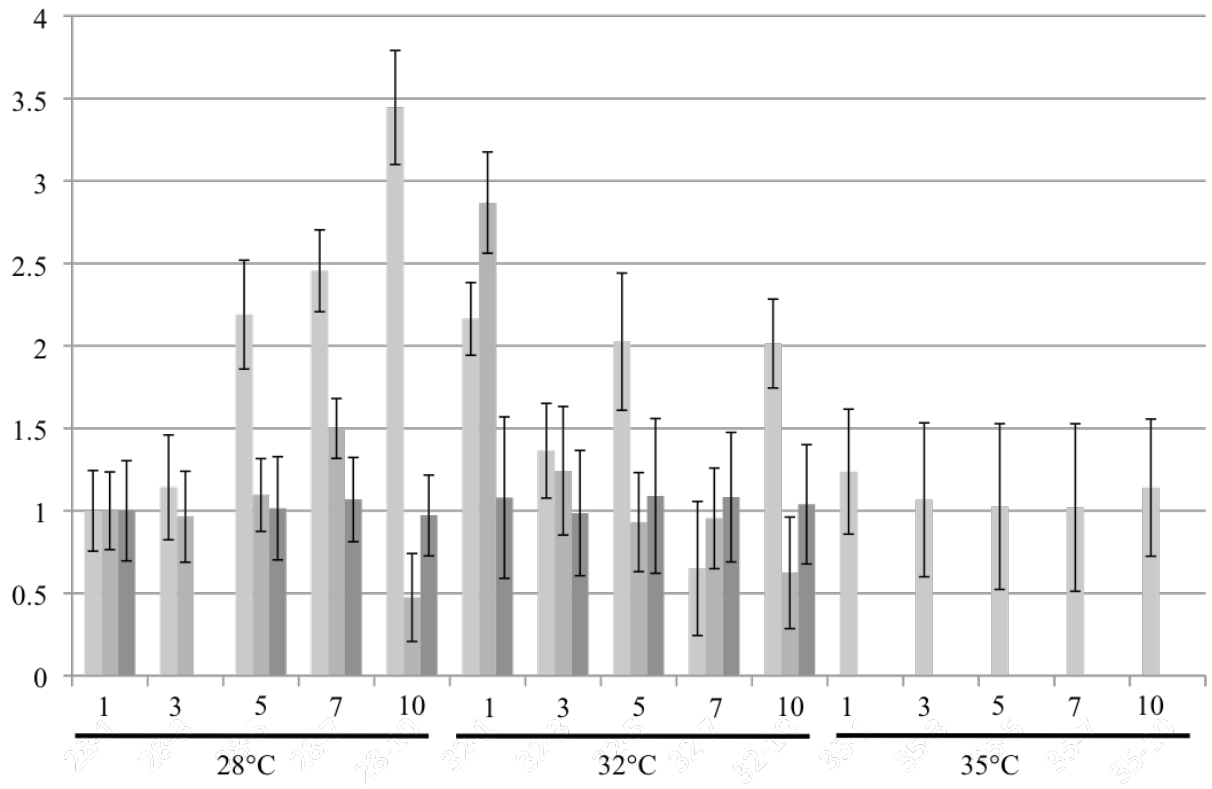


Figure 5.4 Real time analysis of EGFP expression in transgenic Sensor #2 mosquitoes reared at various temperatures. Sensor #2 transgenic *Aedes aegypti* were reared at either 28°C, 32°C, or 35°C and were harvested at 1, 3, 5, 7, or 10 days post eclosion. The relative level of EGFP expression was quantified using real time PCR. Only one replicate was taken at 35°C because the majority of mosquitoes died under these conditions.

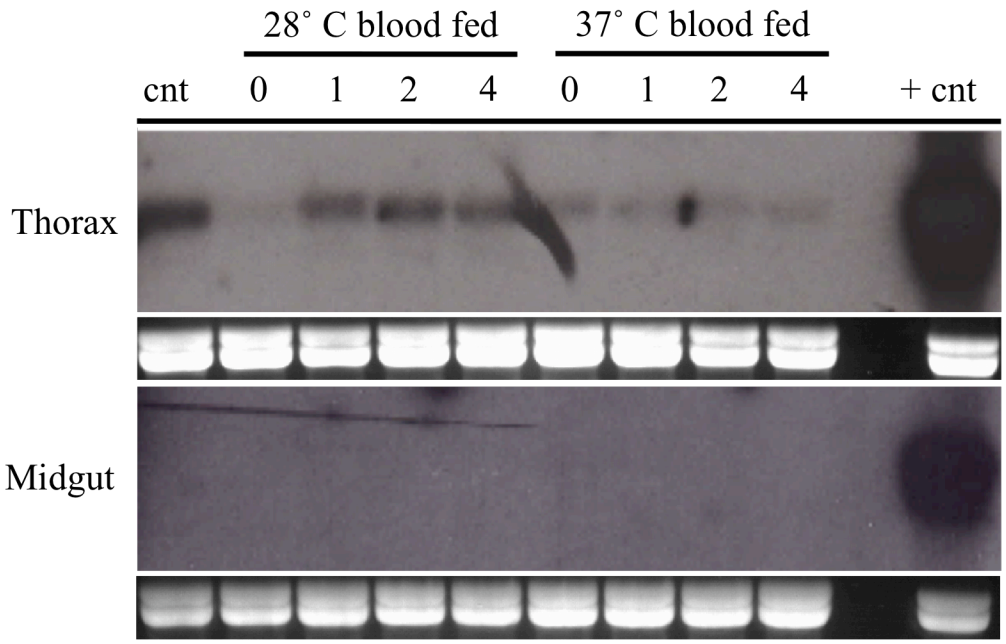


Figure 5.5 Expression of *AaHsp70* genes in response to blood meal. *Aedes aegypti* mosquitoes were fed 28°C or 37°C blood, or 28°C sucrose (cnt) for 30 minutes. Midguts were dissected from engorged mosquitoes immediately after bloodfeeding, 1, 2, and 4 hours post blood feed. Midguts and thoraxes were stored in pools and RNA was extracted. AaHsp70 expression levels were measured using Northern analysis with a probe common to six copies of AaHsp70. Midguts dissected from heat shocked *Aedes aegypti* were used as a positive control (+cnt).

as a control (Figure 5.5). A probe recognizing 6-12 copies of AaHsp70 was used to determine whether blood feeding induces heat shock. No detectable level of AaHsp70 gene expression was noted in any midgut sample. Some expression of AaHsp70 was detectable in thorax samples, though the reason for this is unclear.

