THE USE OF RNA INTERFERENCE AS A TOOL TO EXAMINE GENE FUNCTION, AND ITS POTENTIAL AS A SPECIES-SPECIFIC PESTICIDE IN THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI*

BY

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Abstract

RNA interference (RNAi) is a gene silencing mechanism induced by double-stranded RNA (dsRNA). RNAi has been used extensively to create loss-of-function mutants in many species to identify the functions of genes, but it also has the potential to be used as a species-specific pesticide if the dsRNA can silence essential genes in pests. The mosquito *Aedes aegypti* is a vector of numerous viruses including Dengue and West Nile virus, and is frequently controlled by chemical insecticides. With growing concerns about the extensive use of broad-spectrum pesticides, new control methods are eagerly sought. In this study, I examined the efficacy of feeding pesticidal dsRNAs to mosquito larvae. A dose-dependent RNAi response and mortality was observed when larvae were fed dsRNA targeting several different genes. Unlike RNAi in the related dipteran *Drosophila melanogaster*, RNAi in *A. aegypti* also appeared to be systemic, spreading beyond the gut to other tissues. A degree of species-specificity was also observed, as dsRNA specific to the *D. melanogaster β-tubulin* gene killed *D. melanogaster* larvae but did not kill mosquito larvae.

RNAi was also used to determine the function of a newly-identified *A. aegypti* cytochrome P450 (CYP) gene, *Aacyp*. This gene showed male-biased expression in the mosquitoes, and was expressed primarily in the male abdomen and/or thorax, but unlike some other insect male-biased CYPs, *Aacyp* was not highly expressed in the reproductive structures. While dsRNA injections successfully knocked down expression of *Aacyp*, no discernable change in reproductive or male-specific behaviours were noted. Nevertheless, RNAi is still considered a highly versatile tool for both gene function studies and has promising potential to be developed into a novel class of pesticides.

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Chapter 1: General Introduction

1.1 Mosquito biology

Aedes aegypti, also known as the yellow fever mosquito, is a vector of several important human viruses, including yellow fever and dengue. These diseases mainly affect tropical and subtropical regions, particularly in South America, Africa and India where *A. aegypti* is predominant. On an annual basis, dengue alone affects nearly 100 million people worldwide (Centers for Disease Control and Prevention), and is a threat to nearly 2.5 billion people (World Health Organization). *A. aegypti* also has the potential to transmit West Nile Virus (WNV), cases of which have been steadily increasing since the first North American infection in 1999 (Hayes *et al*., 2005).

The complete life cycle of *A. aegypti* takes about two weeks, which makes it relatively easy to maintain in the lab. The eggs are laid in water and hatch after sensing a decrease in oxygen levels, which is usually indicative of bacterial growth and organic material available as a food source for new larvae (Gjullin *et al*., 1941). Aquatic larvae feed on bacteria and detritus over four instars, and then moult to aquatic pupae, finally emerging as adults a few days later.

The females of most mosquito species require a blood-meal to produce viable eggs. During the feeding process, the insect injects saliva into its host, which may transfer various pathogens that the mosquito harbours. Viruses and other pathogens are usually acquired by the mosquito when feeding on an infected vertebrate host. The pathogen typically replicates in the female's gut cells, then travels from the gut and eventually makes its way to the salivary glands, where it is then transferred to another host when the female feeds again. *A. aegypti* mosquitoes have a preference for human

hosts and when they acquire a virus or other parasite from a blood-meal, the insect typically remains infective for its entire lifespan (Halstead, 2008). These factors can increase transmission rates of viruses in some areas, and the World Health Organization suggests the only way to combat virus transmission is to control the mosquito vectors.

In 2007, a draft of the genome sequence of *A. aegypti* was published in Science (Nene *et al.,* 2007), revealing it to be nearly five times as large as that of the malaria vector *Anopheles gambiae*. Comparisons made to *D. melanogaster* and *A. gambiae* genomes revealed expansions of various protein families within these different dipteran species. The acquisition of different genes in the insects may reflect various biological adaptations of these organisms, and further comparisons to other insect genomes may ultimately reveal the genetic basis of both the phenotypic differences and the vectorial capacities of the mosquito species (Waterhouse *et al*., 2008).

As new genes are identified and their functions validated, it is anticipated that we may also identify new targets for insecticide development or develop novel methods of genetic control of these serious pests. Large research efforts have previously focused on identifying molecular targets within tissues intimately involved in pathogen development and transmission, such as the mosquito's gut and the salivary glands (Abraham *et al*., 2004; Arca *et al*., 2005; Sanders *et al*., 2003; Valenzuela *et al.*, 2002). Only female mosquitoes transmit disease, and consequently, there has been increasing interest in identifying genes and their associated proteins that are involved in sexual development of mosquitoes, as these may also prove particularly useful in understanding how these insects vector the pathogens and in developing novel methods of mosquito control. As part of my research has involved the examination of a gene that displayed a male-biased

expression, it is worth reviewing some aspects of insect and mosquito sexual development.

1.2 Sex determination and differentiation in insects

There are numerous ways to determine the sex of an organism, and all known mechanisms can be observed in different insects (Sanchez, 2008). The signal for sex determination can be zygotic, maternal or environmental. Only a few insects, however, employ maternal or environmental sex-determination systems. The genetics involved in maternal signals are somewhat complex, but in the end, females develop as either gynogenic, producing only female offspring, or androgenic, producing only male offspring. For example, in the blowfly *Chrysomya rufifacies*, gynogenic females express a maternal factor that is incorporated into developing oocytes and forces female development on any zygotes derived from them. Androgenic females do not produce the maternal factor, and therefore only males will develop from their oocytes (Ullerich, 1984).

Environmental factors, such as temperature, can affect sex-determination by inducing the elimination of paternally derived chromosomes in developing offspring, usually increasing the number of females produced (Nigro *et al*., 2007).

Zygotic signals are seemingly the most common mechanism used to determine the sex of an individual. These signals involve chromosome differences between males and females that are usually fixed at fertilization. Normally there is a sex-determining factor based on the presence or absence of specific chromosomes. As in humans, some insects like tephritid and muscid flies possess male-determining factors on their Y

chromosome, making males the heteromorphic sex possessing both an X and Y chromosome. Females are the homomorphic sex and possess two X chromosomes. In lepidopterans, males are the homogametic sex (ZZ) and females are the heterogametic sex (ZW), with Z and W used to distinguish this system from the XY system (Sanchez, 2008). There are also insects whose sex is based on haploid vs. diploid numbers of chromosomes, as in bees, where males are haploid and females are diploid.

For *Drosophila*, whose sex-determining mechanism has been the most thoroughly studied in insects, sex determination is based on chromosome ratios between X chromosomes and autosomes. The homomorphic female possesses a 2X:2A chromosome ratio, where X refers to the X chromosome and A refers to autosomal chromosomes, while the heteromorphic male possesses a 1X:2A ratio. Once the sex of the organism is determined, several genes are turned on or off in order to control development of the soma specific to that sex. Male *Drosophila*, for example, will express sex-specific genes that drive the development of internal and external male body parts, and also lay down the neural networks specific for male courtship behaviours. Female development, however, is the "default setting" and occurs in the absence of the male-specifically spliced genes.

Female *D. melanogaster* express the gene *sex lethal* (*sxl*) to produce a functional SXL protein that signals default female-specific splicing of transformer (*tra*) and doublesex (*dsx*) genes required for differentiation of the body into female specific tissues (Fig. 1).

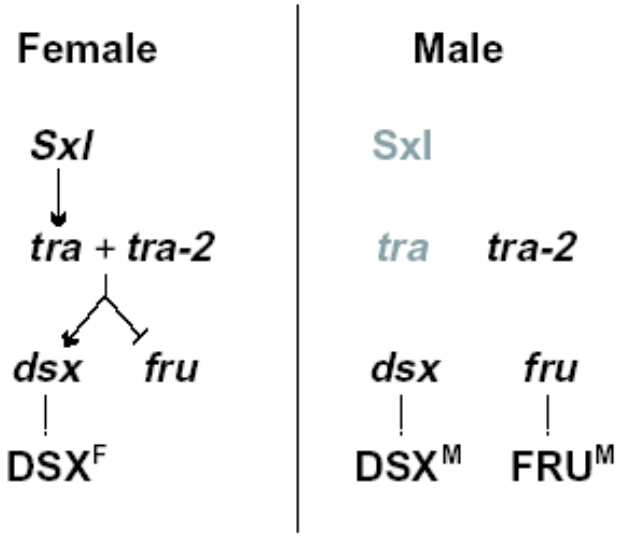

Figure 1. Sex determination pathway in *D. melanogaster* (from Arbeitman *et al*., 2004). Females express *Sxl*, which activates female-specific splicing of *tra*. These genes, along with the sex-non-specific *tra2*, activate female-specific splicing of *dsx* and *fru*, however the female *fru* transcript is not translated. In males, *Sxl* and *tra* are non-functional, leading to male-specific splicing of *dsx* and *fru*. (*Sxl* = sex lethal, *tra*/*tra2* = transformer/transformer 2, *dsx* = doublesex, *fru* = fruitless).

In males, SXL is truncated, and therefore non-functional, which leads to a non-functional TRA and male-specific splicing of *dsx* and *fru* transcripts. *Dsx* encodes sex-specific transcription factors that are responsible for sex-specific development of the soma, while *fru* encodes a transcription factor responsible for male-specific development of the nervous system, controlling male behaviours such as mating and fighting (reviewed in Saccone *et al*., 2002; Sanchez, 2008; Shirangi and McKeown, 2007).

Although the sex-determination pathway is well understood in *D. melanogaster*, there is limited information regarding the pathway in other insects. Originally it was believed that these mechanisms differed greatly among phyla until Raymond *et al*. (1998)

showed that *mab-3* in *C. elegans* and *dsx* in *D. melanogaster*, being similar in sequence, also have similar roles in sexual development, which suggests the possibility of a common evolutionary origin. However, it was later discovered that although several dipteran species possess homologues of *sxl*, the gene at the top of the sex-determination cascade in *Drosophila*, the gene produced proteins of similar size and equal abundance in both males and females, suggesting that it did not play a role in sex determination in these other dipterans (Schütt and Nöthiger, 2000). When *M. domestica sxl* was expressed in *D. melanogaster*, there was no effect on sex ratios, despite the fact that SXL proteins in these two species share 85% of their amino acids (Meise *et al*., 1998). Based on these observations, it has been suggested that SXL acquired a unique sex-determining role in drosophilids, but that it is not the key sex-determining switch in many other insects (Schütt and Nöthiger, 2000; Verhulst *et al*., 2010).

The gene at the end of the sex determination pathway in *D. melanogaster*, *dsx*, appears to be present in other dipteran insect species with a highly conserved structure and expression pattern, with differing male and female-specific transcripts (reviewed in Schütt and Nöthiger, 2000). Homologues of *dsx* have been identified in a range of dipteran species, including *Bactrocera tryoni* (Shearman and Frommer, 1998), *Megaselia scalaris* (Kuhn *et al*., 2000), *Ceratitis capitata* (Saccone, 1998), *M. domestica* (Hediger *et al*., 2004), and *Anopheles gambiae* (Scali *et al*., 2005). More recently, our laboratory has identified the orthologues of *dsx* and *fru* in the mosquito *A. aegypti*, and preliminary evidence suggests that both are alternatively spliced in a sex-specific manner. By using RNA interference (RNAi) to disrupt the expression of a male-specific *dsx* splice variant, it was also observed that normal development of male *A. aegypti* mosquitoes can be

disrupted (Whyard *et al*., unpublished), which suggests that, like *D. melanogaster*, sex differentiation in *A. aegypti* is regulated by this important transcription factor.

1.3 Sex-specific genes and male accessory gland proteins

Apart from genes that regulate sex-specific development, some genes are sex-specifically expressed during maturity of an insect, usually for the purposes of reproduction. Females specifically express genes necessary for egg development, sperm storage and ovipositing, while males typically express genes needed for sperm production, mate-finding, and courtship behaviour.

Arbeitman *et al*. (2004) conducted a genome wide analysis of *Drosophila* genes to determine which genes are sex-specific in somatic tissues. They found 63 genes were differentially expressed between males and females, 11 of those genes being expressed only within internal genital organs and were most likely regulated by *dsx*.

Many uniquely male genes of insects are expressed in the male accessory glands (MAGs); in *D. melanogaster*, Arbeitman *et al*. (2004) found that three genes were expressed solely in MAGs. MAGs are usually paired structures of the male reproductive system that produce all of the components of the seminal fluid. An analysis of *D. melanogaster* MAG gene expression (Chen, 1991; Wolfner, 1997; Wolfner, 2002) revealed that this male tissue produces over 80 proteins, and while the function of many of the proteins have not been fully elucidated, many of them are thought to enhance the male's reproductive success. For the majority of insects, after copulation, the female's fecundity is increased, while her receptivity to mate again is decreased. Several studies

have shown that these changes in the female are caused by secretions of the MAGs transferred to the female during copulation (Chen, 1984).

The most thoroughly studied MAG protein is sex peptide or Acp70A (called Acp for *ac*cessory gland *p*rotein), a small 36 amino acid peptide found in *D. melanogaster*, which when transferred to females, was found to induce the typical post-mating responses of increasing oviposition activity and decreasing receptivity to further mating (Chen *et al*., 1988). Virgin females are known to lay a few eggs per day, but after mating, females may lay up to 60 eggs per day. Virgin females also readily accept courting males whereas after mating, females show specific rejection behaviours such as extruding their ovipositor and closing their genital opening by appressing their vaginal plates. This means that subsequent copulations are mechanically prevented. It has been shown that these behaviours last for about one week, and after that time the female will revert back to a virgin-like state, being receptive to mating again (Kubli, 1992).

Heifetz *et al*. (2000) observed that *D. melanogaster* females have increased numbers of eggs in their oviducts and external openings of the genital tract 6 hours after mating, compared to control females. They also observed that females mated to males that did not produce the MAG protein ovulin possessed half as many eggs passing through their genital tract compared to females mated to control males. Heifetz *et al*. (2005) subsequently found that ectopic expression of ovulin was able to stimulate ovulation in unmated females. They reported that 67% of unmated females ectopically expressing ovulin had an egg in their genital tract, compared to only 8.3% of unmated control females.

Some MAGs have also been shown to produce antibacterial proteins, as there is the potential for micro-organisms to enter the insect's genital tract. Antibacterial proteins may act to protect sperm once it is in the female's body, or protect eggs once in the reproductive tract. There is at least one known antibacterial protein (Acp62F) produced in the MAGs of *D. melanogaster* (Lung *et al*., 2002).

It has also been shown that some MAG proteins are necessary for actual sperm storage within the female's sperm storage organs. Female fruit flies store sperm in their seminal receptacle (a coiled tubule) and their two spermathecae (sac-like organs), from which they can use the sperm for two weeks after mating to fertilize eggs. Tram and Wolfner (1999) counted the number of sperm in females after mating and found significantly less sperm in the seminal receptacle and spermathecae of females mated to males that produced reduced quantities of MAG secretions. Ninety percent of sperm transferred to the female is not properly stored when females are mated to males that do not produce MAG secretions. Neubaum and Wolfner (1999) showed that *D. melanogaster* females mated to males that don't produce the MAG protein Acp36DE have a decreased number of sperm in their sperm storage organs after mating. Twenty-four hours after mating, females mated to control males had more than double the amount of sperm stored in both their seminal receptacle and spermathecae compared to females mated to males that lack Acp36DE. However, the specific role of MAG secretions related to sperm storage is unknown.

When I first started my M.Sc. studies, little was known about the MAG in the mosquito *A. aegypti*, and one of my research objectives was to determine whether *A. aegypti* possessed orthologues to several of the aforementioned MAG proteins. After

starting my research however, two competing research groups published papers describing the complete repertoire of genes expressed in *A. aegypti* MAGs (Sirot *et al*., 2008) and a characterization of sex peptide in the mosquito (Sirot *et al*., 2008; Yapici *et al*., 2008). While my intention had initially been to identify and characterize a subset of the *Aedes* MAG genes, I recognized that these two competing groups were already well ahead of me, and I therefore elected to focus my attention on the role of a novel cytochrome P450 (CYP) gene that I had fortuitously identified using degenerate PCR techniques to isolate MAG gene orthologues in *A. aegypti*.

1.4 Cytochrome P450s

CYPs are a family of enzymes found in all organisms and have the ability to metabolize xenobiotic and endogenous compounds. They were named after a carbon monoxide-binding pigment discovered in rabbit liver microsomes that showed an intense absorption band at 450 nm (Omura and Sato, 1964a; Omura and Sato, 1964b). CYPs can be very diverse, having only three absolutely conserved amino acids. Despite this diverse sequence heterogeneity, their general topography and structural folding patterns are highly conserved. All CYPs contain heme-binding domains near the carboxy terminus, and it is around the heme group that most structural conservation occurs, including the three conserved amino acids (Werck-Reichhart and Feyereisen, 2000).

All CYPs are named beginning with “CYP”, followed by a number for one of over 200 families, characterized by those CYPs with 40% or more amino acid identity, a letter for the subfamily, characterized by greater than 55% identity, and a final number for the gene (e.g. CYP6A1, from the housefly *Musca domestica*). However, since minor

changes in amino acids can alter substrate specificity, a CYP substrate can not be assumed based on its name.

CYPs bind molecular oxygen, introduce one oxygen atom into a substrate, and reduce the other atom to water using two electrons provided by NADPH according to the reaction:

$$\text{Substrate (S)} + (\text{NADPH} + H^+) + O_2 \rightarrow \text{S(O)} + \text{NADP}^+ + H_2O$$

The electrons required for this reaction are transferred from NADPH by an NADPH cytochrome P450 reductase, or by cytochrome b_5 (Bergé *et al*., 1998; Meunier *et al*., 2004). A variety of organic substrates, both small and large molecules, can be used by CYP enzymes, and this high diversity of substrates suggests that large numbers of CYPs may occur in any one individual (Bergé *et al*., 1998).