DISCUSSION

No definitive conclusions can be made about the effect of heat shock on the RNAi pathway without consistent and reproducible results. In order to get consistent results, the real time PCR assay may need to be modified. The portion of EGFP amplified in these real time assays is located 179 bases from the start of transcription and 301 bases from the first polyadenylation signal. The location of the 5' end of the amplicon makes the assay vulnerable to variability due to RNA degradation, as transcripts may be degraded by exonucleases from the 5' and 3' ends. The location of oligonucleotides used in this assay is limited by the inverted repeat portion of the Sensor construct. The transcripts recognized by the real time assay must be unique to be informative, and therefore the oligonucleotides must be located outside of the repeated portion of EGFP. The oligonucleotides used to amplify *Elav* were located close to the middle of the open reading frame, with the amplicon located 1198 bases from start of transcription and 471 bases from the first polyadenylation signal. Therefore, the normalizing portion of the real time assay is not as vulnerable to variability due to degraded RNA. Luciferase assays are another sensitive assay that could be used to monitor the functioning of the RNAi pathway. Currently, a new "Sensor" construct is being built to utilize the sensitivity and convenience luciferase assays. The *polyubiquitin* (*PUb*) promoter (Anderson *et al.* 2010) will be used to drive robust, whole body expression of both a marker gene and luciferase with a complementary inverted repeat

sequence against luciferase in transgenic *Aedes aegypti*. The *PUb* promoter eliminates the need to assay only head tissue and facilitates faster identification of transgenic individuals. Luciferase assays are also faster and less expensive than real time PCR assays.

Recently, a study was published describing the heat shock response elicited in mosquitoes after a blood meal (Benoit *et al.* 2011). Both *hsp70* transcript and protein abundance were assayed in the study after a blood meal, with each indicating an increase as a result of blood feeding. The Benoit study utilized the Rockefeller strain of *Aedes aegypti*, which may explain the contrasting results of Northern analysis as compared to the results described in this study. The published genome sequence for *Aedes aegypti* was assembled using data from the Liverpool strain, and that data was used for the annotation and characterization of *hsp70* genes described in this study. Concerning the Western analysis of *hsp70* abundance in the Benoit study, the specificity of the antibody was not described. In *D. melanogaster*, hsc70 heat shock cognate genes, which are constitutively expressed and slightly upregulated due to stress, share 75 to 80 percent homology to hsp70 heat shock genes (Palter *et al.* 1986). It is possible that the antibody used in the Benoit study was not specific enough to avoid cross-reaction with heat shock cognates. However, a better description of the Hsp70 antibody may reveal high specificity. Blood meal induced expression of *AaHsp70* genes was not detectable using Northern analysis with a probe previously established to be specific to *AaHsp70* genes. However, high-throughput sequencing is much more sensitive and would be useful in determining whether expression is induced. Not only would all AaHsp70 transcripts be included in the sequencing results, the genes could be individually identified by polymorphisms within the open reading frames. Recently a study was published describing changes in gene expression occurring as a result of blood feeding (Bonizzoni *et al.* 2011). The study utilized Illumina high-throughput sequencing

to compare the transcriptome of blood fed versus sugar fed *Aedes aegypti* mosquitoes. *AaHsp70* transcripts were identified in the Illumina sequencing results, but did not appear to be upregulated due to a blood meal.

While the techniques used to study the effect of temperature on the RNAi pathway and the effect of blood feeding on AaHsp70 gene expression may not have been sufficient in these studies, future experiments using the suggested techniques may lead to more definitive conclusions about these questions. The RNAi pathway is complex and more information is being discovered every day. Since temperature changes are an important aspect of arboviral epidemiology, it is important to continue finding ways to tease out the interactions behind heat wave-associated epidemics. Understanding the role heat shock proteins may play in blood feeding is one important aspect of understanding vector competence as a whole.

Chapter 6

Summary

Aedes aegypti is a medically important vector of dengue viruses and yellow fever virus. The publication of the full length genome sequence for *Aedes aegypti* has made comparative genomics and gene function studies possible. Many of these studies have concentrated on genes important to innate immunity and interaction with viruses.

In this study, we sought to identify and characterize heat shock 70 genes in *Aedes aegypti*. Comparative genomics studies with *D. melanogaster* resulted in the identification of 6-12 *AaHsp70* genes. The full length transcripts were described, and expression profiles documented. *AaHsp70* genes were found to be organized and expressed similarly to *Drosophila melanogaster hsp70* genes with expression in larvae, pupae and adults as well as heads, salivary glands, midguts and ovaries. Additionally, *AaHsp70* genes were found to be differentially regulated. Research in *D. melanogaster* has suggested that each copy of the Hsp70 gene may be individually regulated and may respond to various heat stress conditions differently (Krebs and Feder 1997). Expression of *AaHsp70* genes could serve as an important indicator of stress in *Aedes aegypti*.