There are five CYP families that are specific to insects, and one other family that is found in insects as well as other organisms. Many CYPs are necessary for the synthesis and degradation of moulting hormones and juvenile hormone, as well as pheromones. They are also involved in adaptive mechanisms that allow insects to tolerate toxic chemicals produced by plants, where biosynthesis of these enzymes can be triggered by toxins in host plants on which they feed. Because CYPs can metabolize a wide range of compounds, they often contribute to an insect's ability to detoxify insecticides, and consequently, many instances of insecticide resistance are attributed to increased CYP activity (reviewed in Bergé *et al*., 1998). Since there is such diversity in the function of CYPs, the distribution of these enzymes within tissues is also very diverse, seemingly having no specific pattern. This leads to the realization that CYPs can be found in all types of tissues within an organism (Werck-Reichhart and Feyereisen, 2000).

Metabolic resistance of insecticides in insects is often due to enhanced CYP activity. Studies using CYP inhibitors such as piperonyl butoxide have shown reductions, or even complete loss of resistance (Bergé *et al*., 1998). For example, in a pyrethroid-resistant strain of house fly, piperonyl butoxide reduced the resistance factor from 6000 to 32 (Scott and Georghiou, 1986). This same strain of fly shows overproduction of a specific CYP which accounts for 68% of the total P450s, and is 44 times more abundant in the resistant strain relative to a susceptible strain (Wheelock and Scott, 1990). Other studies have also shown that over-expression of specific CYPs in the housefly (Cariño *et al*., 1992; Cariño *et al*., 1994; Liu and Scott, 1998), and in *D. melanogaster* (Amichot *et al*., 2004; Joussen *et al*., 2008) lead to increased metabolism of insecticides. Along with an increase in the CYPs themselves, there is usually an increase in cytochrome P450 reductase or cytochrome b_5 (Bergé *et al*., 1998).

One CYP (CYP6L1) has been found to be sex-specific in the male reproductive system of the German cockroach, *Blattella germanica* (Wen and Scott, 2001). While sex-specific CYPs are common in mammals, none had previously been shown to be sex-specific in any insects, even though it was believed that CYPs could play a role in insect reproduction. CYP6L1 mRNA was only detected in adult male cockroaches, where it was localized to the testes and accessory glands, as it could not be detected in the abdomen when testes and accessory glands had been removed. Wen and Scott (2001) suggested several possible roles for this CYP, including regulation of juvenile hormone in accessory glands and ecdysteroids in testes, as some CYPs have a role in the metabolism of those insect hormones. As mentioned above, the MAGs of insects have the ability to produce numerous proteins involved in altering female physiology and behaviour after mating,

and Wen and Scott (2001) have suggested that this CYP may also have a role in modifying female receptivity or fecundity.

More recently, a sex-specific CYP was characterized in the head of male *D. melanogaster*. This *cyp* gene (*cyp4d21* or *sxe-1*) is regulated by DSX, and significantly reduces mating success when knocked down using RNAi, possibly by affecting circadian rhythms and courting behaviours in males (Fujii *et al*., 2008).

With these exciting discoveries, it opens the door for other sex-specific CYPs to be found in several important insect species. As will be discussed in Chapter 2, a novel CYP with male-biased expression in the mosquito *A. aegypti* is described, and its potential roles in development and reproduction are explored. To assess the functional role of this mosquito CYP, I attempted to use RNAi to create loss-of-function mutants. As RNAi has not been widely used as a research tool in mosquito molecular biology studies, a review of the technology is described below.

1.5 RNA interference

In 1990, Carolyn Napoli and colleagues (Napoli *et al.*, 1990) attempted to enhance *chalcone synthase* gene expression in petunia flowers by the introduction of homologous transgenes into the plants. Instead of enhancing gene expression with extra copies of the transgene, they observed an unexplained post-transcriptional gene silencing (PTGS) phenomenon that resulted in a decrease of the gene's mRNA transcripts. In 2006, Andrew Fire and Craig Mello received the Nobel Prize in Physiology or Medicine for their description in 1998 (Fire *et al.*, 1998) of a similar PTGS mechanism in the nematode *Caenorhabditis elegans*, which they discovered was mediated by double-

stranded RNA (dsRNA). They called the mechanism RNA interference (RNAi), and RNAi is now used for many applications, ranging from a molecular biology tool for examining gene function in many organisms to suppressing disease-causing genes in humans.

1.6 Mechanism of RNAi

When long dsRNA enters a cell, it is recognized by the enzyme Dicer, which initiates RNAi by cleaving the dsRNA into smaller (~21nt) fragments called short interfering RNAs (siRNAs). These siRNAs are then incorporated into an enzyme complex called the RNA-induced silencing complex (RISC), where the siRNA guides RISC to complementary mRNA sequences that are subsequently degraded by an endonuclease associated with RISC (Fig. 2; reviewed in Hannon, 2002).

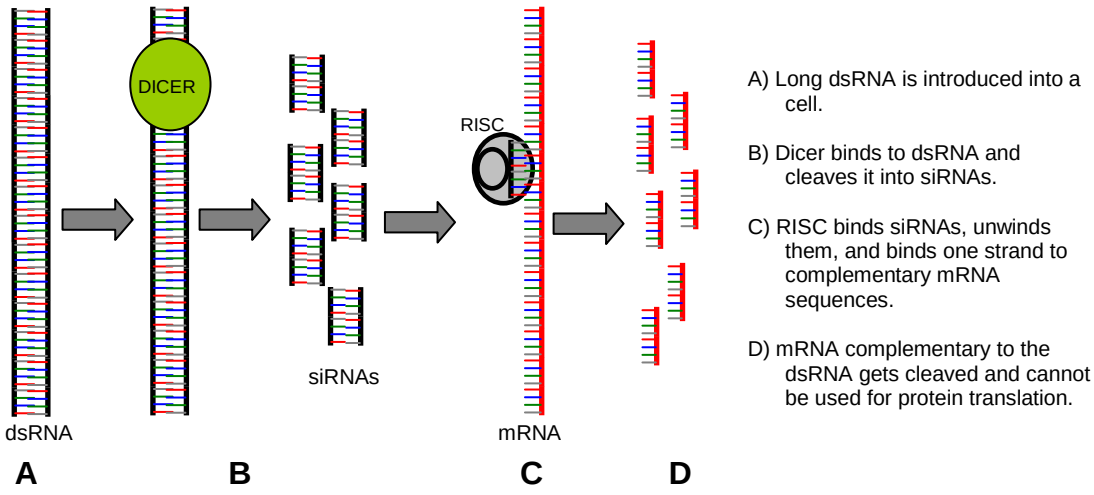

Figure 2. RNAi mechanism in eukaryotes.

Dicer enzymes are one of three classes of RNAse III enzymes that exhibit specific binding and cleavage of dsRNAs, and can therefore use linear dsRNA or RNA hairpins as substrates (Carmell and Hannon, 2004). Dicer itself contains two RNAse III domains, plus a PAZ motif that interacts with 3' ends of single stranded RNA. A helicase domain is also present in some Dicers, particularly in mammals, which may facilitate dsRNA cleavage using energy from ATP hydrolysis (Ketting *et al.*, 2001). The two RNAse III domains of Dicer are connected to the PAZ domain by a large α-helix, and it is suggested that the length of the α-helix will determine the length of siRNAs produced (MacRae *et al.*, 2006).

The siRNAs produced by Dicer possess 2-nucleotide 3' overhangs and 5'-phosphate termini. These properties are significant for incorporation and proper functioning of the RISC complex. The siRNAs are incorporated into a latent form of RISC, which is then activated by unwinding of the siRNAs, and it is the unwound siRNAs that use Watson-Crick base pairing to find complementary mRNAs for degradation (Hannon, 2002). The antisense strand of the siRNA is used as the guide strand to target specific mRNA sequences, whereas the sense strand is simply a "passenger" strand which is later destroyed during RISC assembly (Rana, 2007).

Every RISC must have, at minimum, a small regulatory RNA (i.e. siRNA) and an Argonaute protein (Pratt and MacRae, 2009). Members of the Argonaute protein family are present in virtually all species, but are dependent on their guide RNA in order to function correctly. The largest class of these proteins, the Ago proteins, require a guide RNA generated from dsRNA in the cytoplasm that has been cleaved by Dicer. Most information on Argonaute proteins is based on studies using prokaryotes whose

Argonaute proteins bind to small DNAs as guides. The proteins possess two lobes, one of which contains a PAZ domain to bind the 3' end of the guide strand, while the other lobe binds to the 5'-phosphate. The majority of a DNA guide strand is then in contact with the protein through the phosphodiester backbone, which explains how Argonaute proteins have the ability to bind guide strands regardless of their nucleotide sequences. Perhaps the most important structure of the protein is the PIWI domain, also called the "slicer", which hydrolyzes target RNAs in a manner similar to the enzyme RNase H by generating products with 5'-phosphate and 3'-OH groups (Song *et al.*, 2004). However, not all Argonaute proteins are catalytically active; of the four Ago proteins in humans for example, only one is catalytically active (Liu *et al.*, 2004).

These core components involved in RNAi may be adapted to meet specific biological needs of each organism, suggested by the differences in silencing among different species. For example, RNAi in *C. elegans* is both systemic (capable of inducing RNAi throughout the body) and heritable, whereas in *Drosophila* and mammals RNAi gene silencing appears to be cell-autonomous, meaning the RNAi response will only be seen in those cells possessing dsRNAs (Hannon, 2002). These differences may be either a consequence of differences in the nature of the siRNAs that are produced by Dicer and RISC and/or a consequence of the siRNA transport mechanisms present within different species.

1.7 RNAi in plants

When Napoli *et al.* (1990) attempted to enhance the colour of petunia flowers by overexpressing *chalcone synthase*, the gene encoding the enzyme necessary for the

synthesis of an anthocyanin pigment, they introduced a *chalcone synthase* transgene into the plants, and surprisingly discovered that 42% of the flowers showed completely white petals or petals with white or pale sections. They also found a 50-fold knockdown of *chalcone synthase* mRNA transcripts. At the time, the mechanism of this PTGS was unknown, and because both transgenes and the homologous endogenous genes were silenced they called it "cosuppression" (Vaucheret *et al.*, 2001). It is now believed that dsRNA was produced due to juxtaposed sense-antisense transgenes caused by imperfect integration at the transgene loci, thereby causing an RNAi response in these plants (Broderson and Voinnet, 2006).

Later studies showed that when plants were exposed to RNA viruses, viral mRNA levels were reduced shortly after the initial infection period, but the rate of transcription was not (Jones *et al.*, 2001). This suggested that plants could respond to the presence of viral RNAs by targeting them for destruction (Hannon, 2002). RNA silencing is now considered to be an important part of a plant's defence system, not only against viruses, but against transposable elements as well, and may even be essential for gene expression regulation (Voinnet, 2002).

It is known that plants use RNA-dependant RNA polymerases (RdRPs), similar to those best-characterized in the tomato (Scheibel *et al.*, 1998), in order to enhance gene silencing within the organism. RdRPs use siRNAs as primers to produce secondary siRNAs which can continue to act as guides to silence targeted genes (Ahlquist, 2002; Gordon and Waterhouse, 2007; Vance and Vaucheret, 2001). Other experiments suggest that the spread of silencing in plants could also occur if siRNAs and/or dsRNAs are

transmitted cell to cell over a long distance through the phloem of a plant in the same way systemic viruses move (Mlotshwa *et al.*, 2002; Voinnet *et al.*, 1998).

1.8 RNAi in animals

Guo and Kemphues (1995) observed that sense and antisense RNA could be effective in reducing gene expression in nematodes, but they could not explain precisely how the PTGS occurred. Fire *et al.* (1998) made the discovery that dsRNA was the key molecule that induced the gene silencing, as dsRNA was more than 10 times as effective at gene silencing in *C. elegans* than antisense RNAs alone.

Like plants, *C. elegans* showed the ability to systemically silence targeted genes using small amounts of dsRNA, and it was discovered that they too possess RdRPs which can produce secondary siRNAs for continued gene silencing. Similar RdRP genes have been found in the bread mold *Neurospora crassa* and yeast *Schizosaccaromyces pombe* and it has been suggested that many other eukaryotes possess RdRP-like genes, but their functions have not been validated (Ahlquist, 2002).

In contrast to plants, nematodes also possess a transmembrane protein called SID-1 that is located at the periphery of cells and is sensitive to RNAi. SID-1 is suggested to act as a channel to transport siRNAs or dsRNAs between cells (Winston *et al.*, 2002). The presence of SID-1 in the nematode helped explain how injection of dsRNA into the worm could spread from cell to cell. Interestingly, neuronal cells in nematodes are refractory to injected dsRNA, and they lack SID-1 (Tavernarakis *et al*., 2000; Winston *et al*., 2002). The function of this putative RNA transporter at the periphery of cells is still unknown, but it has been speculated that it could assist in the detection of microbial and

viral pathogens (Winston *et al*., 2002). Based on a limited survey of animal species, it appears that SID-1 genes are present in most animal genomes, with the curious exception of dipteran insects (Gordon and Waterhouse, 2007).

While invertebrate species can induce RNAi following exposure to dsRNAs of any length greater than or equal to 21 nt, dsRNAs longer than 30 nt within vertebrate cells will induce the protein kinase R (PKR) pathway, which is an interferon-inducible pathway that can protect vertebrate cells from possible viral infections (Feng *et al*., 1992). The PKR pathway, activated by long dsRNAs, first inhibits the cell's translational machinery, and can ultimately induce apoptosis. In vertebrates therefore, RNAi can be induced only if dsRNAs less than 30 bp in length are present or introduced into the cells (Gil and Esteban, 2000).

Since RNAi responses can be elicited in virtually all eukaryotic species, its use as a tool to examine the function of newly discovered genes has dramatically increased, and with the continued sequencing of many species' genomes, higher throughput RNAi screening tools are being developed (Hannon, 2002; Kuttenkeuler and Boutros, 2004).

1.9 RNAi in insects

Insects affect humans in many ways, particularly as vectors of disease and as pests of agricultural crops. Billions of dollars are spent annually around the world to produce and distribute pesticides to control insect pests, but with increasing concerns about insecticide resistance and the negative impact of current pesticides on non-target species (reviewed in Nicholson, 2007; Paoletti and Pimental, 2000), it is important that we continue to search for new, and hopefully more selective, pesticides to control pest

insects. As more pest insects' genomes are sequenced, it is becoming easier to identify new insect genes and gene products that could be targeted by a new generation of pesticides. RNAi can be used as a tool for both identifying genes and analyzing various gene functions, as well as silencing target genes to aid in the development of species-specific insecticides to control pest species.

Many insect species have already been studied using RNAi techniques. Knockdown of target mRNA sequences have been demonstrated in several insect orders including Lepidoptera, Hymenoptera, Coleoptera, Hemiptera and some Diptera, as well as other related pest arthropods such as ticks (reviewed in Gordon and Waterhouse, 2007). However, studies on various species have revealed that RNAi responses are not equal in all insects.

While all species studied appear to have the basic components of RNAi such as Dicer and RISC, not all species respond equally to dsRNA treatments (reviewed in Gordon and Waterhouse, 2007). It is clear from various studies that the delivery methods of dsRNA to insects can affect the RNAi response and it is also evident that the uptake mechanisms in different species may also differ. Within the order Lepidoptera (moths and butterflies) for example, similar methods of dsRNA delivery can have very different effects in different species (Terenius *et al*., 2010).

Most insects are known to possess SID-1-like proteins to passively transport dsRNAs, preferentially those ~500 bp long, and allow for systemic gene silencing within the organism (Weiner, 2003). Curiously, dipteran insects (flies) are the only animal species apparently lacking these transport proteins, which is consistent with reports confirming an absence of systemic silencing in the model fly species *Drosophila*

melanogaster. However, the fact that dsRNA can enter cells of this species suggests there may be a different method for dsRNA uptake such as receptor-mediated endocytosis (Saleh *et al.*, 2006; Ulvila *et al.*, 2006).

1.10 DsRNA delivery methods

Numerous studies have shown that insects can elicit an RNAi response caused by the presence of dsRNA. There are several methods of delivering dsRNA to an insect, all with their advantages and disadvantages. Traditionally, the method to deliver dsRNA to most organisms has been by direct injection of a dsRNA solution. This method has been used to deliver dsRNA to most stages of insects, from embryos to adults. One of the earliest studies of injections of dsRNA to an insect was in *D. melanogaster* embryos. Misquitta and Paterson (1999) showed that both full length and partial dsRNA sequences specific to *nautilus*, a gene involved in embryonic muscle formation, could cause severe disruption of muscle patterns, and suggested RNAi as an easy alternative to traditional mutation methods involving a combination of genetic selection, P-element insertion and deficiency analyses after gamma-irradiation.

Several other species have shown susceptibility to dsRNA injections as well. The honeybee *Apis mellifera* produces relatively large insect eggs (1.6 mm long), making injection of these embryos easier than in other species, but only partial silencing has been observed, possibly because there was limited diffusion of the dsRNA through the embryos (Beye *et al.*, 2002). Adult honeybees can be injected intra-abdominally to disrupt gene expression, even in organs distant from the injection site. However, differences in the persistence of dsRNA have been noted, with some researchers finding

that the dsRNA could be detected in the hemolymph for only three days after injection (Gatehouse *et al.*, 2004), while others found that the dsRNA persisted 15 days after injection (Amdam *et al.*, 2003). The reasons for these differences are unknown, but it is possible that some dsRNAs may be sequestered into cells faster than others. The RNAi response may also be affected by several factors including the nature of the target gene, the site of dsRNA accumulation, the tissue where RNAi activity occurs, the delivery method, and the stage of the insect (Gatehouse *et al.*, 2004).

Tribolium castaneum (flour beetle) larvae and pupae dsRNA injections have also proven effective in silencing the insect's genes (Bucher *et al.*, 2002; Tomoyasu and Denell, 2004), and in these insects, it was first shown that RNAi responses in female pupae can be passed on to their offspring, but the mechanism for how dsRNA might enter oocytes has not yet been demonstrated (Bucher *et al.*, 2002).

Lepidopterans have also been injected with various dsRNAs and displayed RNAi responses. Larvae have shown nearly 80% reductions in target gene transcripts (Rajagopal *et al.*, 2002), and even pupae in diapause injected with full-length dsRNAs have shown gene silencing continuing to the adult stage (Bettencourt *et al.*, 2002).

Injection of dsRNA is probably one of the most widely used methods for inducing transient gene silencing in insects, however it is possible to create transgenic insects that consistently express dsRNA. Two large research consortiums have developed transgenic *D. melanogaster* strains that express hairpin-dsRNAs that are under the control of the upstream activating sequence (*UAS*) of the yeast transcriptional activator GAL4 (Piccin *et al.*, 2001), and it is now possible to acquire transgenic fruit flies that express hairpin

dsRNA transgenes under the control of tissue-specific, inducible promoters (available at the Japanese National Institute of Genetics and The Vienna Drosophila RNAi Center).

Another method that may be used to deliver dsRNA is the biolistic approach, where microscopic, dsRNA-coated beads can be blasted at high-pressure using a gene gun into dechorionated embryos of some species (Yuen *et al.*, 2008). In this study, silencing was observed from embryos up to the 3rd instar stage, indicating that the RNAi effect can persist through larval development using this method.

The methods stated above are effective, but can also be difficult and time consuming. A simpler method of delivery involves feeding, or even soaking, an organism in a solution of dsRNA to cause an RNAi response. The soaking method has been successfully used in *C. elegans* (Tabara *et al.*, 1998) and planarians (Orii *et al.*, 2003). A few insect species have been subjected to dsRNA soaking with varying results: *D. melanogaster* embryos can be dechorionated and successfully soaked in a dsRNA solution (Eaton *et al.*, 2002), and neonate larvae also showed a high degree of gene silencing when soaked in dsRNA (Whyard *et al.*, 2009). In contrast, Rajagopal *et al.* (2002) found that soaking of the moth *Spodoptera litura* larvae was unsuccessful at knocking down gut-specific genes.

Feeding artificial diets of dsRNA to organisms has been successful in knocking down specific gene expression in a number of species. In the light brown apple moth *Epiphyas postvittana*, 3rd instar larvae that were droplet fed dsRNA showed reduced gene expression in the larval midgut, but more amazingly showed that larvae fed dsRNA had reduced expression of genes in adult antennae, which indicated that the dsRNA persisted across life stages for approximately 18 days (Turner *et al.*, 2006). Diamondback

moth larvae (*Plutella xylostella*) can also be droplet fed dsRNA to cause gene knockdown (Bautista *et al.*, 2009).

In the hemipteran *Rhodnius prolixus*, feeding 2nd instar nymphs resulted in a 42% reduction of a salivary protein gene (Araujo *et al.*, 2006). Zhou *et al.* (2008) showed that feeding termites (*Reticulitermes flavipes*) high doses of dsRNA targeting genes required for moulting can result in death of those insects. Walshe *et al.* (2009) showed that dsRNA can be delivered in a bloodmeal to the tse tse fly *Glossina morsitans morsitans* to knockdown midgut specific genes; however, feeding dsRNA was unable to induce knockdown of genes specific to the fat body. Whyard *et al.* (2009) showed that species-specific gene silencing of a gut specific vATPase gene was induced by feeding species-specific dsRNAs to first instar pea aphids (*Acyrthosiphon pisum*), larval *Tribolium castaneum* and tobacco hornworm (*Manduca sexta*) larval neonates.

Although it is not an insect, the pest tick species *Ixodes scapularis*, which transmits Lyme disease to humans, showed systemic gene silencing when nymphs were capillary fed a dsRNA solution specific to the *isac* gene, which is required for processing bloodmeals (Soares *et al.*, 2005).

Feeding dsRNA in a solution is clearly effective at silencing genes in many arthropod species, however the current costs to produce such solutions is quite high. A cheaper alternative to producing synthetic dsRNAs is to induce bacteria or other rapidly dividing microorganisms to generate dsRNAs that can be cheaply extracted from them, or can be fed directly to the insect. Feeding bacteria that express dsRNAs to organisms has already been achieved in *C. elegans* (Timmons *et al.*, 2001) and planarians (Newmark *et al.*, 2003; Reddien *et al.*, 2005). So far, this method has not been attempted in insects, but

it may be particularly useful for delivering dsRNAs to some insect larvae that naturally feed on bacteria or other microorganisms.

Recently, transgenic plants have been created to express hairpin-dsRNAs that will silence endogenous plant genes or the genes of insect species that may feed on them (Baum *et al.*, 2007; Mao *et al.*, 2007). Given that insects will eventually develop resistance to the current pesticides used to protect crop plants, dsRNA-based pesticides may offer plant technologists a new set of alternative pesticides that could be designed to selectively target specific insect species.

If dsRNA soaking and/or feeding delivery methods can be developed for other insect species, it would allow for higher throughput screening of the increasing collection of genes being discovered from various genome sequencing projects, and possibly aid in the development of insecticides targeting specific pest species.

1.11 RNAi in mosquitoes

Although RNAi has been widely used in a variety of species, including insects, its use in dipteran insects has been dominated by the vinegar fly *D. melanogaster*. Very few RNAi studies have been done using mosquitoes, and most of those involve transfection of mosquito cell lines with dsRNAs, rather than the intact organism. Studies using live mosquitoes have focused primarily on silencing genes that are endogenous to the pathogens transmitted by these vectors, rather than genes expressed by the mosquitoes themselves.

The *Aedes albopictus* cell line, C6/36, a commonly used, immortalized cell line that was originally derived from larval homogenates, has been used in several studies to

determine if various Dengue virus-specific genes could be silenced in infected cells by the addition of siRNAs (Adelman *et al*., 2002; Caplen *et al*., 2002; Scott *et al*., 2010; Wu *et al*., 2010). These studies have been quite successful, showing that viral replication is reduced in dsRNA treated cells. Recently, however, it has been shown that C6/36 cells may possess a Dicer protein that cleaves dsRNAs irregularly (Brackney *et al*., 2010). Cells infected with one of West Nile, Sindbis or La Crosse viruses were screened for the presence of small viral RNA molecules, under the notion that viral RNAs will be processed by the RNAi pathway. However, the majority of small viral RNAs were not the expected average of 21 nt lengths, but were an average of 25.2 nt long. In contrast, *Drosophila* S2 cells used as a control produced the typical 21 nt siRNAs. It is unclear whether this phenomenon really affects the efficiency of RNAi induced silencing in this cell line as Brackney *et al*. (2010) suggest that only siRNAs will induce gene silencing in C6/36 cells despite that fact that other experiments have shown differently.

Other mosquito cell lines that have shown typical RNAi responses have been the *A. aegypti* cell line Aag2, derived from embryos (Scott *et al*., 2010), as well as a larval cell line called Ag55 (Smith and Linser, 2009) and a hemocyte cell line, both from *Anopheles gambiae* (Hoa *et al*., 2003).

In embryonic and adult mosquitoes, experiments using RNAi have been done to test the function of genes by looking for phenotypic changes after silencing. Many of these experiments have used microinjections of dsRNAs on various species, including *A. aegypti* (Attardo *et al*., 2003; Clemons *et al*., 2010; Roy *et al*. 2007; Zhu *et al*., 2003), *An. gambiae* (Boisson *et al*., 2006), and *Culex pipiens* (Kim *et al*., 2010).

Some groups have also made transgenic mosquitoes that express hairpin RNA molecules to induce gene silencing. Brown *et al*. (2003) created transgenic *Anopheles stephensi* to determine if this species would be a good model for using RNAi as a method to test parasite transmission, and Travanty *et al*. (2004) have created transgenic *A. aegypti* that express hairpin RNAs specific to silence Dengue virus genes and reduce transmission of the disease.

1.12 Thesis objectives

The initial main objective of this project was to examine sex-specific proteins produced by MAGs in the mosquito *A. aegypti*, particularly those involved in reducing mating receptivity and increasing egg production in females when transferred during mating. However, with the recent publication of this information (Sirot *et al*., 2008; Yapici *et al*., 2008), I decided to focus on a novel, sex-biased gene, using the relatively new tool, RNAi, to analyze its function.

As RNAi has rarely been used in mosquitoes, and is usually performed using traditional methods (i.e. injections), it was worth considering whether oral delivery could be a viable method for delivering dsRNA to mosquitoes, as it had been shown successful in other insects, particularly lepidopterans. Therefore, the objectives for my M.Sc. were:

1. *Functional characterization of a novel cytochrome P450 gene using RNAi*

CYP proteins have typically been associated with metabolizing exogenous compounds within a wide range of insects, and most studies have focused on their role in insecticide resistance. In this study, a putative, male-biased, *cyp* gene was identified in

the mosquito *A. aegypti* and RNAi was used to analyze its possible role in ecdysis and male reproductive success.

2. *Determine the effectiveness of oral delivery of dsRNA to induce an RNAi response*

In addition to validating the function of newly discovered genes, there is the possibility to use RNAi as a biological pesticide. During my thesis, I examined the potential for delivering dsRNA in oral formulations to *A. aegypti* larvae, and assessed its toxicity on developing larvae by attempting to silence a small number of genes considered either essential for normal cell functioning or mosquito growth and development.

Chapter 2: A novel, sex-biased cytochrome P450 gene in *A. aegypti*

2.1 Introduction

Insects express numerous sex-specific genes during development, but also throughout adulthood, usually with the intention of enhancing reproductive success. For example, females express genes for egg development, sperm storage and ovipositing, while males typically express genes for sperm production, mate-finding, and courtship behaviour.

Sixty-three genes have been found to be sex-specifically expressed in various tissues within *D. melanogaster*. Fifty-two of these genes are sex-specifically expressed in various tissues of the body, and although the functions of these genes have not been fully elucidated, they presumably regulate the differential growth, development and/or function of tissues and organs in the two different sexes. Eleven genes are sex-specifically expressed in internal reproductive organs, and three of those are found solely in the MAGs (Arbeitman *et al*., 2004).

The MAGs produce many uniquely male genes in many insects, but have been most thoroughly studied in *D. melanogaster*. Many of the proteins examined have no specifically defined function, yet most are considered to play a role in improving the reproductive success of the male. One of the original goals of this research project had been to identify MAG genes in the pest mosquito *Aedes aegypti* using a low stringency PCR method with degenerate primers that were based on a number of previously identified MAG genes from *Drosophila* species. However, during the initial phase of this project, another research group published a paper describing the MAG proteins in *A. aegypti* (Sirot *et al*., 2008) and another group described the MAG sex peptide and its

receptor in *D. melanogaster* and *A. aegypti* (Yapici *et al*., 2008). Given that two competing research groups had achieved some of the initial project's goals, attention was directed to another MAG protein, Acp26Aa (also called ovulin), which in *D. melanogaster*, is associated with increased egg laying behaviour in females following mating (Herndon and Wolfner, 1995). While attempting to amplify the *A. aegypti* homologue of the *Acp26Aa* gene, a PCR fragment with similarity to the *D. melanogaster* cytochrome P450 gene *cyp4g15* was identified. In *D. melanogaster*, this gene has a potential role in ecdysteroid synthesis (Maïbèche-Coisne *et al*., 2000), and as a portion of this gene was fortuitously isolated from *A. aegypti*, the focus of this research changed to examine the expression and explore the possible functions of this gene in the mosquito.

2.2 Methods

2.2.1 Mosquito Rearing

McAllen strain *Aedes aegypti* were reared at 25°C, 50% humidity, on a 16h light: 8h dark photoperiod. Eggs were placed in small tubs of dechlorinated tap water with ground desiccated liver tablets to induce hatching. Larvae were maintained in these tubs until pupation. Pupae were then collected and placed in large cages to eclose, and adults were maintained on a 10% sucrose solution. Females were blood fed once a week by creating a "blood bag" from stretched Nescofilm (Karlan Research Products) containing ~2 ml of blood obtained from cows maintained at the Gleanlea Research Station, Manitoba, or from rats maintained in the Animal Holding Facilities of the University of Manitoba, Fort Garry Campus. Blood was warmed to 42°C prior to feeding, and females were allowed to lay eggs on dampened paper towels. Laid eggs were dried and stored in humid containers prior to hatching.

2.2.2 Isolation of the CYP gene fragment from cDNA

RNA was extracted from 40 dissected MAGs and from 10 adult male and 10 adult female *A. aegypti* using a Qiagen RNeasy RNA extraction kit according to the manufacturer's instructions. Firstly, mosquitoes or the dissected tissues were placed in 1.5 ml microfuge tubes and crushed using a plastic pestle in 600 µl of RLT lysis buffer containing 1% β-mercaptoethanol. The mixture was then placed in a Qiagen QiaShredder column for tissue homogenization and the resulting lysate was used with the RNeasy extraction kit. Extracted RNA was eluted in 60 µl of RNase-free water and concentrations

were determined using either an Ultrospec 3100 pro (GE Healthcare), or a NanoVue (GE Healthcare) spectrophotometer.

RNA was then treated with amplification-grade DNase I (Invitrogen) for subsequent use as a template for cDNA synthesis. cDNA was synthesized using an Invitrogen First Strand cDNA synthesis kit with oligo dT primers and 0.3-1 μg of RNA as template. Samples were incubated with Superscript II reverse transcriptase (Invitrogen) for 50 min and then the reaction was terminated by incubating at 70°C for 15 min. Finally, RNAse H (Invitrogen) was added to remove RNA duplexed to the cDNA.

Degenerate primers were originally designed to target possible homologues of the *Drosophila* male accessory gland (MAG) gene *ovulin* (*Acp26Aa*) for a study on male reproductive genes in *A. aegypti*. These primers (DegOvF 5' CKGCKGYTKWYRCARWGGYW, DegOvR 5' RGCAKGTGSKGYGMTAKCCTT) amplified a PCR product (using the following cycles: 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 1 min, followed by a final extension at 72°C for 10 min) from cDNA derived from MAG tissue that was then sequenced (as below) and used to design new primers specific to the *A. aegypti* sequence. The newly designed primers (AedesUnknownF 5' CGGCGGTTGGTCGCAGTGG, AedesUnknownR 5' GGCAGGTGGGGCGATATCTT) were used in a standard 25 μl PCR reaction that was programmed as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, followed by a final extension at 72°C for 10 min.

The PCR reactions were resolved on a 1% agarose gel, and the band was excised from the gel and purified with Qiagen's QIAquick Gel Extraction Kit. Gel purified

samples were ligated to Qiagen's cloning vector pDrive (Fig. 3) that possesses U overhangs (rather than T overhangs) to allow for more efficient insertion of PCR fragments generated by *Taq* DNA polymerase.

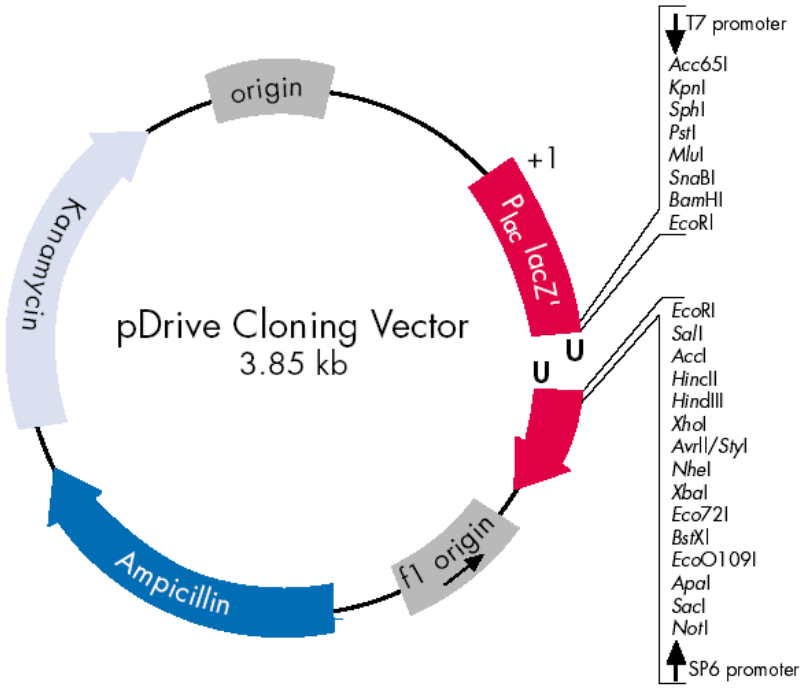

Figure 3. Plasmid map of Qiagen's Cloning Vector pDrive. Note the U overhangs, multiple cloning site, and T7 and Sp6 promoter sites. (From Qiagen's PCR Cloning Handbook, April 2001).

Subcloning Efficiency™ DH5α Chemically Competent *E. coli* cells (Invitrogen) were transformed with the ligated plasmids using a heat-shock method as described by the manufacturer. The cells were then plated on LB agar (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 1.5% bacto-agar) plates with ampicillin (50 mg/ml).

Transformed bacterial colonies were checked for the presence of PCR fragment inserts using a PCR colony screening method where bacterial colonies are picked and dotted into PCR tubes, then microwaved for 2 min to lyse cells. The lysed cells are then

used as template in a PCR reaction similar to that used above, but scaled down to 15 µl. For these reactions, T7 (5' TAATACGACTCACTAGGG) and Sp6 (5' GATTTAGGTGACACTATAG) primers were used to determine if plasmids within the bacteria contained the desired insert, and an annealing temperature of 40°C was used during the PCR cycles. PCR reactions were resolved on a 1% agarose gel to identify any colonies containing plasmids with appropriate-sized inserts. The bacterial colonies were then grown in 4 ml of LB broth with ampicillin (50 mg/ml) overnight at 37°C with shaking at 225 rpm to give adequate aeration to the cells. The plasmid DNA was purified from the bacteria using Qiagen's QIASpin Miniprep kit.

Plasmids were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The cycle sequencing reaction was prepared by adding the following components to 1 µl of plasmid DNA: 3.5µl of 5x BigDye® Sequencing Buffer, 1µl BigDye® Terminator Ready Reaction Mix, 1.6 µl T7 or Sp6 primer (2 µM) and 11.9µl Nanopure H_2O. Sequencing products were then amplified as follows: denaturation at 96ºC for 1 minute, followed by 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes. Amplified products were then precipitated by adding 5 µl of 125 mM EDTA and 60 µl of 100% ethanol, and incubated at room temperature for 15 minutes. Tubes were then centrifuged at 13,000 rpm for 15 minutes at 4°C, and the pellet was washed with 70% ethanol. The pellets were then dried for 10 minutes in a DNA 120 SpeedVac Concentrator (Savant) and resuspended in 20 µl of Hi-Di™ formamide (Applied Biosystems). DNA was denatured at 94ºC for 5 minutes in a heating block then immediately placed on ice to prevent re-annealing. Samples were then loaded onto a 96-

well plate and sequenced using a Genetic Analyzer 3130 sequencer (Applied Biosystems).