Putative promoter sequences were identified upstream of the most highly expressed genes, and were shown to act as inducible promoters in *Ae. aegypti* cells and embryos. Putative heat shock transcription factor binding sites, required for heat inducible expression, were identified in these upstream regions. Additional promoter constructs were designed containing optimal amounts of upstream sequence to maintain heat inducibility while reducing the overall construct size. These constructs were then stably transformed to produce transgenic *Ae. aegypti*.

Expression of both *AaHsp70* promoter constructs was documented in transgenic larvae, pupae and adults, and multiple tissues. These results were consistent with endogenous hsp70 expression patterns in *D. melanogaster*.

No detectable change in the status of the RNA interference pathway was documented as a result of heat shock. Also, no *AaHsp70* gene expression after blood feeding was detectable using Northern analysis. However, it is possible that the techniques used in these studies were not sensitive enough or appropriate to measure changes. High-throughput sequencing techniques may be able to detect changes in the functioning of the RNAi pathway due to heat shock and blood feeding, as well as document individual *AaHsp70* gene expression.

The *AaHsp70* promoters characterized in this study would be useful for driving precisely timed gene knockdown and expression of transgenes throughout the *Aedes aegypti* mosquito. Gene function studies requiring tightly regulated spatial and temporal gene expression will be possible in the same way they were made possible in *Drosophila melanogaster* using the *DmHsp70* promoter. This will lead to further annotation of the *Aedes aegypti* genome. Additionally, the *AaHsp70* promoter may be used to drive transgene expression as a result of different sources of stress, allowing for the investigation of stress-inducing conditions in *Aedes aegypti*. This information may contribute to a better understanding of the effect of stress conditions on the susceptibility of mosquitoes to pathogens.

References Cited

2008. Dengue and dengue haemorrhagic fever, Fact Sheets. WHO.

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y. H. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, K. H. Wan, C. Doyle, E. G. Baxter, G. Helt, C. R. Nelson, G. L. Gabor, J. F. Abril, A. Agbayani, H. J. An, C. Andrews-Pfannkoch, D. Baldwin, R. M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E. M. Beasley, K. Y. Beeson, P. V. Benos, B. P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M. R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K. C. Burtis, D. A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J. M. Cherry, S. Cawley, C. Dahlke, L. B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A. D. Mays, I. Dew, S. M. Dietz, K. Dodson, L. E. Doup, M. Downes, S. Dugan-Rocha, B. C. Dunkov, P. Dunn, K. J. Durbin, C. C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A. E. Gabrielian, N. S. Garg, W. M. Gelbart, K. Glasser, A. Glodek, F. Gong, J. H. Gorrell, Z. Gu, P. Guan, M. Harris, N. L. Harris, D. Harvey, T. J. Heiman, J. R. Hernandez, J. Houck, D. Hostin, K. A. Houston, T. J. Howland, M. H. Wei, C. Ibegwam, et al. 2000. The genome sequence of Drosophila melanogaster. Science 287: 2185-95.

Adelman, Z. N., M. A. Anderson, E. M. Morazzani, and K. M. Myles. 2008. A transgenic sensor strain for monitoring the RNAi pathway in the yellow fever mosquito, Aedes aegypti. Insect Biochem Mol Biol 38: 705-13.

Adelman, Z. N., N. Jasinskiene, S. Onal, J. Juhn, A. Ashikyan, M. Salampessy, T. MacCauley, and A. A. James. 2007. nanos gene control DNA mediates developmentally regulated transposition in the yellow fever mosquito Aedes aegypti. Proc Natl Acad Sci U S A 104: 9970-5.

Adelman, Z. N., N. Jasinskiene, K. J. Vally, C. Peek, E. A. Travanty, K. E. Olson, S. E. Brown, J. L. Stephens, D. L. Knudson, C. J. Coates, and A. A. James. 2004. Formation and loss of large, unstable tandem arrays of the piggyBac transposable element in the yellow fever mosquito, Aedes aegypti. Transgenic Res 13: 411-25.

Aigaki, T., I. Fleischmann, P. S. Chen, and E. Kubli. 1991. Ectopic expression of sex peptide alters reproductive behavior of female D. melanogaster. Neuron 7: 557-63.

Amin, J., J. Ananthan, and R. Voellmy. 1988. Key features of heat shock regulatory elements. Mol Cell Biol 8: 3761-9.

Amin, J., R. Mestril, R. Lawson, H. Klapper, and R. Voellmy. 1985. The heat shock consensus sequence is not sufficient for hsp70 gene expression in Drosophila melanogaster. Mol Cell Biol 5: 197-203.

Amin, J., R. Mestril, P. Schiller, M. Dreano, and R. Voellmy. 1987. Organization of the Drosophila-Melanogaster Hsp70 Heat-Shock Regulation Unit. Molecular and Cellular Biology 7: 1055-1062.

Anderson, M. A., T. L. Gross, K. M. Myles, and Z. N. Adelman. 2010. Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic Aedes aegypti. Insect Mol Biol 19: 441-9.

Arensburger, P., K. Megy, R. M. Waterhouse, J. Abrudan, P. Amedeo, B. Antelo, L. Bartholomay, S. Bidwell, E. Caler, F. Camara, C. L. Campbell, K. S. Campbell, C. Casola, M. T. Castro, I. Chandramouliswaran, S. B. Chapman, S. Christley, J. Costas, E. Eisenstadt, C. Feschotte, C. Fraser-Liggett, R. Guigo, B. Haas, M. Hammond, B. S. Hansson, J. Hemingway, S. R. Hill, C. Howarth, R. Ignell, R. C. Kennedy, C. D. Kodira, N. F. Lobo, C. Mao, G. Mayhew, K. Michel, A. Mori, N. Liu, H. Naveira, V. Nene, N. Nguyen, M. D. Pearson, E. J. Pritham, D. Puiu, Y. Qi, H. Ranson, J. M. Ribeiro, H. M. Roberston, D. W. Severson, M. Shumway, M. Stanke, R. L. Strausberg, C. Sun, G. Sutton, Z. J. Tu, J. M. Tubio, M. F. Unger, D. L. Vanlandingham, A. J. Vilella, O. White, J. R. White, C. S. Wondji, J. Wortman, E. M. Zdobnov, B. Birren, B. M. Christensen, F. H. Collins, A. Cornel, G. Dimopoulos, L. I. Hannick, S. Higgs, G. C. Lanzaro, D. Lawson, N. H. Lee, M. A. Muskavitch, A. S. Raikhel, and P. W. Atkinson. 2010. Sequencing of Culex quinquefasciatus establishes a platform for mosquito comparative genomics. Science 330: 86-8.

Benedict, M. Q., A. F. Cockburn, and J. A. Seawright. 1993. The Hsp70 heat-shock gene family of the mosquito Anopheles albimanus. Insect Mol Biol 2: 93-102.

Benoit, J. B., G. Lopez-Martinez, K. R. Patrick, Z. P. Phillips, T. B. Krause, and D. L. Denlinger. 2011. From the Cover: Drinking a hot blood meal elicits a protective heat shock response in mosquitoes. Proc Natl Acad Sci U S A 108: 8026-9.

Berger, E. M., G. Marino, and D. Torrey. 1985. Expression of Drosophila hsp 70-CAT hybrid gene in Aedes cells induced by heat shock. Somat Cell Mol Genet 11: 371-7.

Bettencourt, B. R., and M. E. Feder. 2001. Hsp70 duplication in the Drosophila melanogaster species group: How and when did two become five? Molecular Biology and Evolution 18: 1272-1282.

Bettencourt, B. R., and M. E. Feder. 2002. Rapid concerted evolution via gene conversion at the Drosophila hsp70 genes. Journal of Molecular Evolution 54: 569-586.

Blair, C. D., Z. N. Adelman, and K. E. Olson. 2000. Molecular strategies for interrupting arthropod-borne virus transmission by mosquitoes. Clin Microbiol Rev 13: 651-61.

Bonizzoni, M., W. A. Dunn, C. L. Campbell, K. E. Olson, M. T. Dimon, O. Marinotti, and A. A. James. 2011. RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, Aedes aegypti. BMC Genomics 12: 82.

Calvo, E., M. Walter, Z. N. Adelman, A. Jimenez, S. Onal, O. Marinotti, and A. A. James. 2005. Nanos (nos) genes of the vector mosquitoes, Anopheles gambiae, Anopheles stephensi and Aedes aegypti. Insect Biochem Mol Biol 35: 789-98.

Campos, A. R., D. Grossman, and K. White. 1985. Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis. J Neurogenet 2: 197-218.

Chambeyron, S., and W. A. Bickmore. 2004. Does looping and clustering in the nucleus regulate gene expression? Current Opinion in Cell Biology 16: 256-262.

Cho, K. H., H. M. Cheon, V. Kokoza, and A. S. Raikhel. 2006. Regulatory region of the vitellogenin receptor gene sufficient for high-level, germ line cell-specific ovarian expression in transgenic Aedes aegypti mosquitoes. Insect Biochem Mol Biol 36: 273-81.

Coates, C. J., N. Jasinskiene, L. Miyashiro, and A. A. James. 1998. Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti. Proc Natl Acad Sci U S A 95: 3748-51.

Coates, C. J., N. Jasinskiene, G. B. Pott, and A. A. James. 1999. Promoter-directed expression of recombinant fire-fly luciferase in the salivary glands of Hermes-transformed Aedes aegypti. Gene 226: 317-25.

Coates, C. J., C. L. Turney, M. Frommer, D. A. O'Brochta, and P. W. Atkinson. 1997. Interplasmid transposition of the mariner transposable element in non-drosophilid insects. Mol Gen Genet 253: 728-33.

Collins, P. L., and L. E. Hightower. 1982. NEWCASTLE-DISEASE VIRUS STIMULATES THE CELLULAR ACCUMULATION OF STRESS (HEAT-SHOCK) MESSENGER-RNAS AND PROTEINS. Journal of Virology 44: 703-707.

Craig, E. A. 1985. The heat shock response. CRC Crit Rev Biochem 18: 239-80.

Dahlgaard, J., V. Loeschcke, P. Michalak, and J. Justesen. 1998. Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult Drosophila melanogaster. Functional Ecology 12: 786-793.

Dai, H., R. Jiang, J. Wang, G. Xu, M. Cao, Z. Wang, and J. Fei. 2007. Development of a heat shock inducible and inheritable RNAi system in silkworm. Biomol Eng 24: 625-30.

de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol 7: 725-37.

Dellavalle, R. P., R. Petersen, and S. Lindquist. 1994. Preferential deadenylation of Hsp70 mRNA plays a key role in regulating Hsp70 expression in Drosophila melanogaster. Mol Cell Biol 14: 3646-59.

Ding, S. W. 2010. RNA-based antiviral immunity. Nat Rev Immunol 10: 632-44.

Evgen'ev, M. B., O. G. Zatsepina, D. Garbuz, D. N. Lerman, V. Velikodvorskaya, E. Zelentsova, and M. E. Feder. 2004. Evolution and arrangement of the hsp70 gene cluster in two closely related species of the virilis group of Drosophila. Chromosoma 113: 223-232.

Feder, J. H., J. M. Rossi, J. Solomon, N. Solomon, and S. Lindquist. 1992. The Consequences of Expressing Hsp70 in Drosophila Cells at Normal Temperatures. Genes & Development 6: 1402-1413.

Feder, M. E., N. V. Cartano, L. Milos, R. A. Krebs, and S. L. Lindquist. 1996. Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of Drosophila melanogaster. J Exp Biol 199: 1837-44.