The identity of the sequence was analyzed by comparing it to the genome databases available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) using the basic local alignment search tool (BLAST), as well as the *Aedes aegypti* genome database at the Vectorbase website (http://www.vectorbase.org/). Alignments of sequences were performed using the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) online alignment program ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). As the gene fragment showed a high degree of sequence identity to that of a cytochrome-P450 gene (see Results), the gene hereafter will be referred to as *Aacyp*.

2.2.3 Expression profile of the novel CYP gene in *A. aegypti*

2.2.3.1 General expression within different life stages of A. aegypti

Quantitative reverse-transcriptase PCR (qRT-PCR) was used to determine when the *Aacyp* gene was being expressed during development of the mosquitoes. First, all life stages of the mosquito were collected, including eggs, 1st instar larvae, 2nd instar larvae, 3rd instar larvae, male 4th instar larvae, female 4th instar larvae, male pupae, female pupae, male adults and female adults. Male 4th instar larvae were identified using a compound microscope and observing the ventral side of the anal segment for the developing ejaculatory ducts (Chambers, 2005). Larvae with an absence of these structures were considered as females. For male and female pupae, 4th instar larvae were sexed and then

allowed to pupate before collection. RNA extractions and cDNA syntheses were performed as described in section 2.2.2.

For each cDNA sample, qRT-PCR was performed in triplicate using a BioRad iQ5 Real-Time PCR Detection System using 96-well plates with 20 µl reactions containing ~10 ng of cDNA, 10 µl of SYBR Green Supermix (BioRad), 1 µl each of forward and reverse primers (10 µM), and Nanopure water, using the following program: 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, then 55°C for 30 sec, followed by a melt curve analysis to confirm that only a single PCR product was amplified.

qRT-PCR primer sets were designed using the Beacon Designer^{TM} program (Premier Biosoft), which generates ideal primer sequences from a given gene sequence. In general, sets of good qRT-PCR primers should have annealing temperatures within 1°C of each other and produce relatively small amplicons of fewer than 200 bp long. When specifically designing qRT-PCR primers to measure expression of a gene that was potentially knocked down using dsRNA, it is also ideal to design primers that will amplify a portion of the gene outside of the region to which the dsRNA specifically targets. For this experiment, one set of primers was designed to target the ribosomal protein S7RP (S7RPqPCRFwd: 5' AAATAAATTCGCTATGGTTTTCGG and S7RPqPCRRev: 5' CCTTCTTGCTGTTGAACTCG, 182 bp) as an internal reference gene for comparing quantities of *Aacyp* among the different life stages. The following primers were used to determine expression of the unknown *A. aegypti* CYP: AeCYPqPCRFwd: 5' GAAAGGAGGTGGGGAAGG and AeCYPqPCRRev: 5' CACAGCCATCGCATAGTC, amplifying 134 bp.

Cycling conditions and dissociation curve analysis were performed on the iQ5 Thermal Cycler (BioRad) according to the manufacturer's instructions. The relative amount of *Aacyp* transcripts in the mosquito samples was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) where *Aacyp* transcript levels are normalized to the internal standard (*S7rp*) using the following equation:

Fold change in *Aacyp* expression = $2^{-\Delta\Delta CT}$, where

$\Delta\Delta CT = (CT,_{Aacyp}\text{-}CT,_{S7rp})_{Time\ x} - (CT,_{Aacyp}\text{-}CT,_{S7rp})_{Time\ 0}$

In this case, "Time 0" is equivalent to the egg samples, and "Time *x*" refers to any other samples being compared to the egg samples.

2.2.3.2 Aacyp expression in specific tissues

Virgin mosquitoes were collected at days 0, 3 and 7 post-eclosion and dissected to collect heads, reproductive systems and the remaining carcass. In males, the reproductive systems were separated into the MAGs and remaining reproductive system. Heads were collected in an attempt to isolate nervous tissue that could be involved in the control of mating behaviours, and were removed from the thorax using ultra fine forceps. The remaining carcass, after removal of reproductive systems and heads, was stored separately, as were the other tissues, in appropriate volumes of the RNAse inhibitor solution *RNAlater* (Ambion) until RNA extraction.

To collect mated mosquitoes, virgin adults were collected at 3 and 7 days post-eclosion, mated to adults of the same age, then left overnight before dissection, which was performed similar to the virgin mosquito dissections. Freshly eclosed (0 days post-eclosion) adults were not mated because they are unable to copulate at that time. Males

must undergo a 180° rotation of their genitalia, which takes 15-24 hr and females are refractory to insemination for the first 48 hrs after eclosion (Hartberg, 1971). Matings occurred by placing a single pair of virgin mosquitoes in a small mosquito cage (approx. 30 cm x 30 cm x 30 cm), then tapping the sides to get the mosquitoes flying, as this species of mosquito must initiate mating while in flight. Usually within a few minutes the mosquitoes could be observed in a brief coupling, which is typical of a mating bout for this species, and then the pair was placed in small plastic vials until dissection the following day.

RNA extractions, cDNA synthesis, and qRT-PCR were performed as above (section 2.2.2) using the same qRT-PCR primers listed in section 2.2.3.1.

2.2.4 Cloning *A. aegypti* cytochrome P450 for dsRNA delivery

The *Aacyp* gene fragment was excised from the pDrive plasmid using two restriction enzymes, *ApaI* and *PstI*, and then ligated to the dual T7 vector pL4440 (Fig. 4), which was kindly provided by Andrew Fire, using T4 ligase (Invitrogen). The convergent T7 promoters possessed by pL4440 allows for *in vitro* transcription of dsRNA. The ligated DNA was used to transform DH5α cells as described above (section 2.2.2), and putative transformed bacterial colonies were PCR screened (using pL4440 specific primers: pL4440Fwd: 5'ACCTGGCTTATCGAA and pL4440Rev: 5' TAAAACGACGGCCAGT, and an annealing temperature of 57°C in the PCR cycles) for the appropriate-sized inserts. DNA sequencing was performed to confirm that the genes of interest were in the pL4440 plasmid using a Genetic Analyzer 3130 Sequencer

(Applied Biosystems), as described above (section 2.2.2), and sequence identities were confirmed using NCBI and Vectorbase's BLAST programs.

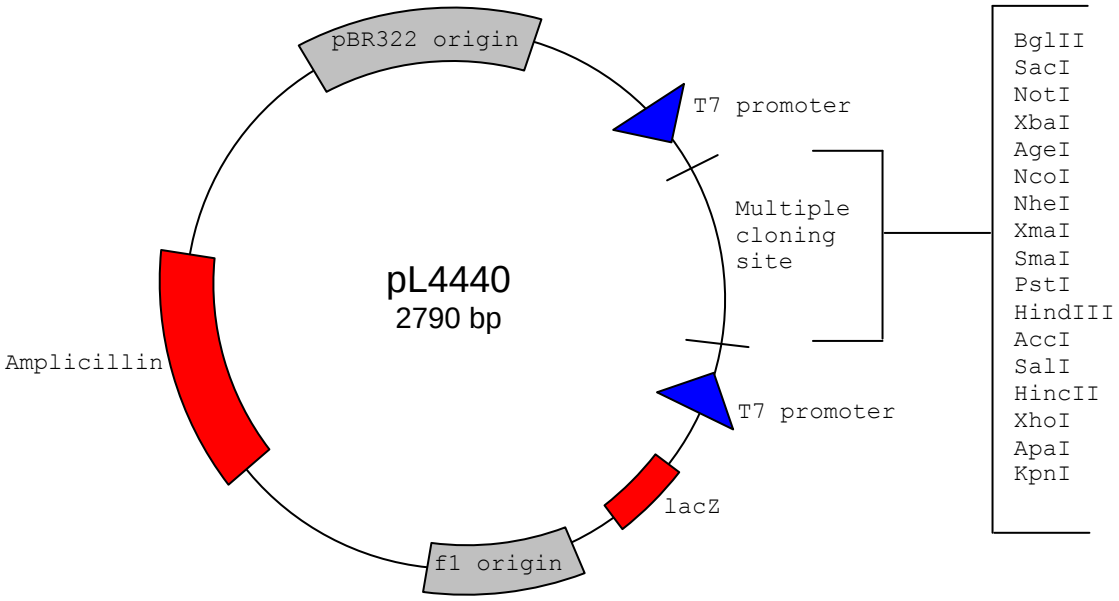

Figure 4. Plasmid map of the dual-T7 vector, pL4440. Note the opposing T7 promoters and multiple cloning site.

2.2.5 *In vitro* transcription of dsRNA

To obtain sufficient DNA template for *in vitro* transcription from each of the pL4440 plasmids, the gene fragment cloned into pL4440 (*Aacyp*) was PCR-amplified along with the flanking convergent T7 promoter sequences from the dsRNA expression plasmid, using the specially designed pL4440 primers listed above in section 2.2.4. To obtain *gus*-dsRNA, pL4440 containing a 1.8 kb *gus* gene fragment was provided by Dr. Steven Whyard. Five standard 25 µl PCR reactions (using the following program: 94°C for 3 min, followed by 30 cycles of 94°C for 30sec, 57°C for 30 sec, 72°C for 1.5 min, followed by a final extension of 72°C for 10 min) for each gene were tested for proper

amplification by resolving 5 µl of each PCR reaction on a 1% agarose gel. The remaining ~100 µl of PCR reaction for each gene was then pooled together and purified using Qiagen's QIAquick PCR Purification kit to remove unused PCR reagents such as excess primers and dNTPs.

The purified PCR template (1 µg) was used in 20 µl *in vitro* transcription reactions with the MEGAscript® RNAi kit (Ambion) according to the manufacturer's specifications to produce concentrated dsRNA complementary to each gene of interest (*Aacyp* and *gus*). Briefly, this kit synthesizes complementary single-stranded RNA (ssRNA) by incubating the template, along with NTPs, buffer, water and T7 RNA polymerase at 37°C for 2-4 hr, depending on the size of the gene of interest. Four hour incubations were used for *gus* dsRNA synthesis, whereas *Aacyp* was incubated only two hours.

The complementary strands were then annealed by first denaturing the strands at 75°C for 5 min, then allowing the reactions to gradually cool to room temperature. The dsRNA reactions were then treated with DNAse to remove the template, and RNAse to remove any ssRNA, and then finally purified using a filtered column.

A 10-fold diluted sample of purified dsRNA was resolved on a 1% agarose gel to check for size and purity of the sample. Concentrations of dsRNA were measured using an Ultrospec 3100 spectrophotometer (GE Healthcare) or a NanoVue Spectrophotometer (GE Healthcare).

2.2.6 Cytochrome P450 injections and bioassay

Cytochrome P450 dsRNA was diluted to 0.5 μg/μl for injection into 4th instar *A. aegypti* larvae and pupae. Injections were performed using a mouth pipette and glass needles. Needles were pulled on a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments) using 50 μl glass capillary tubes and a program of: one cycle of heat = ramp + 5, pressure = 500, pull = 30, velocity = 120, time = 125.

Approximately 250-500 ηl of either *gus*- or *Aacyp*-dsRNA were injected into the side of 4th instar larvae, usually between the 5th and 8th abdominal segment. Pupae were injected into the ventral side, usually in the 5th or 6th segment. After injection, mosquitoes were placed in Petri plates or plastic vials containing dechlorinated tap water plus ampicillin (final volume, approx. 0.25 μg/ml). The insects were then observed for further development into adulthood, and for the survivors, their ability to reproduce was assessed by allowing the insects to mate with untreated insects, followed by blood-feeding of the females and then allowing them to lay their eggs and observe the hatch rates.

Subsets of injected larvae and individual pupae and developed adults were collected 2 days after injection (or the day of mating for adults) for testing of RNAi efficiency. Samples were placed in *RNAlater* until RNA extraction and cDNA synthesis. Knockdown of the CYP gene was determined using qRT-PCR as described in section 2.2.3.1. *S7rp* primers were again used to amplify the reference gene, however a new set of CYP primers were designed that correspond to sequences found outside of the dsRNA target region as follows: AeCYPqRToutF: 5’ CCTCGGGCTGATAAGATGTC and AeCYPqRToutR: 5’ GACCTGGCAATCGGGAAC, amplifying 127 bp.

2.2.7 Phylogenetic analysis of *A. aegypti* cytochrome P450

To determine how similar this novel CYP from *A. aegypti* is to CYPs of other species, a multiple sequence alignment was performed using ClustalX and *cyp* sequences from various insect species (see Results, Table 4), most being from the CYP4 family. ClustalX uses the neighbour-joining method to calculate a phylogenetic tree, and bootstrap analysis (1000 bootstraps) was performed. A phylogram was created from this data using TreeView (see Results, Fig.16).

2.3 Results

2.3.1 A novel CYP gene in the *A. aegypti* genome

Degenerate primers were originally used to amplify a putative homolog of *D. melanogaster*'s MAG gene *ovulin*, but the amplified gene fragment, once sequenced, showed no identity to ovulin from any other insect. Instead, a BLAST search of the *A. aegypti* genome (Vectorbase) showed that the PCR product was an exact match to an uncharacterized gene (Gene: AAEL006824) found in supercontig 1.222 that showed high identity to a cytochrome P450 gene, and hence was tentatively named *Aacyp*. Interestingly, the translated protein sequence of *Aacyp* is a near perfect match to a putative CYP gene named *cyp4g35* (Gene: AAEL008345), found in supercontig 1.318 (Fig. 5). Although the predicted protein sequences are virtually the same, differing in only one amino acid, the nucleotide sequences of the two genes differ at 55 nucleotide sites (Fig. 6). Both genes have three exons, all of the same predicted lengths, but *cyp4g35* has a first intron that is nearly 2000 bp longer than that observed in *Aacyp* (Table 1). A global, pairwise alignment of these two intron sequences showed only 44.2% overall similarity between them (alignment not shown). The only interesting aspect about these first introns in the two genes is that they appear to contain remnants of different transposable elements. A nucleotide BLAST search of the *Aacyp* first intron against the NCBI database identified a ~300 bp sequence with high identity (94%) to a MosquI-Aa5 retrotransposon (NCBI accession: AF134903). Likewise, a BLAST of *cyp4g35*'s 8561 bp long intron identified three partial sequences with high identities (ranging from 79 to 91%) to the transposon AeTRC1 (NCBI accession: BN000947). Approximately the first 600 bp of both the 5' and 3' sequences flanking the start and stop codons of each gene are

nearly 100% identical, and then the sequences differ considerably beyond those limits. In all likelihood, these two genes are paralogs, and although it is unknown whether both are transcribed, if translated, they would generate virtually identical proteins.

```
4g35            -------MSAEIVAERGSSLVSLAVPMVIFMTLVLVASALFHFWMISRRYVQLGNKIPGP 53
Aacyp           VSGVIYKMSAEIVAERGSSLVSLAVPMVIFMTLVLVASALFHFWMISRRYVQLGNKIPGP 60
                       **************************************************

4g35            RAYPFIGNANMLLGMNHNEIMERAMQLSYIYGSVARGWLGYHLVVFLTEPADIEIILNSY 113
Aacyp           RAYPFIGNANMLLGMNHNEIMERAMQLSYIYGSVARGWLGYHLVVFLTEPADIEIILNSY 120
                ************************************************************

4g35            VHLTKSSEYRFFKPWLGDGLLISSGEKWRSHRKLIAPAFHMNVLKTFVDVFNDNSLAVVE 173
Aacyp           VHLTKSSEYRFFKPWLGDGLLISSGEKWRSHRKLIAPAFHMNVLKTFVDVFNDNSLAVVE 180
                ************************************************************

4g35            RMRKEVGKEFDVHDYMSEVTVDILLETAMGSQRTSESKEGFDYAMAVMKMCDILHSRQLK 233
Aacyp           RMRKEVGKEFDVHDYMSEVTVDILLETAMGSQRTSESKEGFDYAMAVMKMCDILHSRQLK 240
                ************************************************************

4g35            FHLRMDSVFNFTKIKQEQERLLGIIHGLTRKVVKQKKELFEKNFADGKLPSPSLSEIIAK 293
Aacyp           FHLRMDSVFNFTKIKQEQERLLGIIHGLTRKVVKQKKELFEKNFADGKLPSPSLSEIIAK 300
                ************************************************************

4g35            EESESKEPLPVISQGSLLRDDLDFNDENDIGEKRRLAFLDLMIETAKSGADLTDEEIKEE 353
Aacyp           EESESKESLPVISQGSLLRDDLDFNDENDIGEKRRLAFLDLMIETAKSGADLTDEEIKEE 360
                *******.****************************************************

4g35            VDTIMFEGHDTTAAGSSFVLCLLGIHQDVQDRVYKEIYQIFGNSKRKATFNDTLEMKYLE 413
Aacyp           VDTIMFEGHDTTAAGSSFVLCLLGIHQDVQDRVYKEIYQIFGNSKRKATFNDTLEMKYLE 420
                ************************************************************

4g35            RVIFETLRMYPPVPVIARKVTQDVRLASHDYVVPAGTTVVIGTYKVHRRADIYPNPDVFN 473
Aacyp           RVIFETLRMYPPVPVIARKVTQDVRLASHDYVVPAGTTVVIGTYKVHRRADIYPNPDVFN 480
                ************************************************************

4g35            PDNFLPERTQNRHYYSYIPFSAGPRSCVGRKYAMLKLKVLLSTILRNYRVVSNLKESDFK 533
Aacyp           PDNFLPERTQNRHYYSYIPFSAGPRSCVGRKYAMLKLKVLLSTILRNYRVVSNLKESDFK 540
                ************************************************************

4g35            LQGDIILKRTDGFRIQLEPRV 554
Aacyp           LQGDIILKRTDGFRIQLEPRV 561
                *********************
```

Figure 5. Clustal alignment of CYP4G35 and Aacyp protein sequences. Blue highlights indicate differences between the amino acid sequences.