Fernandes, M., H. Xiao, and J. T. Lis. 1995. Binding of heat shock factor to and transcriptional activation of heat shock genes in Drosophila. Nucleic Acids Res 23: 4799-804.

Fulton, R. E., M. L. Salasek, N. M. DuTeau, and W. C. t. Black. 2001. SSCP analysis of cDNA markers provides a dense linkage map of the Aedes aegypti genome. Genetics 158: 715-26.

Glotzer, J. B., M. Saltik, S. Chiocca, A. I. Michou, P. Moseley, and M. Cotten. 2000. Activation of heat-shock response by an adenovirus is essential for virus replication. Nature 407: 207-211.

Golic, K. G., and S. Lindquist. 1989. The Flp Recombinase of Yeast Catalyzes Site-Specific Recombination in the Drosophila Genome. Cell 59: 499-509.

Gong, W. J., and K. G. Golic. 2004. Genomic deletions of the Drosphila melanogaster Hsp70 genes. Genetics 168: 1467-1476.

Gratz, N. G. 1999. Emerging and resurging vector-borne diseases. Annu Rev Entomol 44: 51-75.

Gross, T. L., K. M. Myles, and Z. N. Adelman. 2009. Identification and characterization of heat shock 70 genes in Aedes aegypti (Diptera: Culicidae). J Med Entomol 46: 496-504.

Gubler, D. J. 1998. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-96.

Gubler, D. J. 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 10: 100-3.

Halstead, S. B. 1984. Selective primary health care: strategies for control of disease in the developing world. XI. Dengue. Rev Infect Dis 6: 251-64.

Halstead, S. B. 1988. Pathogenesis of dengue: challenges to molecular biology. Science 239: 476-81.

Hardy, M. E., L. V. Ross, and C. B. Chien. 2007. Focal gene misexpression in zebrafish embryos induced by local heat shock using a modified soldering iron. Dev Dyn 236: 3071-6.

Harrington, L. C., J. D. Edman, and T. W. Scott. 2001. Why do female Aedes aegypti (Diptera : Culicidae) feed preferentially and frequently on human blood? Journal of Medical Entomology 38: 411-422.

Hay, B. A., C. H. Chen, C. M. Ward, H. Huang, J. T. Su, and M. Guo. 2010. Engineering the genomes of wild insect populations: challenges, and opportunities provided by synthetic Medea selfish genetic elements. J Insect Physiol 56: 1402-13.

Heinemeyer, T., E. Wingender, I. Reuter, H. Hermjakob, A. E. Kel, O. V. Kel, E. V. Ignatieva, E. A. Ananko, O. A. Podkolodnaya, F. A. Kolpakov, N. L. Podkolodny, and N. A. Kolchanov. 1998. Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res 26: 362-7.

Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab, D. R. Nusskern, P. Wincker, A. G. Clark, J. M. Ribeiro, R. Wides, S. L. Salzberg, B. Loftus, M. Yandell, W. H. Majoros, D. B. Rusch, Z. Lai, C. L. Kraft, J. F. Abril, V. Anthouard, P. Arensburger, P. W. Atkinson, H. Baden, V. de Berardinis, D. Baldwin, V. Benes, J. Biedler, C. Blass, R. Bolanos, D. Boscus, M. Barnstead, S. Cai, A. Center, K. Chaturverdi, G. K. Christophides, M. A. Chrystal, M. Clamp, A. Cravchik, V. Curwen, A. Dana, A. Delcher, I. Dew, C. A. Evans, M. Flanigan, A. Grundschober-Freimoser, L. Friedli, Z. Gu, P. Guan, R. Guigo, M. E. Hillenmeyer, S. L. Hladun, J. R. Hogan, Y. S. Hong, J. Hoover, O. Jaillon, Z. Ke, C. Kodira, E. Kokoza, A.

Koutsos, I. Letunic, A. Levitsky, Y. Liang, J. J. Lin, N. F. Lobo, J. R. Lopez, J. A. Malek, T. C. McIntosh, S. Meister, J. Miller, C. Mobarry, E. Mongin, S. D. Murphy, D. A. O'Brochta, C. Pfannkoch, R. Qi, M. A. Regier, K. Remington, H. Shao, M. V. Sharakhova, C. D. Sitter, J. Shetty, T. J. Smith, R. Strong, J. Sun, D. Thomasova, L. Q. Ton, P. Topalis, Z. Tu, M. F. Unger, B. Walenz, A. Wang, J. Wang, M. Wang, X. Wang, K. J. Woodford, J. R. Wortman, M. Wu, A. Yao, E. M. Zdobnov, H. Zhang, Q. Zhao, et al. 2002. The genome sequence of the malaria mosquito Anopheles gambiae. Science 298: 129-49.

Horn, C., B. Jaunich, and E. A. Wimmer. 2000. Highly sensitive, fluorescent transformation marker for Drosophila transgenesis. Dev Genes Evol 210: 623-9.

Ingolia, T. D., E. A. Craig, and B. J. McCarthy. 1980. Sequence of three copies of the gene for the major Drosophila heat shock induced protein and their flanking regions. Cell 21: 669-79.

Isoe, J., S. Kunz, C. Manhart, M. A. Wells, and R. L. Miesfeld. 2007. Regulated expression of microinjected DNA in adult Aedes aegypti mosquitoes. Insect Mol Biol 16: 83-92.

James, A. A., K. Blackmer, and J. V. Racioppi. 1989. A salivary gland-specific, maltase-like gene of the vector mosquito, Aedes aegypti. Gene 75: 73-83.

Jasinskiene, N., C. J. Coates, M. Q. Benedict, A. J. Cornel, C. S. Rafferty, A. A. James, and F. H. Collins. 1998. Stable transformation of the yellow fever mosquito, Aedes aegypti, with the Hermes element from the housefly. Proc Natl Acad Sci U S A 95: 3743-7.

Kalosaka, K., G. Chrysanthis, A. P. Rojas-Gill, M. Theodoraki, P. Gourzi, A. Kyriakopoulos, M. Tatari, A. Zacharopoulou, and A. C. Mintzas. 2006. Evaluation of the activities of the medfly and Drosophila hsp70 promoters in vivo in germ-line transformed medflies. Insect Mol Biol 15: 373-82.

Kampmueller, K. M., and D. J. Miller. 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. J Virol 79: 6827-37.

Kang, S., C. Sim, B. D. Byrd, F. H. Collins, and Y. S. Hong. 2008. Ex vivo promoter analysis of antiviral heat shock cognate 70B gene in Anopheles gambiae. Virol J 5: 136.

Karpov, V. L., O. V. Preobrazhenskaya, and A. D. Mirzabekov. 1984. Chromatin Structure of Hsp-70 Genes, Activated by Heat-Shock - Selective Removal of Histones from the Coding Region and Their Absence from the 5' Region. Cell 36: 423-431.

Kokoza, V., A. Ahmed, E. A. Wimmer, and A. S. Raikhel. 2001. Efficient transformation of the yellow fever mosquito Aedes aegypti using the piggyBac transposable element vector pBac[3xP3-EGFP afm]. Insect Biochem Mol Biol 31: 1137-43.

Kokoza, V., A. Ahmed, S. Woon Shin, N. Okafor, Z. Zou, and A. S. Raikhel. 2010. Blocking of Plasmodium transmission by cooperative action of Cecropin A and Defensin A in transgenic Aedes aegypti mosquitoes. Proc Natl Acad Sci U S A 107: 8111-6.

Krebs, R. A., and M. E. Feder. 1997. Tissue-specific variation in Hsp70 expression and thermal damage in Drosophila melanogaster larvae. J Exp Biol 200: 2007-15.

Lam, G., and C. S. Thummel. 2000. Inducible expression of double-stranded RNA directs specific genetic interference in Drosophila. Curr Biol 10: 957-63.

Lawson, D., P. Arensburger, P. Atkinson, N. J. Besansky, R. V. Bruggner, R. Butler, K. S. Campbell, G. K. Christophides, S. Christley, E. Dialynas, D. Emmert, M. Hammond, C. A. Hill, R. C. Kennedy, N. F. Lobo, M. R. MacCallum, G. Madey, K. Megy, S. Redmond, S. Russo, D. W. Severson, E. O. Stinson, P. Topalis, E. M. Zdobnov, E. Birney, W. M. Gelbart, F. C. Kafatos, C. Louis, and F. H. Collins. 2007. VectorBase: a home for invertebrate vectors of human pathogens. Nucleic Acids Res 35: D503-5.

Lebedeva, L. A., E. N. Nabirochkina, M. M. Kurshakova, F. Robert, A. N. Krasnov, M. B. Evgen'ev, J. T. Kadonaga, S. G. Georgieva, and L. Tora. 2005. Occupancy of the Drosophila hsp70 promoter by a subset of basal transcription factors diminishes upon transcriptional activation. Proceedings of the National Academy of Sciences of the United States of America 102: 18087-18092.

Lehmann, R., and C. Nusslein-Volhard. 1991. The maternal gene nanos has a central role in posterior pattern formation of the Drosophila embryo. Development 112: 679-91.

Li, G. C., and Z. Werb. 1982. Correlation between Synthesis of Heat-Shock Proteins and Development of Thermotolerance in Chinese-Hamster Fibroblasts. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 79: 3218-3222.

Lindquist, S. 1980. Varying Patterns of Protein-Synthesis in Drosophila During Heat-Shock - Implications for Regulation. Developmental Biology 77: 463-479.

Lindquist, S. 1986. The heat-shock response. Annu Rev Biochem 55: 1151-91.

Liu, R. Y., P. M. Corry, and Y. J. Lee. 1994. Regulation of chemical stress-induced hsp70 gene expression in murine L929 cells. J Cell Sci 107 (Pt 8): 2209-14.

Lobo, N. F., K. S. Campbell, D. Thaner, B. Debruyn, H. Koo, W. M. Gelbart, B. J. Loftus, D. W. Severson, and F. H. Collins. 2007. Analysis of 14 BAC sequences from the Aedes aegypti genome: a benchmark for genome annotation and assembly. Genome Biol 8: R88.

Lowenberger, C., M. Charlet, J. Vizioli, S. Kamal, A. Richman, B. M. Christensen, and P. Bulet. 1999. Antimicrobial activity spectrum, cDNA cloning, and mRNA expression of a newly isolated member of the cecropin family from the mosquito vector Aedes aegypti. J Biol Chem 274: 20092-7.

Lynch, M., and A. Force. 2000. The probability of duplicate gene preservation by subfunctionalization. Genetics 154: 459-473.

Maside, X., C. Bartolome, and B. Charlesworth. 2002. S-element insertions are associated with the evolution of the Hsp70 genes in Drosophila melanogaster. Current Biology 12: 1686-1691.

Mathur, G., I. Sanchez-Vargas, D. Alvarez, K. E. Olson, O. Marinotti, and A. A. James. 2010. Transgene-mediated suppression of dengue viruses in the salivary glands of the yellow fever mosquito, Aedes aegypti. Insect Mol Biol 19: 753-63.

Monath, T. P. 1994. Dengue - The risk to developed and developing-countries. Proceedings of the National Academy of Sciences of the United States of America 91: 2395-2400.

Moreira, L. A., M. J. Edwards, F. Adhami, N. Jasinskiene, A. A. James, and M. Jacobs-Lorena. 2000. Robust gut-specific gene expression in transgenic Aedes aegypti mosquitoes. Proc Natl Acad Sci U S A 97: 10895-8.