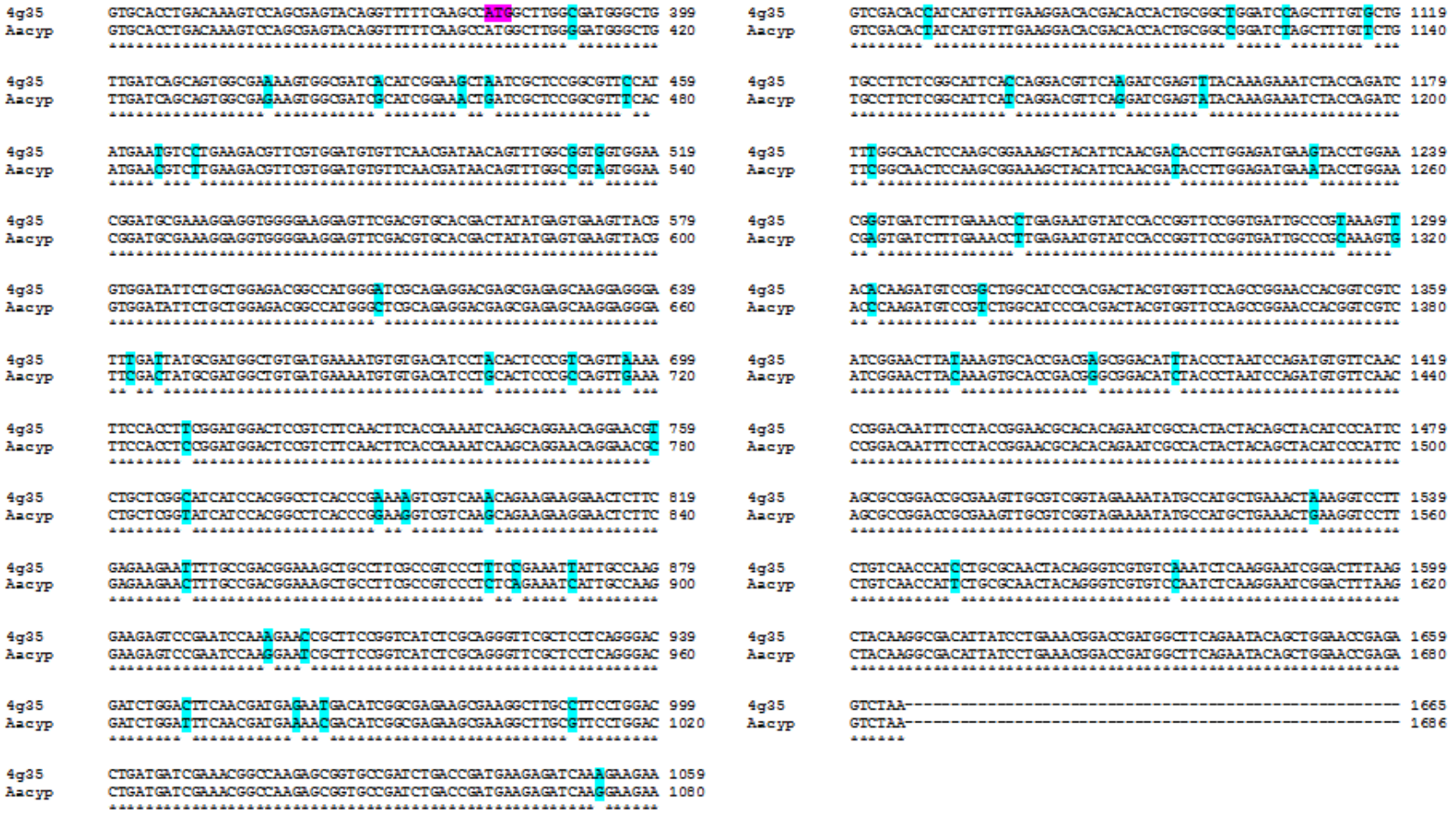

Figure 6. Clustal alignment of *Aacyp* and *cyp4g35* nucleotide sequences. Blue highlights indicate differences in nucleotides (55 differences), and pink indicates the start codon for *cyp4g35*.

Table 1. Comparison of intron and exon sizes in *Aacyp* and *cyp4g35*.

	Size (nt)	
Gene segment	*Aacyp*	*Cyp4g35*
Exon 1	665	665
Intron 1	6725	8651
Exon 2	839	839
Intron 2	73	73
Exon 3	161	161

The Vectorbase website also provides information on the expression of many of the *A. aegypti* genes based on microarray analyses. Curiously, there are two different microarray analyses performed by Harker *et al*. (2007, unpublished) that report conflicting gene expression levels of *cyp4g35*; one microarray experiment suggests that there is nearly a 4-fold reduction in expression of *cyp4g35* in females compared to males, while the other microarray experiment showed no significant difference in expression between the sexes. At this time, it is unclear whether this discrepancy reflects a weakness in the microarray experiment or may reflect different expression levels of the two closely-related CYP genes.

A BLAST analysis of the *Aacyp* sequence revealed that it is most similar to *D. melanogaster*'s *cyp4g15* gene, sharing 60.5% identity of the nucleotide sequence and 55.6% identity of the predicted protein sequence (Fig. 7 and 8).

```
Aacypfrag       -----------------TGGGCTGTTGATCAGCAGTGGCGAGAAGTGGCGATCGCATCGG 43
Cyp4g15         AAGCCCTGGCTGGGCGACGGACTGCTGATCAGCACAGGTCAAAAATGGCGATCGCACCGC 420
                      ** *** *********  **  * ** ********** **

Aacypfrag       AAACTGATCGCTCCGGCGTTTCACATGAACGTCTTGAAGACGTTCGTGGATGTGTTCAAC 103
Cyp4g15         AAACTGATTGCACCCACATTCCATCTGAATGTCTTGAAGAGCTTCATCGAGTTGTTCAAC 480
                ******** ** **  * ** **  **** **********  *** * **  ********

Aacypfrag       GATAACAGTTTGGCCGTAGTGGAACGGATGCGAAAGGAGGTGGGGAAGGAGTTCGACGTG 163
Cyp4g15         GAGAACTCGCGTAATGTGGTGAGGAAACTGCGTGCGGAGGATGGTCGCACTTTTGATTGC 540
                ** ***         ** ***       ****   *****  **       ** **

Aacypfrag       CACGACTATATGAGTGAAGTTACGGTGGATATTCTGCTGGAGACGGCCATGGGCTCGCAG 223
Cyp4g15         CATGATTACATGAGCGAGGCAACCGTGGAGATTCTATTGGAGACTGCGATGGGTGTGTCG 600
                ** ** ** ***** ** *  ** ***** *****  ******* ** *****   *  *

Aacypfrag       AGGACGAGCGAGAGCAAGGAGGGATTCGACTATGCGATGGCTGTGATGAAAATGTGTGAC 283
Cyp4g15         AAGAAAACGCAGGACAAATCGGGATTCGAGTATGCGATGGCCGTGATGCGGATGTGCGAC 660
                * **  *   **  ***   ********* ********** ******   ***** ***

Aacypfrag       ATCCTGCACTCCCGCCAGTTGAAATTCCACCTCCGGATGGACTCCGTCTTCAACTTCACC 343
Cyp4g15         ATCCTTCACGCCCGCCATCGGAGCATCTTTCTGCGCAACGAGTTCGTGTTCACCCTGACG 720
                ***** *** *******   **   **   ** ** *  ** * *** **** * * **

Aacypfrag       AAAATCAAGCAGGAACAGGAACGCCTGCTCGGTATCATCCACGGCCTCACCCGGAAGGTC 403
Cyp4g15         CGCTACTACAAGGAGCAGGGTCGCCTGCTGAACATCATCCACGGCCTGACCACCAAGGTG 780
                     * *  **** ****  ********    ************** ***   *****

Aacypfrag       GTCAAGCAGAAGAAGGAACTCTTCGAGAAGAAC------------TTTGCCGA------- 444
Cyp4g15         ATCCGCAGCAAGAAGGCGGCCTTTGAGCAGGGCACCCGTGGATCCCTTGCCCAGTGCGAG 840
                 **      *******    *** *** **  *             ***** *

Aacypfrag       CGGAA---AGCTGCCTTCG--------------------------------CCGTCCCTC 469
Cyp4g15         CTGAAGGCAGCCGCCTTAGAGCGGGAACGGGAACAGAATGGTGGTGTTGACCAGACCCCA 900
                * ***   *** ***** *                                 * * ***

Aacypfrag       TCAGAAATCATTGCCAAGGAAGAGTCCGAATCCAAGGAATCGCTTCCGGTCATCTCGCAG 529
Cyp4g15         TCAACAGCTGGCAGCGATGAGAAGGATCGGGAGAAGGATAAGGAGAAGGCCAGTCCGTGG 960
                ***  *        * * **  **         *****   *     ** **   **  *

Aacypfrag       -GGTTCGCTCCTCAGGGACGATCTG--GATTTCAACGATGAA---------------AAC 571
Cyp4g15         CGGGACCTGTCCTATGGACAGTCGGCTGGTCTCAAGGATGACCTGAATGTGGAGGACAAT 1020
                 **  *    *  * ****  ** *  * * **** *****               **

Aacypfrag       GACATCGGCGAGAAGCGAAGGCTTGCGTTCCTGGACCTGATGATCGAAACGGCCAAGAGC 631
Cyp4g15         GATATTGGTAAGAAGAAGCGGCTGGCCTTCCTTGATTTGATGCTCGAAAGTGCACAGAAT 1080
                ** ** **  *****    **** ** ***** **  ***** ******  **  ***

Aacypfrag       GGTGCCGATCTGACCGATGAAGAGATCAAGGAAGAAGTCGACACTATCATGTTTGAAGGA 691
Cyp4g15         GGAGCACTCATTACGGACACGGAAATTAAGGAGCAGGTGGACACGATCATGTTCGAGGGA 1140
                ** **     * ** **    ** ** *****  * ** ***** ******** ** ***

Aacypfrag       CACGACACCACTGCGGCC---------------------------------------- 709
Cyp4g15         CACGACACCACGGCAGCCGGCTCCTCGTTCTTTCTCTCGCTGATGGGCATCCACCAGGAC 1200
                ********** ** ***
```

Figure 7. Clustal alignment of the novel *Aacyp* fragment, labelled Aacypfrag, from *A. aegypti* and the coding sequence of *cyp4g15* (NCBI accession: AF159624) from *D. melanogaster*. *indicate exact matches between the sequences.

```
Cyp4g15         KPWLGDGLLISTGQKWRSHRKLIAPTFHLNVLKSFIELFNENSRNVVRKLRAEDGRTFDC 180
Aacypfrag       ------GLLISSGEKWRSHRKLIAPAFHMNVLKTFVDVFNDNSLAVVERMRKEVGKEFDV 54
                      *****:*:***********:**:****:*:::**:**  **.::* * *: **

Cyp4g15         HDYMSEATVEILLETAMGVSKKTQDKSGFEYAMAVMRMCDILHARHRSIFLRNEFVFTLT 240
Aacypfrag       HDYMSEVTVDILLETAMGSQRTSESKEGFDYAMAVMKMCDILHSRQLKFHLRMDSVFNFT 114
                ******.**:******** .:.::.*.**:******:******:*: .:.** : **.:*

Cyp4g15         RYYKEQGRLLNIIHGLTTKVIRSKKAAFEQGTRGSLAQCELKAAALERE-REQNGGVDQTP 300
Aacypfrag       KIKQEQERLLGIIHGLTRKVVKQKKELFEK----------------------NFADGKLP 152
                :  :** ***.****** **::.**  **:                      * . .: *

Cyp4g15         STAGSDEKDREKDKEKASPWRDLSYGQSAGLKDDLNVED-NDIGKKKRLAFLDLMLESAQ 359
Aacypfrag       SPSLSEIIAKEESESKES---LPVISQGSLLRDDLDFNDENDIGEKRRLAFLDLMIETAK 209
                *.: *:   :*:.:.* *       .*.: *:***:.:* ****:*:********:*:*:

Cyp4g15         NGALITDTEIKEQVDTIMFEGHDTTAAGSSFFLSLMGIHQDIQDRVLAELDSIFGDSQRP 419
Aacypfrag       SGADLTDEEIKEEVDTIMFEGHDTTAA-------------------------------- 236
                .** :** ****:**************
```

Figure 8. Clustal alignment of the protein sequences of the novel Aacyp fragment labelled Aacypfrag, and CYP4G15 (NCBI accession: AF159624) from *D. melanogaster*. * indicate an exact match in sequence, colons indicate conserved substitutions and periods indicate semi-conserved substitutions.

Cyp4g15 is expressed in the nervous tissue of 3rd instar *D. melanogaster* larvae, however its function is unknown (Maïbèche-Coisne *et al*., 2000). A similar gene is also expressed in the steroidogenic glands of the crayfish *Orconectes limosus*, and is believed to be involved in ecdysteroid synthesis (Dauphin-Villemant *et al.*, 1999). Given the high similarity to *cyp4g15*, expression of this novel CYP (*Aacyp*) in *A. aegypti* was examined further.

2.3.2 *Aacyp* is sex-biased in *A. aegypti* males

The developmental expression profile of *Aacyp* was examined using qRT-PCR with cDNA derived from various life stages of *A. aegypti* mosquitoes. Expression in juvenile stages (*i.e.* eggs, 1st, 2nd, 3rd and 4th instar larvae) was fairly low compared to pupal and adult stages, which were nearly 250-fold higher than in eggs (Fig. 9). Expression in males was approximately 2-fold higher than in females, suggesting that this

CYP is indeed sex-biased, and perhaps has a role in male reproductive function(s). It is worth noting that for this and all subsequent qRT-PCR analyses, the primers used to measure gene expression would not discriminate between *Aacyp* and *cyp4g35* gene transcripts.

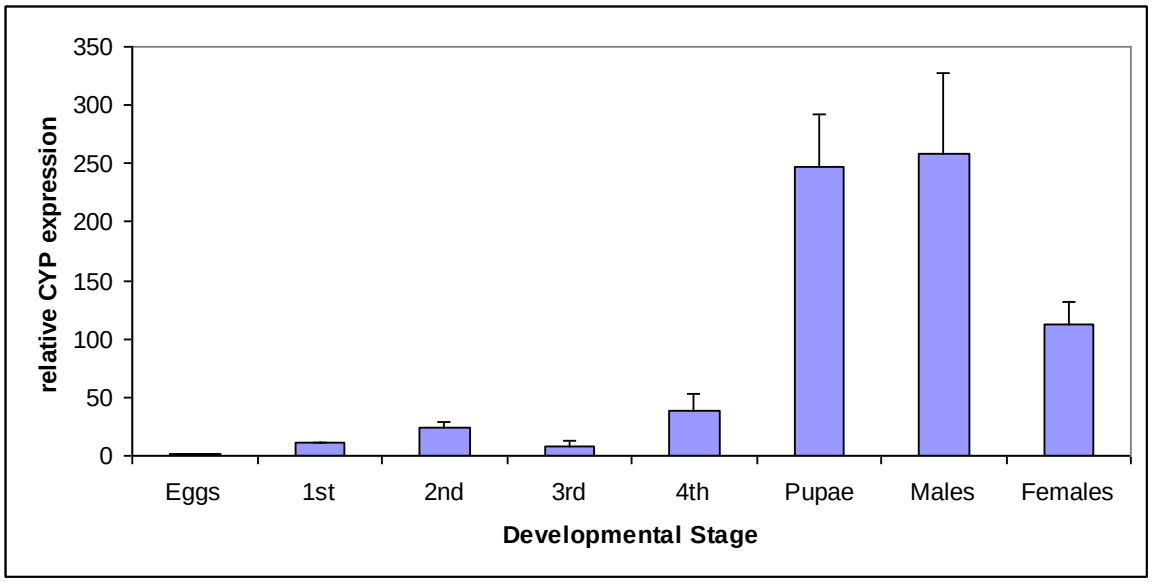

Figure 9. *Aacyp* expression in different life stages of *A. aegypti*. Changes in gene expression are relative to expression in eggs, and calculations were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Values represent the means and standard errors from 3 different replicate experiments. Expression in males is significantly different from expression in females (ANOVA, $p<0.05$).

Aacyp was also highly expressed in pupae, but it is worth noting that the cDNAs used in the qRT-PCR analyses of Figure 9 were derived from pools of male and female pupae mixed together. Therefore, further experiments were performed to determine if expression was male-biased in 4th instar larvae and pupae by sex-sorting larvae according to a method developed by Chambers (2005). *Aacyp* expression was then examined in male and female larval and pupal samples (Fig. 10), and although the male pupae

appeared to have approximately 2.5-fold higher expression than female pupae, the differences between the sexes was not statistically significant (ANOVA, $p > 0.05$). While male 4th instar larvae also showed slightly higher expression than females (Fig. 10), the difference was not statistically significant and was not as pronounced as the differences observed in adult males and females.

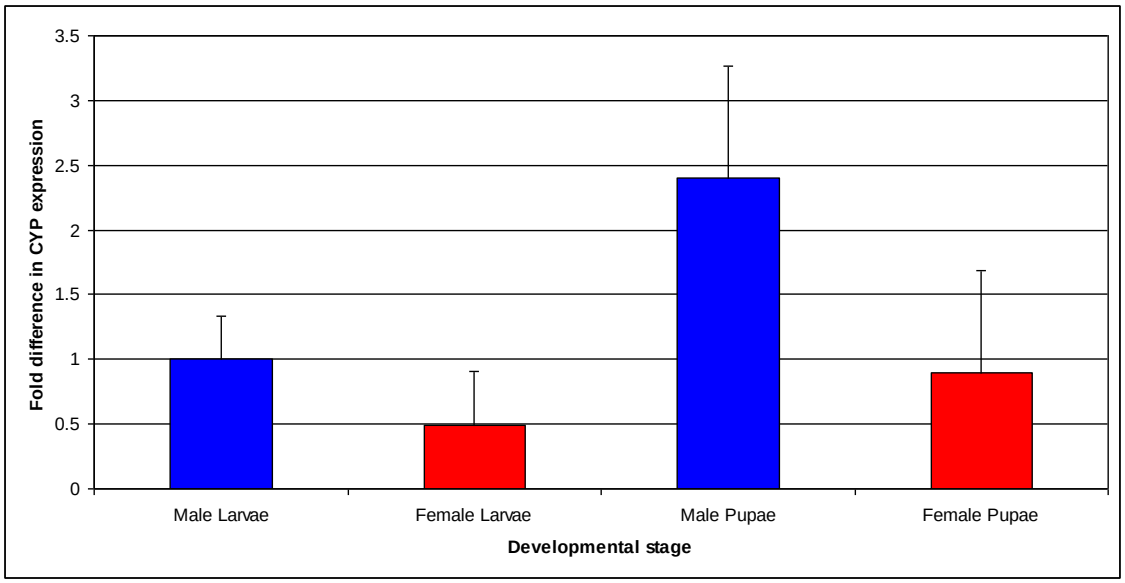

Figure 10. *Aacyp* expression in male and female larvae and pupae. Calculations were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Values represent the means and standard errors from 3 replicate experiments.