Morris, A. C., T. L. Schaub, and A. A. James. 1991. FLP-mediated recombination in the vector mosquito, Aedes aegypti. Nucleic Acids Res 19: 5895-900.

Mourya, D. T., P. Yadav, and A. C. Mishra. 2004. Effect of temperature stress on immature stages and susceptibility of Aedes aegypti mosquitoes to chikungunya virus. American Journal of Tropical Medicine and Hygiene 70: 346-350.

Mukhopadhyay, I., D. K. Saxena, and D. K. Chowdhuri. 2003. Hazardous effects of effluent from the chrome plating industry: 70 kDa heat shock protein expression as a marker of cellular damage in transgenic Drosophila melanogaster (hsp70-lacZ). Environmental Health Perspectives 111: 1926-1932.

Murphy, B. R., and S. S. Whitehead. 2011. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol 29: 587-619.

Nene, V., J. R. Wortman, D. Lawson, B. Haas, C. Kodira, Z. J. Tu, B. Loftus, Z. Y. Xi, K. Megy, M. Grabherr, Q. H. Ren, E. M. Zdobnov, N. F. Lobo, K. S. Campbell, S. E. Brown, M. F. Bonaldo, J. S. Zhu, S. P. Sinkins, D. G. Hogenkamp, P. Amedeo, P. Arensburger, P. W. Atkinson, S. Bidwell, J. Biedler, E. Birney, R. V. Bruggner, J. Costas, M. R. Coy, J. Crabtree, M. Crawford, B. deBruyn, D. DeCaprio, K. Eiglmeier, E. Eisenstadt, H. El-Dorry, W. M. Gelbart, S. L. Gomes, M. Hammond, L. I. Hannick, J. R. Hogan, M. H. Holmes, D. Jaffe, J. S. Johnston, R. C. Kennedy, H. Koo, S. Kravitz, E. V. Kriventseva, D. Kulp, K. LaButti, E. Lee, S. Li, D. D. Lovin, C. H. Mao, E. Mauceli, C. F. M. Menck, J. R. Miller, P. Montgomery, A. Mori, A. L. Nascimento, H. F. Naveira, C. Nusbaum, S. O'Leary, J. Orvis, M. Pertea, H. Quesneville, K. R. Reidenbach, Y. H. Rogers, C. W. Roth, J. R. Schneider, M. Schatz, M. Shumway, M. Stanke, E. O. Stinson, J. M. C. Tubio, J. P. VanZee, S. Verjovski-Almeida, D. Werner, O. White, S. Wyder, Q. D. Zeng, Q. Zhao, Y. M. Zhao, C. A. Hill, A. S. Raikhel, M. B. Soares, D. L. Knudson, N. H. Lee, J. Galagan, S. L. Salzberg, I. T. Paulsen, G. Dimopoulos, F. H. Collins, B. Birren, C. M. Fraser-Liggett, and D. W. Severson. 2007. Genome sequence of Aedes aegypti, a major arbovirus vector. Science 316: 1718-1723.

Nguyen, V. T., M. Morange, and O. Bensaude. 1988. Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. Anal Biochem 171: 404-8.

Nover, L. 1991. Heat Shock Response. CRC Press, Boca Raton.

Nusslein-Volhard, C., H. G. Frohnhofer, and R. Lehmann. 1987. Determination of anteroposterior polarity in Drosophila. Science 238: 1675-81.

O'Brochta, D. A., N. Sethuraman, R. Wilson, R. H. Hice, A. C. Pinkerton, C. S. Levesque, D. K. Bideshi, N. Jasinskiene, C. J. Coates, A. A. James, M. J. Lehane, and P. W. Atkinson. 2003. Gene vector and transposable element behavior in mosquitoes. J Exp Biol 206: 3823-34.

Palter, K. B., M. Watanabe, L. Stinson, A. P. Mahowald, and E. A. Craig. 1986. Expression and localization of Drosophila melanogaster hsp70 cognate proteins. Mol Cell Biol 6: 1187-203.

Pauli, D., A. P. Arrigo, and A. Tissieres. 1992. Heat shock response in Drosophila. Experientia 48: 623-9.

Pelham, H. R. 1982. A regulatory upstream promoter element in the Drosophila hsp 70 heat-shock gene. Cell 30: 517-28.

Perisic, O., H. Xiao, and J. T. Lis. 1989. Stable binding of Drosophila heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. Cell 59: 797-806.

Petersen, R. B., and S. Lindquist. 1989. Regulation of Hsp70 Synthesis by Messenger-Rna Degradation. Cell Regulation 1: 135-149.

Phillips, B., K. Abravaya, and R. I. Morimoto. 1991. Analysis of the specificity and mechanism of transcriptional activation of the human hsp70 gene during infection by DNA viruses. Journal of Virology 65: 5680-5692.

Pinkerton, A. C., K. Michel, D. A. O'Brochta, and P. W. Atkinson. 2000. Green fluorescent protein as a genetic marker in transgenic Aedes aegypti. Insect Mol Biol 9: 1-10.

Ramos, D. M., F. Kamal, E. A. Wimmer, A. N. Cartwright, and A. Monteiro. 2006. Temporal and spatial control of transgene expression using laser induction of the hsp70 promoter. Bmc Developmental Biology 6.

Rasmussen, E. B., and J. T. Lis. 1993. In-Vivo Transcriptional Pausing and Cap Formation on 3 Drosophila Heat-Shock Genes. Proceedings of the National Academy of Sciences of the United States of America 90: 7923-7927.

Read, D., M. Levine, and J. L. Manley. 1992. Ectopic expression of the Drosophila tramtrack gene results in multiple embryonic defects, including repression of even-skipped and fushi tarazu. Mech Dev 38: 183-95.