2.3.3 *Aacyp* expression in specific tissues of *A. aegypti* adults

In an attempt to establish *Aacyp*'s possible role in male reproduction, expression was measured in various tissues of virgin and mated adult *A. aegypti* at different ages. At 0, 3 and 7 days post-eclosion, tissues from virgin males and females were collected and used for qRT-PCR experiments. Mosquitoes were separated into heads (to isolate the

majority of nervous tissue), reproductive tracts (to isolate reproductive tissues), and the remaining carcass. Male reproductive tracts were further dissected to isolate MAGs to determine if *Aacyp* could be a MAG-specific gene. The same tissues were isolated in three and seven day-old mated mosquitoes, then qRT-PCR was performed to determine the expression of *Aacyp* in each of those tissues.

There was no significant difference in the expression of *Aacyp* in heads of male and female virgin and mated mosquitoes, however expression was slightly higher (approximately 3-fold) in 3 day old virgin mosquitoes compared to other ages (Fig. 11).

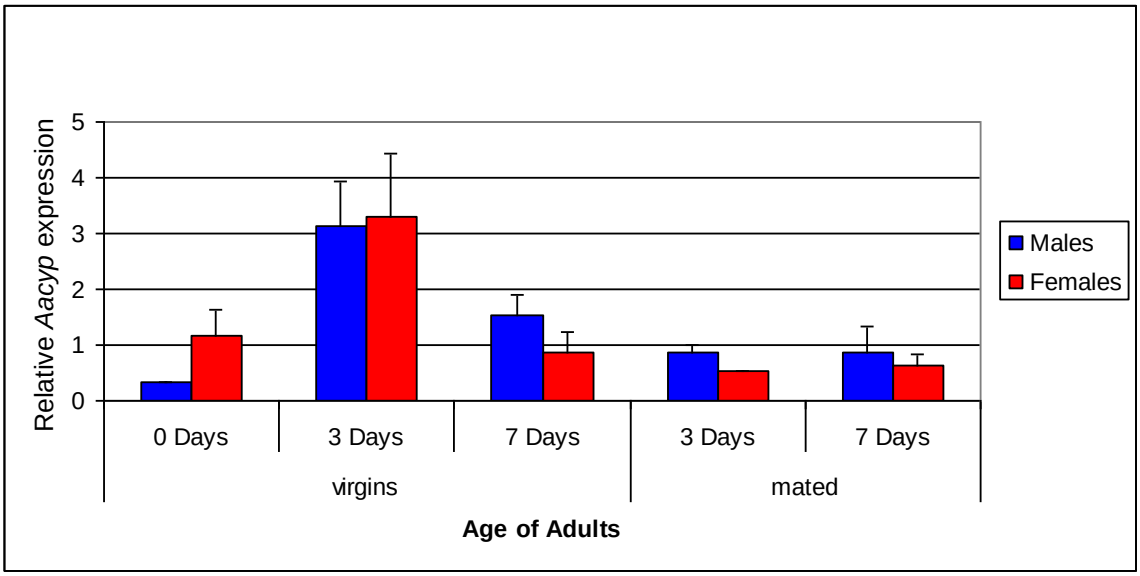

Figure 11. *Aacyp* expression in male and female head tissues at various ages. Values are not significantly different between males and females. Changes in gene expression are relative to expression in 0 day old females, and calculations were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Relative expression was not significantly different between males and females of the same age (Student's t-test, $p>0.05$). Values represent the means and standard errors from 3 replicate experiments.

In the carcass of mosquitoes (bodies, minus the head and reproductive tracts) *Aacyp* expression was significantly higher (about 4 to 5-fold higher) in males than females in both virgin and mated mosquitoes at 3 and 7 days old (Fig. 12). Newly eclosed males had nearly 15-fold higher expression than newly eclosed females (not shown).

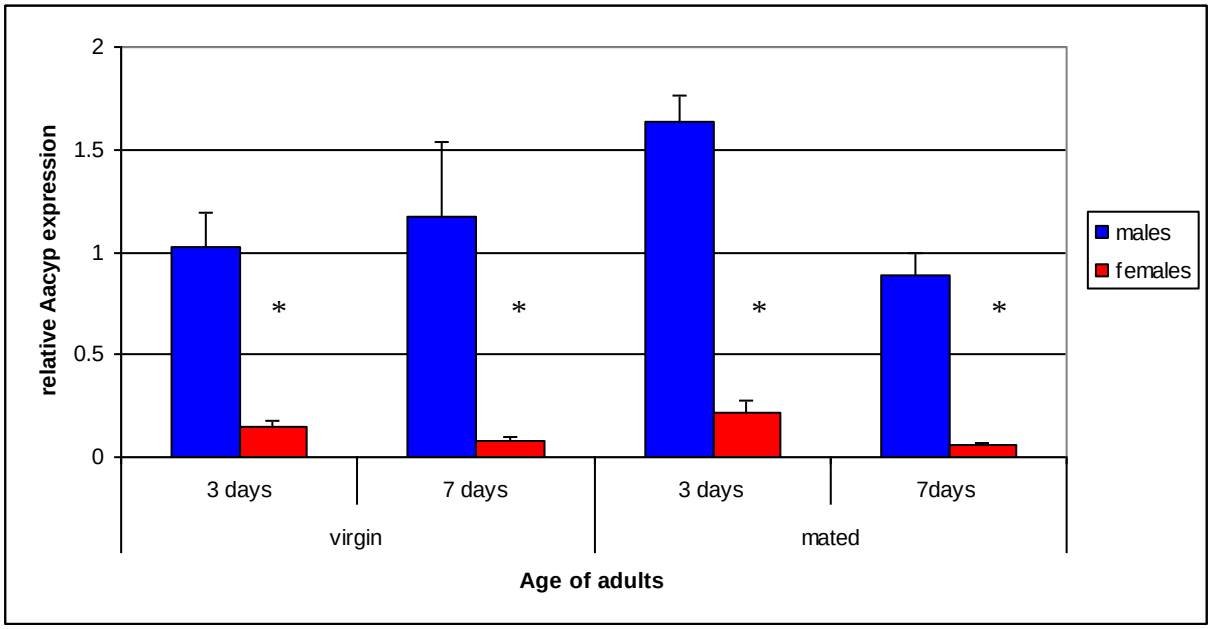

Figure 12. *Aacyp* expression in male and female bodies (minus reproductive tissues) at various ages. Values are significantly higher in males than females. Changes in gene expression are relative to expression in 3 day old virgin males, and calculations were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Stars indicate significant differences between male and female expression (Student's t-tests, $p<0.05$). Expression in males was not significantly different from one another, and neither was expression in females (ANOVA, $p<0.05$).

When reproductive tissues were examined for *Aacyp* expression, varying results were observed. Expression in male reproductive tracts and MAG tissues were not significantly different from each other at any age (Fig. 13), suggesting there may be equal expression of *Aacyp* throughout the male reproductive tract. Expression in female

reproductive tracts were a bit more varied, being relatively high in 0 day old virgins and mated 7 day old mosquitoes, and low in all other females tested.

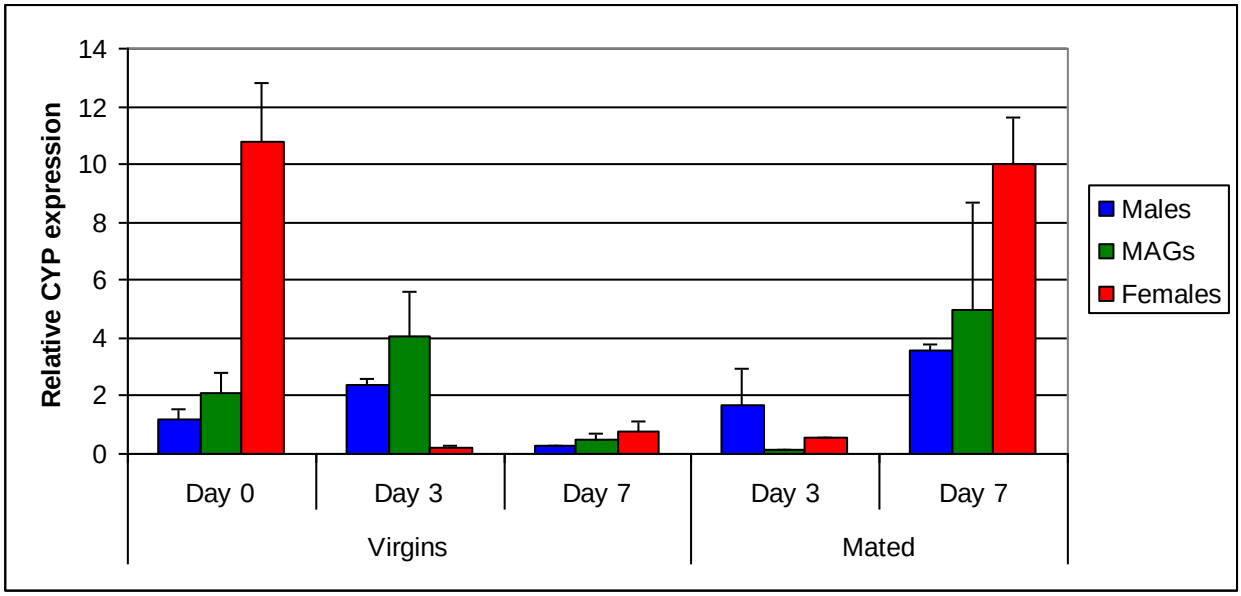

Figure 13. *Aacyp* expression in male and female reproductive tissues at various ages. Changes in gene expression are relative to expression in 0 day old virgin males, and calculations were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

However, when *Aacyp* expression is compared across all tissues, it is clear that there is very little expression of *Aacyp* in heads and reproductive tracts of *A. aegypti* relative to the expression in male mosquito bodies (abdomens and thoraxes; Fig. 14).

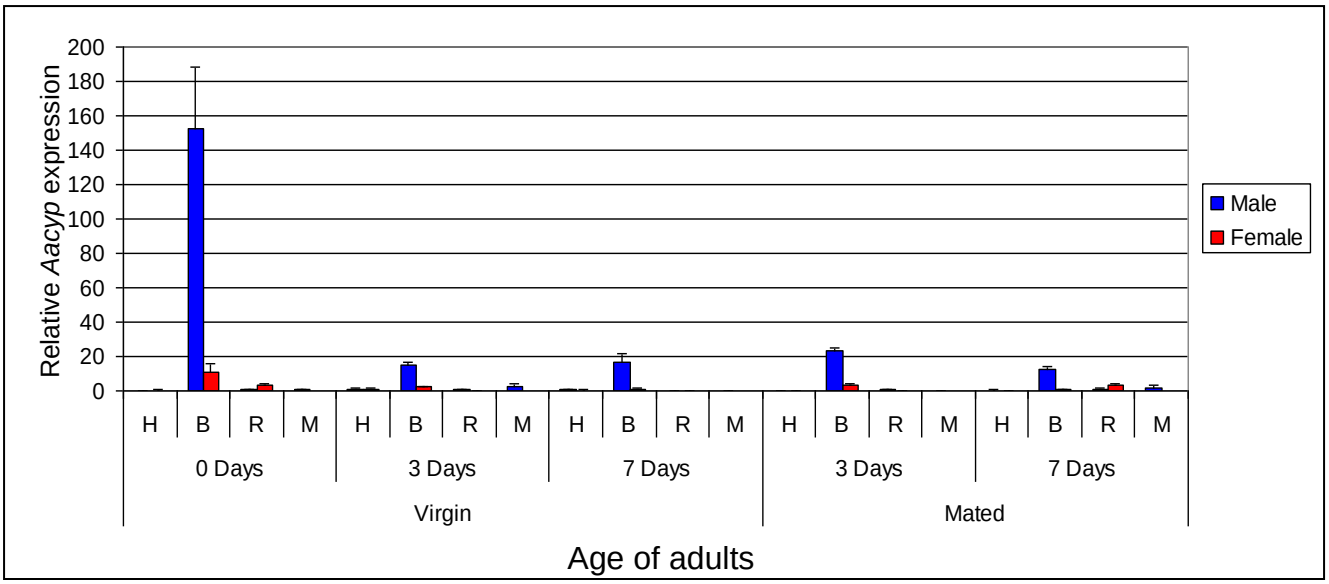

Figure 14. Comparison of *Aacyp* expression in all tissues tested. Note the expression in most tissues is minimal when compared to expression in male bodies of *A. aegypti*. (H = heads, B = bodies, R = reproductive system, M = MAGs).

2.3.4 *Aacyp* dsRNA injections and mating bioassays

To test if the ability to moult was affected by *Aacyp*, mosquitoes were injected at both the larval and pupae stages with dsRNA targeting the *Aacyp* gene in *A. aegypti*. It appears that 4th instar larvae were much more likely to suffer from the injection process compared to pupae, as ~40% of the larvae died within a day of the injections, regardless of which dsRNA was injected; in contrast, only 9% of the pupae died from the injection process (Table 2).

The numbers of males and females that developed from injected larvae and pupae were very similar in both *gus*- and *Aacyp*-dsRNA treatments (Table 2). Injections of *gus*-dsRNA served as a negative control, as the *gus*-dsRNA shows no homology to an insect gene and has been used in previous studies of RNAi in other insects (Whyard *et al*., 2009;

Yuen *et al*., 2008) and mosquitoes (see Chapter 3). The fact that the *Aacyp*-dsRNA had no effect on survival in either sex suggests that either the gene's expression was not sufficiently silenced in these treatments or that the gene is not essential for survival at the stages of development examined.

Table 2. Mortality and moulting success of individual *A. aegypti* injected with dsRNA.

Life stage injected	dsRNA injected	Total injected	% mortality 1 day after injection*	Surviving injectants	Developed to adulthood		
					Males	Females	Total
4th instar larvae	*gus*	324	43.6%	171	34	66	100 (58.5%)
	Aacyp	323	41.4%	177	38	51	89 (50.3%)
Pupae	*gus*	160	9.6%	142	59	46	105 (73.9%)
	Aacyp	160	7.7%	146	65	52	117 (80.1%)

* no significant difference in mortality between *gus*- and *Aacyp*-dsRNA injected mosquitoes (chi-square test, $p>0.05$)

Injected pupae were allowed to develop to adulthood and single pair matings were set up in small plastic vials for 2 days. Females were then blood-fed and allowed to lay eggs. Eggs were laid approximately two to three weeks after the initial injection, and due to this long duration, more mosquitoes died before their fecundity could be assessed, and hence, the data set was unfortunately rather small (Table 3). Similar numbers of eggs were laid and hatched in the *gus*- and *Aacyp*-dsRNA treatment groups.

Table 3. Fertility data obtained from mated individuals injected with dsRNA at the pupal stage.

Treatment	# male pupae injected	# bloodfed females*	# females that laid eggs	Total eggs laid	Total eggs hatched[a]
gus-dsRNA	32	16	10	301	217 (72.1%)
Aacyp-dsRNA	44	18	7	209	161 (77.0%)

* uninjected females that were mated to dsRNA injected males.

[a] no significant difference in percent eggs hatched between treatments (chi-square test, $p>0.05$).

qRT- PCR was performed on individual pupae and individual males that had been injected as pupae to determine if RNAi of the *Aacyp* gene had occurred (Figure 15).

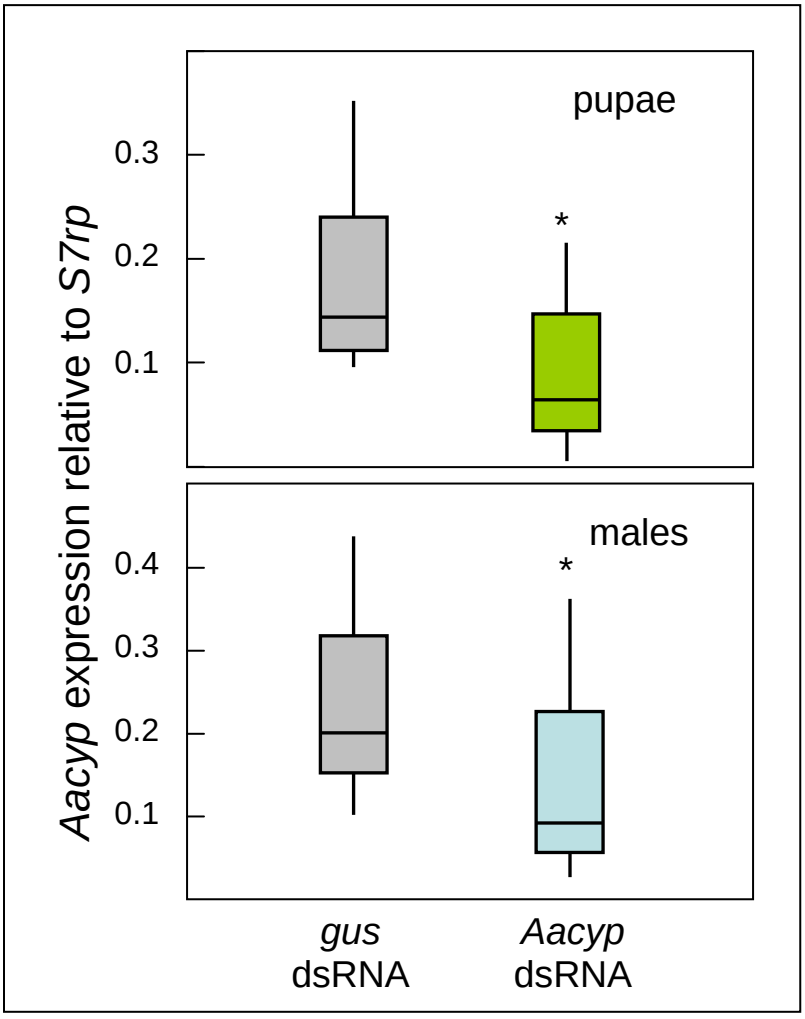

Figure 15. Box plot showing the range of *Aacyp* expression in dsRNA-injected individuals. Changes in *Aacyp* expression in individual pupae or males (injected at the pupal stage) injected with *Aacyp*-dsRNA are compared to individuals injected with *gus*-dsRNA. * indicates a significant difference between the medians of gus-dsRNA treated individuals and *Aacyp*-dsRNA treated individuals (Mann-Whitney test, $p<0.05$).

There was a high degree of variation in *Aacyp* expression of dsRNA-injected individuals, with a few showing no significant level of RNAi, but nevertheless, most pupae injected with *Aacyp*-dsRNA showed a readily detectable level of RNAi. The median values of *Aacyp* expression revealed a significant knockdown of the gene's expression relative to the negative controls in both the injected pupae (*gus*-dsRNA injected median = 0.14, *Aacyp*-dsRNA injected median = 0.07) and the adults that developed from them (*gus*-dsRNA injected median = 0.20, *Aacyp*-dsRNA injected median = 0.095).

Based on this small sampling, it appears that RNAi is more potent soon (48 hr) after dsRNA injections, and as the insects age and develop into adults, a greater number of them showed less pronounced RNAi of the target gene.

2.3.5 Phylogenetic analysis of *Aacyp*

A more comprehensive BLAST search identified a number of insect CYP proteins (Table 4), three of which appear to have a male-biased expression. A phylogenetic analysis (Fig. 16) was created using ClustalX, and as expected, the *Aedes Aacyp* (*cyp4g35*) was most closely related to *cyp4g15* of *D. melanogaster*, however it is unexpectedly closely related to *cyp4g19*, a gene associated with permethrin resistance in the cockroach *B. germanica*.

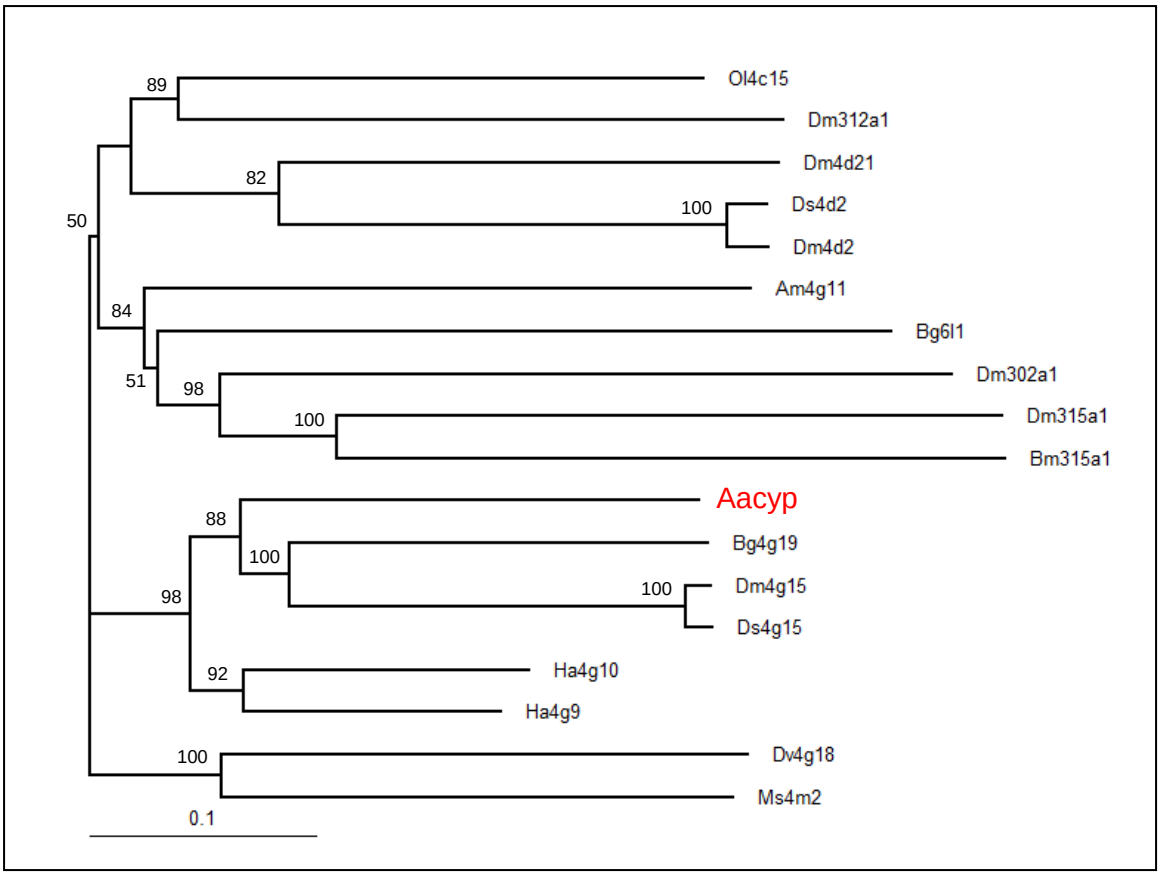

Figure 16. Phylogenetic tree comparing *Aacyp* with *cyp* genes from various insects (see Table 4). Bootstrap values (percentage out of 1000 replicates) are shown at each node. Species names have been abbreviated as two letters followed by *cyp* gene names, and *Aacyp* is shown in red. The scale bar represents the number of nucleotide substitutions per site.

Table 4. Cytochrome P450 genes used for comparison with *Aacyp*. See phylogenetic tree above (Fig. 16).

Species name	Abbreviation in Figure 16	Gene	NCBI accession	Specific tissue expression	Possible function	Reference
D. melanogaster	Dm	*cyp4g15*	AF159624	Third instar, neural tissue	Ecdysteroid synthesis	Maïbèche-Coisne *et al*., 2000
		cyp4d21 (*sxe-1*)	NM_135285	Male head, sensory structures	Male courtship and mating	Fujii *et al*., 2008
		*cyp4d2**	AF017018	DDT-resistant strain	Detoxification	Dunkov *et al*., 1996
		cyp312a1	NM_140773	Male abdomen	Ecdysteroid degradation	Kasai and Tomita, 2003
		cyp302a1 (*dib*)	NM_080071	Embryos, female follicle cells	Ecdysone synthesis	Warren *et al*., 2002 Kasai and Tomita, 2003
		cyp315a1 (*sad*)	NM_141866	Embryos, female follicle cells	Ecdysone synthesis	Warren *et al*., 2002 Kasai and Tomita, 2003
D. simulans	Ds	*cyp4g15*	XM_002106649	-	-	NCBI
		*cyp4d2**	AF017019	-	-	NCBI
B. germanica	Bg	*cyp6l1*	AF227531	Male reproductive tissue	Regulation of juvenile hormone and ecdysteroids	Wen and Scott, 2001
		cyp4g19	AY176056	Permethrin resistant strain	Insecticide resistance	Pridgeon *et al*., 2003
H. armigera	Ha	*cyp4g9**	U86003	Pyrethroid-resistant strain	Pyrethroid metabolism	Pittendrigh *et al*., 1997
		*cyp4g10**	U86004	Pyrethroid resistant strain	Pyrethroid metabolism	Pittendrigh *et al*., 1997
D. virgifera virgifera	Dv	*cyp4g18*	AF243506	Adults	Insecticide resistance	Scharf *et al*., 2001
A. mellifera	Am	*cyp4g11**	AF207948	Adults	unknown	Tares *et al*., 2000
B. mori	Bm	*cyp315a1*	NM_001112750	larvae	Ecdysteroid synthesis	Niwa *et al*., 2004
O. limosus	Ol	*cyp4c15*	AF091117	Steroidogenic gland (Y-organ)	Steroid hormone biosynthesis	Dauphin-Villemant *et al*., 1999
M. sexta	Ms	*cyp4m2*	L38671	Larval fat body	Xenobiotic response	Snyder *et al*., 1995

* indicates only partial gene sequences available for comparison.

2.4 Discussion

Cytochrome P450 enzymes (CYPs or P450s) are present in all phyla and play a major role in the metabolism of various compounds. They are used to catalyze highly specific oxidative attacks of non-activated hydrocarbons at physiological temperatures, reactions that would be unspecific and require high temperatures if uncatalyzed (Werck-Reichhart and Feyereisen, 2000). With a high degree of variability in the sequence and structure of P450s, even a single amino acid substitution can drastically alter the substrate specificity of the enzyme. This has made the determination of function of most CYPs unpredictable (Feyereisen, 1999).

In insects, there are six families of CYP genes - five are insect specific (CYP6, 9, 12, 18 and 28) while the sixth family (CYP4) is also found in vertebrate species (Feyereisen, 1999). The majority of CYPs that have been analyzed in insects are considered to have a role in the metabolism of important endogenous compounds, but many have also been associated with the detoxification of insecticides (reviewed in Feyereisen, 1999; Scott, 1999). Therefore, the discovery of a sex-biased CYP, particularly male-biased, is somewhat unusual.

Only a few male-specific CYPs have been found in insects. The first was discovered in the cockroach *Blattella germanica* (CYP6L1), and was speculated to have a role in regulating juvenile hormone (JH) or ecdysteroids in male reproductive tissues (Wen and Scott, 2001), although no experimental evidence for this possible function was provided. The first report of a male-specific CYP in *D. melanogaster* was that of c*yp312a1*. This gene was found to be expressed predominantly in the abdomen of male flies, and based on this distribution the authors suggested that its encoded protein could

have a role in male reproduction (Kasai and Tomita, 2003). These two CYPs share little similarity in their sequences and their actual functions have yet to be established. More recently, *cyp4d21* (also known as *sxe-1*) was identified in *D. melanogaster* and is highly expressed in the head and thorax of males, but not in the abdomen (Fujii *et al*., 2008). Specifically, it is found in cells associated with mechanosensory bristles, as well as maxillary and labial palps. It has been shown to have characteristics similar to a fatty-acid ω-hydroxylase, and knockdown of this gene affects courtship behaviour and causes significant reduction in the mating success of male flies, suggesting that courtship and mating may be regulated by small compounds generated from this enzyme.

According to my results, the CYP gene isolated from *A. aegypti* has a strong male-biased expression, particularly in the adults. The obvious differences between male and female insects are differences in sexual development, particularly differences in reproductive structures and their associated requirements (e.g. many female mosquitoes require a blood-meal to produce eggs). The fact that a CYP has a sex-biased expression suggests that it has a role in that gender's sexual function, and in the case of this male-specific CYP in *A. aegypti*, it may have a role in the development and functioning of reproductive structures or in male-specific processes such as courtship or mate-seeking behaviours.

A BLAST search of the *A. aegypti* genome database (Vectorbase) for sequences similar to *Aacyp* identified a *cyp* gene (*cyp4g35*) that is nearly identical to *Aacyp* in protein sequence, but has several nucleotide substitutions. The microarray analysis reported on Vectorbase, while being somewhat ambiguous, suggests that the expression

of one or both of these genes is male-biased, but a specific function of these two related genes is unknown.

Since many of *D. melanogaster*'s genes have been annotated, *Aacyp* was first compared to CYPs found in the *D. melanogaster* genome database. The most similar CYP to *Aacyp* in *D. melanogaster*, is *cyp4g15*, which in the flies, is present in the brains of third instar larvae, but is also found in pupae and adults. Maïbèche-Coisne *et al*. (2000) speculated that this CYP may have a role in ecdysteroid synthesis, given its similarity to another CYP involved in ecdysteroid biosynthesis, *cyp4c15*, found in crayfish, but the function for the *D. melanogaster* CYP4G15 enzyme has not yet been confirmed. In my study, I attempted to knock down the expression of *Aacyp* using RNAi and examined the ability of the *Aacyp*-dsRNA injected larvae and pupae to moult, anticipating that if the Aacyp protein had a role in metamorphosis, I would observe that the injectants would fail to moult.

Injections of *A. aegypti* larvae were technically challenging, with many simply dying from the stress of the injection process. Nevertheless, enough larvae and considerably more pupae were successfully injected and survived the dsRNA injections to assess the effects of the *Aacyp*-dsRNA on moulting. No significant difference in moulting success or adult survival was observed between the *gus*-dsRNA controls and the *Aacyp*-dsRNA injectants, which suggests that this CYP does not have an important role in metamorphosis, and is not likely directly involved in the metabolism of moulting hormones such as ecdysone, as has been suggested for some male-specific CYPs found in other insects.

The qRT-PCR analyses revealed that *Aacyp* was predominantly expressed in adult males, but not in their reproductive tissues. Instead, the bulk of the expression was found in the male's body (i.e. the thorax and abdomen), excluding the head. While the *Aacyp* was similarly mostly localized to the bodies of the females, the female's expression level was less than 10% that of males. From this, it appears that *Aacyp* is not like that of the other insect male-biased CYPs, in that it is neither head- nor reproductive tract-specific.

Despite a lack of expression in reproductive tissues, the sex-biased expression of this gene may still reflect a role in male reproduction. It is possible that *Aacyp* could affect a male's ability to transfer sperm to the female during mating, if for example, *Aacyp* is a component of the male copulatory structures that were not isolated along with the testes and MAGs, or perhaps *Aacyp* affects courtship behaviour. To test this, pupae injected with *Aacyp*-dsRNA were allowed to develop to adulthood, mated, and the females were then allowed to lay eggs, which were counted and hatched to check for evidence of fertilization. Unfortunately, despite injecting over 100 pupae with *gus*- or *Aacyp*-dsRNA, less than 20 individuals from either dsRNA injection survived to test their fertility. No significant impact of the *Aacyp*-RNAi on male fertility or ability to mate was observed. Similarly, injections of females with *Aacyp* dsRNA did not affect their ability to mate or produce viable eggs. Given the small sample size however, it is difficult to make any definitive conclusions as to whether *Aacyp* influences any male-specific mating capability or fertility. Some changes to the experimental design to assess particular aspects of the male's reproductive capacities will be considered later in this discussion.

qRT-PCR was used to measure *Aacyp* transcript levels in injected larvae, pupae and adults to determine if knockdown of the *Aacyp* transcripts could be detected in

individual insects. For the majority of tested mosquitoes, there was significantly lower expression of *Aacyp* in *Aacyp*-dsRNA injected individuals compared to *gus*-dsRNA injected individuals. However, there were some individuals that showed no evidence of RNAi after dsRNA treatment. This could be due to several factors. For instance, injections were performed by mouth pipetting using glass needles, and some variation in the volume of dsRNA may have occurred. The needles used for injections were relatively large compared to the body size of injected individuals. Injected larvae and pupae were therefore usually left with considerably large holes in their bodies, which may have caused some leakage of injected dsRNAs, decreasing the efficacy of the treatment. There is also the possibility that some *Aacyp*-dsRNA-injected individuals may have experienced an initial knockdown of the gene's expression, but this could have been followed by a subsequent increase in expression if the affected cells tried to compensate for the initial loss in expression.

Interestingly, the RNAi effect could still be detected in adults that were up to 7 days post-injection, albeit the RNAi level was not as strong as in pupae, 2 days post-injection. This suggests that for most of the insects used in these bioassays, some degree of transcript reduction persisted over the bioassay period. As the half-life of Aacyp protein is unknown, and I did not quantify its levels, the lack of an observable phenotype in the bioassays still makes it difficult to make any conclusions about this protein's function.

As stated earlier, there are some additional experiments that could be performed to clarify if *Aacyp* really does play a role in male reproductive success. Even though *Aacyp* was not expressed in the testes or MAGs, it may be involved in regulating the

function of the copulatory apparatus. It would therefore have been informative to determine if there was definite copulation and transfer of sperm to females from *Aacyp*-dsRNA injected males. One way to determine this is by dissecting spermathecae from recently mated females and looking for stored sperm. Sperm within a female's spermathecae could also be stained to make visualization easier, possibly allowing estimates of the number of sperm transferred (Clements, 1992).

To determine if *Aacyp* was affecting regulation of spermatogenesis, qRT-PCR could be performed on samples derived from *Aacyp*-dsRNA-injected pupae and adults to test for changes in expression levels of genes involved in the spermatogenesis pathway. Also, 4th instar larvae could be sexed prior to injection so that equal numbers of males and females could be tested for changes in expression levels using qRT-PCR.

As noted, results from qRT-PCR analyses suggest that the male body has the highest expression of *Aacyp*, but which tissues in the body specifically expressing this CYP are still unknown. In order to more accurately determine which tissues are expressing *Aacyp*, *in situ* hybridization could be used to show which cells are expressing *Aacyp*. Knowing that the body of male mosquitoes highly express this P450 gene, it is worth focusing on which tissues within the thorax or abdomen are more likely to express genes in a sex-specific manner. Tissues of particular interest are the digestive organs, fat body, neural tissue, and muscle.

It is reasonable to suspect that the gut tissue could differ between the sexes, as the females require a blood-meal and males do not. Clements (1999) suggests that plant fluids, including sugars and water, are essential to male survival, whereas females acquire more nutrients from their blood meals. Male *A. aegypti* will naturally feed on nectar from

plants, primarily at night (Clements, 1999). It is possible that *Aacyp* may be necessary for proper digestion or detoxification of compounds in plant nectar, and given that males may feed more on plant nectar than females, there is a greater need for this enzyme in male gut cells. In our lab-bred mosquitoes, the insects were fed 10% sucrose, and it is unlikely that the Aacyp would be needed to deal with such a simple diet. Hence, if Aacyp's role is one of metabolizing a plant-derived compound in the diet, no differential survival following RNAi would be detected in my experiments.