Reyes-Del Valle, J., S. Chavez-Salinas, F. Medina, and R. M. Del Angel. 2005. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol 79: 4557-67.

Serva, S., and P. D. Nagy. 2006. Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. J Virol 80: 2162-9.

Severson, D. W., D. L. Knudson, M. B. Soares, and B. J. Loftus. 2004a. Aedes aegypti genomics. Insect Biochem Mol Biol 34: 715-21.

Severson, D. W., B. deBruyn, D. D. Lovin, S. E. Brown, D. L. Knudson, and I. Morlais. 2004b. Comparative genome analysis of the yellow fever mosquito Aedes aegypti with Drosophila melanogaster and the malaria vector mosquito Anopheles gambiae. Journal of Heredity 95: 103-113.

Sim, C., Y. S. Hong, K. A. Tsetsarkin, D. L. Vanlandingham, S. Higgs, and F. H. Collins. 2007. Anopheles gambiae heat shock protein cognate 70B impedes o'nyong-nyong virus replication. Bmc Genomics 8.

Sim, C., Y. S. Hong, D. L. Vanlandingham, B. W. Harker, G. K. Christophides, F. C. Kafatos, S. Higgs, and F. H. Collins. 2005. Modulation of Anopheles gambiae gene expression in response to o'nyong-nyong virus infection. Insect Mol Biol 14: 475-81.

Smartt, C. T., A. P. Kim, G. L. Grossman, and A. A. James. 1995. The Apyrase gene of the vector mosquito, Aedes aegypti, is expressed specifically in the adult female salivary glands. Exp Parasitol 81: 239-48.

Smith, R. C., M. F. Walter, R. H. Hice, D. A. O'Brochta, and P. W. Atkinson. 2007. Testis-specific expression of the beta2 tubulin promoter of Aedes aegypti and its application as a genetic sex-separation marker. Insect Mol Biol 16: 61-71.

Solomon, J. M., J. M. Rossi, K. Golic, T. McGarry, and S. Lindquist. 1991. Changes in Hsp70 Alter Thermotolerance and Heat-Shock Regulation in Drosophila. New Biologist 3: 1106-1120.

Sorger, P. K. 1991. Heat shock factor and the heat shock response. Cell 65: 363-6.

Sutherst, R. W. 2004. Global change and human vulnerability to vector-borne diseases. Clin Microbiol Rev 17: 136-73.

Tamura, K., Dudley, J, Nei, M and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Tang, H. B., Y. Y. Liu, L. Madabusi, and D. S. Gilmour. 2000. Promoter-proximal pausing on the hsp70 promoter in Drosophila melanogaster depends on the upstream regulator. Molecular and Cellular Biology 20: 2569-2580.

Tanguay, R. M. 1988. Transcriptional Activation of Heat-Shock Genes in Eukaryotes. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire 66: 584-593.

Tatem, A. J., S. I. Hay, and D. J. Rogers. 2006. Global traffic and disease vector dispersal. Proc Natl Acad Sci U S A 103: 6242-7.

Terenius, O., O. Marinotti, D. Sieglaff, and A. A. James. 2008. Molecular genetic manipulation of vector mosquitoes. Cell Host Microbe 4: 417-23.

Thompson, J. F., L. S. Hayes, and D. B. Lloyd. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 103: 171-7.

Topol, J., D. M. Ruden, and C. S. Parker. 1985. Sequences required for in vitro transcriptional activation of a Drosophila hsp 70 gene. Cell 42: 527-37.

Uhlirova, M., M. Asahina, L. M. Riddiford, and M. Jindra. 2002. Heat-inducible transgenic expression in the silkmoth Bombyx mori. Development Genes and Evolution 212: 145-151.

Waterhouse, R. M., S. Wyder, and E. M. Zdobnov. 2008. The Aedes aegypti genome: a comparative perspective. Insect Mol Biol 17: 1-8.

Weaver, S. C., and W. K. Reisen. 2010. Present and future arboviral threats. Antiviral Res 85: 328-45.

Weeks, S. A., and D. J. Miller. 2008. The heat shock protein 70 cochaperone YDJ1 is required for efficient membrane-specific flock house virus RNA replication complex assembly and function in Saccharomyces cerevisiae. J Virol 82: 2004-12.

Weeks, S. A., W. P. Shield, C. Sahi, E. A. Craig, S. Rospert, and D. J. Miller. 2010. A targeted analysis of cellular chaperones reveals contrasting roles for heat shock protein 70 in flock house virus RNA replication. J Virol 84: 330-9.

Wilson, R., J. Orsetti, A. D. Klocko, C. Aluvihare, E. Peckham, P. W. Atkinson, M. J. Lehane, and D. A. O'Brochta. 2003. Post-integration behavior of a Mos1 mariner gene vector in Aedes aegypti. Insect Biochem Mol Biol 33: 853-63.

Wimmer, E. A. 2003. Innovations: applications of insect transgenesis. Nat Rev Genet 4: 225-32.

Wise de Valdez, M. R., D. Nimmo, J. Betz, H. F. Gong, A. A. James, L. Alphey, and W. C. t. Black. 2011. Genetic elimination of dengue vector mosquitoes. Proc Natl Acad Sci U S A 108: 4772-5.

Yadav, P., P. V. Barde, M. D. Gokhale, V. Vipat, A. C. Mishra, J. K. Pal, and D. T. Mourya. 2005. Effect of temperature and insecticide stresses on Aedes aegypti larvae and their influence on the susceptibility of mosquitoes to dengue-2 virus. Southeast Asian J Trop Med Public Health 36: 1139-44.

Zhao, Y. G., and P. Eggleston. 1999. Comparative analysis of promoters for transient gene expression in cultured mosquito cells. Insect Mol Biol 8: 31-8.