The fat body of the two sexes may also differ, although at first glance, sex-specific differences may not be obvious, as the fat body is essential for energy use and storage in both sexes (reviewed in Arrese and Soulages, 2010). Cells of the fat body control the synthesis and utilization of fat and glycogen, which are used as energy reserves in insects, and they also synthesize the majority of hemolymph proteins and circulating metabolites. While both sexes would require the fat body to serve a number of common functions, it is essential for many other processes, as it co-ordinately stores or releases compounds during metamorphosis and reproduction, acting as an endocrine organ, as well as a detoxification organ (Arrese and Soulages, 2010). Female insects, for instance, use the fat body to produce many nutrients for egg production, including synthesis of the yolk proteins (Raikhel *et al*., 2002). While little is known of male-specific fat body functions, it is possible that a variety of males-specific proteins involved in male reproductive physiologies or male-specific activities could be produced in this highly versatile organ. If *Aacyp* is expressed differentially in the fat body of the two sexes, it may be playing a sex-specific role in the synthesis or breakdown of a male-specific metabolite.

Another possible function for *Aacyp* could be in controlling the mosquito's flight. Mosquitoes possess two types of muscles for flight, direct and indirect, both of which are mainly found in the thorax. Direct muscles are responsible for extending and flexing the wings, and controlling their angle of attack, while indirect muscles are responsible for powering flight itself (Clements, 1999). The main fuel to power flight is proline, which is abundantly found in the mosquito. Proline is synthesized in the fat body from acetyl-CoA and alanine, then released into the hemolymph. The mitochondria of flight muscles possess enzymes that are able to oxidize the proline (Arrese and Soulages, 2010). As both sexes must fly to find food and mates, it may at first seem unlikely that *Aacyp* could be required for the basic mechanics of flight, as both sexes undoubtedly have similar requirements for this process. However, it is very possible that sex-specific differences in flight are essential, particularly in mating courtship behaviour.

Flight in males is essential for mating and courtship behaviour. Hartberg (1971) observed *A. aegypti* in the wild, and observed that females and males would surround a blood meal host, and the majority of males would copulate quickly with females in flight, usually only one metre above the ground. Occasionally, copulations would continue briefly on the ground. Although it is unknown whether specific flight patterns are involved in the courtship of these mosquitoes, it is possible that males have subtle differences in their flight muscles or how they use these muscles to seek or display to their mates, and Aacyp could potentially mediate some of those differences.

Virgin male and female mosquitoes will respond to each other's wing beat frequencies (Cator *et al*., 2009). The fundamental frequency of a female's wing beat is 400-Hz, whereas a male's is 600-Hz, and it was found that couples will shift their flight

tones to match a frequency of 1200-Hz, a frequency originally thought to exceed the upper hearing limits of *A. aegypti* (Cator *et al*., 2009). If pairs are unable to match this frequency, it is less likely that they will mate, since it was observed that mated females were not willing to shift their flight tones to match male wing beat frequencies (Cator *et al*., 2009).

During my mating and fertility bioassay experiments, males and females were either placed in small mating cages or even smaller plastic vials in order to mate. Even if *Aacyp* is used to regulate flight or wing beat frequencies, the space limitations of our mating cages may have compromised the mosquitoes' abilities to perform normal courtship and mating behaviours. As a consequence, our attempts to use RNAi to decrease *Aacyp* transcripts would fail to detect any role that this protein may have in regulating male-specific courtship activities.

A labour intensive, yet much more informative method for determining the true function for *Aacyp* would be to create a transgenic strain of mosquitoes that either does not express the gene, or overexpresses the gene in specific tissues such as reproductive tissue, wing muscles, digestive tissues, or fat body tissues, to establish its exact role in the mosquito.

Several CYP gene sequences from different species were aligned using ClustalX, and a phylogenetic tree was created (Fig. 16). A list of the species and their genes can be found in Table 4. The majority of sequences used for comparison belong to the CYP4 family of P450 genes because the most similar *Drosophila* gene to *Aacyp* (*cyp4g15*) also belongs to the CYP4 family. This protein's function is unknown, but given that it is a neurally-expressed CYP in larvae, it is unlikely that it shares a similar function to that of

Aacyp. Interestingly, the one other protein with the highest sequence similarity was one in the cockroach that conferred increased resistance to the pesticide permethrin. It will be of interest to determine whether the mosquito Aacyp has any affinity for this pesticide. Of course, permethrin is unlikely to be its primary substrate in the insects, but identifying natural substrates of CYPs is particularly challenging as many are capable of interacting with a range of compounds. From this preliminary phylogenetic analysis, it is quite evident that the sequence of a CYP gene is very little help in determining its function. However, with tools like RNAi, particularly high-throughput RNAi methods, establishing definite roles for the vast numbers of CYPs could be made far easier.

Chapter 3: Ingested double-stranded RNA can act as an insecticide in *A. aegypti* larvae

3.1 Introduction

RNAi has been used to silence genes in a growing number of invertebrates, including numerous insect species. Most double-stranded RNA (dsRNA) delivery methods for invertebrates involve injection directly into the body cavity of the organism, but other methods have shown promise as well. In the roundworm *Caenorhabditis elegans* and the planarian *Schmidtea mediterranea*, soaking the worms in dsRNA solutions can effectively elicit an RNAi response (Orii *et al*., 2003; Tabara *et al*., 1998). Oral delivery (i.e. feeding) of dsRNA has also been successful in many species. In some arthropods, such as apple moths (Turner *et al*., 2006), aphids, fruit flies (Whyard *et al*., 2009), and ticks (Soares *et al*., 2005), dsRNA can be fed in a liquid solution. In some terrestrial insects, such as beetles and moth larvae, dsRNA can also be orally delivered simply by adding it to a semi-dry diet (Whyard *et al*., 2009). Given these early successes in delivering dsRNA simultaneously to moderately large numbers of invertebrates, it may also be possible to deliver dsRNA by similar methods to aquatic insect larvae, such as mosquitoes, to induce an RNAi response. The gene silencing abilities of dsRNA has the potential for many applications including high-throughput screening of gene functions and the development of species-specific pesticides (Huvenne and Smagghe, 2010; Whyard *et al.*, 2009).

Currently, chemical pesticides are the main method to control insects that are detrimental to both agriculture and human health. The majority of the chemicals used, however, have off-target affects, killing organisms other than the pest, as well as

beneficial species. This is usually because the chemicals act on particular proteins required for essential processes in a broad range of species, including vertebrates. A well cited example is the excessive use of the insecticide DDT, which was used to eradicate different insect disease vectors in many countries world-wide, but was later directly linked to the decline of many endangered birds species (reviewed in Bernanke and Köhler, 2009). More recently, it has been suggested that off-target effects of insecticides may be a contributing factor to colony collapse disorder (CCD) in honeybees by making them more susceptible to pathogens (Ratnieks and Carreck, 2010).

The reason that these chemical pesticides have such a dramatic impact on numerous species is because many of them work by targeting the nervous system, acting on either neurotransmitters or the enzymes involved in the breakdown of neurotransmitters. High doses of these chemicals can cause serious health problems in humans, especially in the workers applying the chemicals, and it is estimated that in developing nations, approximately 3 million workers suffer from severe pesticide poisoning each year (World Health Organization).

With the relatively recent incursion of West Nile virus to North America in 1999 (Komar *et al*., 2003), there has been an even greater use of insecticides to control the mosquito vectors in order to reduce the risk of transmission of this serious disease. Of particular relevance to Canada, and specifically Manitoba, mosquito populations are routinely being controlled with the organophosphate malathion. Like most organophosphates, malathion inhibits acetylcholinesterase, an enzyme necessary for the breakdown of the neurotransmitter acetylcholine. In insects, excess acetylcholine affects the central nervous system, causing paralysis and death. Acetylcholinesterase, however,

is a highly conserved enzyme found in many organisms, including mammals. There are growing concerns that excess exposure to chemicals like malathion may cause serious health problems in humans (Gilden *et al*., 2010). In addition, the routine use of these pesticides will inevitably lead to the development of resistant populations of insects, for which the typical response is to increase the use of the pesticides further (Rivero *et al*., 2010).

The development of species-specific pesticides that kill only the pest insect is one possible solution to decrease the negative affects that chemical insecticides have on humans and other non-target organisms. Given that RNAi can be designed to selectively silence a single gene within a species, it may also be possible to develop insecticidal dsRNAs that are species-specific and have little or no effect on other non-target organisms. In this project, oral delivery of dsRNA specific to three genes was tested in first instar *A. aegypti* larvae to determine the efficiency of soaking aquatic insects to induce an RNAi response, and its possible use as a species-specific insecticide.

3.2 Methods

3.2.1 Cloning *A. aegypti β-tubulin* for dsRNA delivery

RNA was extracted from 10 adult *A. aegypti* using a Qiagen RNeasy RNA extraction kit and cDNA was produced as in Chapter 2, section 2.2.2.

A long (785 bp; *β-tubL*) and a short (328 bp; *β-tubS*) fragment of the *β-tubulin* (*β-tub*) gene (Fig. 17) from *A. aegypti* (NCBI accession: XM_001655975) were independently PCR-amplified from adult *A. aegypti* cDNA, using 1 µl each, of the following primers (20µM): Aedesβ-tubLFor: 5'GGAAATCATCTCCGACGAAC and Aedesβ-tubLRev: 5'CACGGTACTGTTGCGATCC or Aedesβ-tubSFor: 5'GGAAATCATCTCCGACGAA and Aedesβ-tubSRev: 5'CAGGCAGTCGCACGATTC in a standard 25 µl PCR reaction containing 1µl cDNA template, 17.3 µl H_2O, and the following reagents from Invitrogen: 1 µl 10 mM dNTPs, 1 µl 50 mM $MgCl_2$, 2.5 µl 10x PCR reaction buffer, and 0.2 µl recombinant *Taq* DNA polymerase. The following program was used to amplify the DNA sequences: 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, followed by a final extension of 72°C for 10 min.

ACTAGTCCCCTTCAGAGCAGCTCATTTAGTGTGTGATCGTCAAGGAGTACAGTCTCGGCGTTCTTTGATT
GTTGCCCGTTTGTGTCTTCTTTCTGTGCTTTGAGGAGAGAAAAGCAGCAGAAGAACAGAAAAAAAGGAGT
GAAGTGTGAAAAACAGACAAGTTTTAAAACTCGAATTTAAGAGCAGCCCTCGCCAAAGGCTACGCCGAAG
TTTCTCTGTTAATTTGTTAAGAAACAAAAAAAAACCTTTCACCATGAGAGAAATCGTCCACATCCAAGCC
GGTCAGTGCGGAAACCAAATTGGAGCTAAGTTTTG*GGAAATCATCTCCGACGAAC*ATGGAATCGACGCCA
CCGGAGCCTACCATGGTGACTCAGACCTGCAGCTGGAACGCATCAACGTGTACTACAATGAAGCCTCCGG
CGGCAAATACGTGCCACGTGCCGTGCTAGTCGATCTGGAACCCGGTACCATGGACTCCGTCCGCTCGGGG
CCATTCGGACAGATCTTCCGCCCGGACAACTTCGTCTTCGGACAGTCCGGTGCCGGTAACAACTGGGCCA
AGGGACACTACACCGAGGGTGCCGAACTGGTCGATTCAGTGTTGGACGTTGTCCGCAAAGAAGCC*GAATC*
*GTGCGACTGCCTG*CAAGGATTCCAGCTGACCCACTCGCTCGGAGGTGGTACCGGCTCCGGTATGGGCACA
CTGTTGATCTCGAAAATCCGCGAAGAATATCCCGACAGAATCATGAACACATACTCAGTTGTCCCCTCGC
CAAAAGTATCAGACACCGTCGTAGAACCGTACAACGCCACCCTCTCAGTGCACCAGCTGGTCGAAAACAC
CGACGAGACGTACTGTATCGACAATGAAGCCCTGTATGATATCTGCTTCCGCACCCTGAAGCTCACAACC
CCAACCTACGGTGATCTGAACCATCTCGTGTCACTGACCATGTCCGGAGTTACCACCTGCCTGCGTTTCC
CTGGTCAATTGAATGCTGATCTCCGAAAACTGGCTGTCAACATGGTTCCATTCCCACGTCTGCACTTCTT
CATGCCTGGATTTGCCCCACTCACCTCCCGC*GGATCGCAACAGTACCGTG*CCCTCACCGTCCCAGAACTG
ACCCAACAGATGTTCGATGCCAAGAACATGATGGCCGCCTGCGACCCACGACATGGACGTTACCTGACAG
TTGCCGCCGTTTTCCGAGGACGCATGTCGATGAAGGAAGTCGATGAACAGATGCTGAACATCCAAAACAA
GAACAGCAGCTACTTCGTTGAATGGATCCCCAACAACGTTAAGACCGCCGTCTGTGATATTCCTCCACGA
GGACTGAAGATGTCTGCCACCTTCATCGGTAACTCGACCGCCATCCAGGAACTGTTCAAGCGTATCTCCG
AACAATTCACTGCTATGTTCCGTCGTAAGGCTTTCTTGCATTGGTACACTGGCGAGGGTATGGATGAGAT
GGAATTCACTGAAGCCGAAAGCAACATGAACGATCTGGTGTCCGAATATCAGCAATACCAGGAAGCCACC
GCCGACGAGGATGCTGAATTCGACGAAGAACAGGAAGCTGAAGTTGACGAAAACTAAACTAATTGAGCTC
TCACTCACACACACGAACCTGCCTCCCCTTCTATACAAATCTCCCCATCCCCCTCAAAGGGAAACTCTAC
TCTCTCATTCCAAAAAAAAAAAAACTTTCCTCTATCTGCGCCACTTCTACTACTAATCTCAAAAAGTACC
AATTCAGAGAGAATGCAACGTTCTTTTTTCGAAAAGAAAAACGAAAAAGTATCGATCAGGAGAGAATACA
ACATCATCAAGCGAAAACCACAAAACAACAGCAGAAATGTGAAGAAAAAAACGCAGCAGCAGTAACACCA
ACAAAACAGCCAGCGCAGCAAAAAAAAATCCTACAAAACAACTAAAAAAGAGTCGAAAAATAGCAAGAGA
AAAGTCGCAAAATTAGTAACCACTGCCAGCTCAGCAAAAAAGAAAAGAATAAAAGTGAAGTAATTTAAAA
AAAAACGGAAAACAAACTAAAATCAATTTCCTCTTGTTTGATTTTATTCTTTAGTGCACTTTTTTGCTTC
AAAAACCCCCCAACAAGAGAAACTGCCATTTCGTTCGTTAGGTTTGTTCGGAGATCCCATCATTCCACAC
CGCCTATCCAAGCGAACTCTCTTCTCTGATTTGTGTTGATTTCGTGTTTTGCATATTTCTTCCCACTTCT
CTCTTCCCACACTTTCGTATTCGTCTCTCTTCACAGGCACGTGTGCAAAAGAGATGTAAAATCGTTATAT
CGTAGCAGAAAGTACATTACTTTTCTCTTATAATTATTGATCAGCTAATTTTCTTCATTACTAATT

Figure 17. *A. aegypti* partial *β-tubulin* mRNA sequence from NCBI's gene database (Accession: XM_001655975). Red sequence represents *β-tubS*. Red and blue sequences combined represent *β-tubL*. Underlined and italicized sequences represent primer sequences used to amplify gene fragments.

The PCR products were resolved on a 1.2% low melt agarose gel, visualized using SYBR Gold (Invitrogen) stain and gel-purified using a QIAQuick Gel Extraction kit (Qiagen). Gel purified samples were ligated to Qiagen's cloning vector pDrive and plasmids were purified as in Chapter 2, section 2.2.2.

To confirm that the plasmids contained the correct inserts, they were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as in Chapter 2, section 2.2.2.

The identity of the sequences was confirmed by comparing them to the genome databases available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) using the basic local alignment search tool (BLAST).

3.2.2 Preparation of double-stranded RNA

β-tubulin gene fragments were excised from pDrive (Qiagen) using the restriction enzymes *Apa*I and *Pst*I (Invitrogen) for *β-tubS*, and *Not*I (Invitrogen) and *Mlu*I (New England Biolabs) for *β-tubL*. Excised fragments were then ligated into the dual-T7 vector pL4440 and sequenced, then dsRNA was prepared as in Chapter 2, section 2.2 5. Using the MEGAscript RNAi kit, four hour incubations were used for *gus* (as obtained in Chapter 2, section 2.2.5) and *β-tubL* dsRNA synthesis, whereas *β-tubS* was incubated longer (~8 hr).

3.2.3 Bioassays

3.2.3.1 Survival bioassays

A. aegypti eggs were allowed to embryonate for a minimum of one week, and stored for up to two months in humidified plastic containers. Eggs were then submerged in dechlorinated tap water with a liver powder solution to induce hatching of larvae. First instar larvae were collected in groups of 50 and treated with 75 µl of dsRNA at various

concentrations. To produce the dsRNA dilutions, the dsRNA was mixed with appropriate amounts of either dechlorinated tap water that had been filter sterilized using a 0.2 μm syringe filter or molecular-grade water from Sigma.

Liposomes have been shown to aid in the delivery of dsRNA to *D. melanogaster* larvae (Whyard *et al*., 2009), most likely by fusing with an organism's cell membrane and depositing its contents into the cell. Therefore, some preliminary survival bioassays were performed to test the efficiency of liposomes with *A. aegypti* larvae. For these bioassays, 1 μl of Lipofectamine 2000 (Invitrogen) was added to 75 μl of dsRNA at concentrations of 0.2 μg/μl, 0.1 μg/μl and 0.05 μg/μl for each gene of interest. Larvae were soaked in the dsRNA solutions in 2 ml microfuge tubes for 2 hours then transferred to 100 mm Petri-dishes with ~30 ml of dechlorinated tap water and 1 ml of liver powder solution (approx. 1 g liver powder per 25 ml of water) as a food source. The next day, larvae were transferred to individual wells of 48-well plates for easier scoring of survival. Each well contained ~1.5 ml of dechlorinated tap water and 30 μl of liver powder solution was added to the wells every few days. Survival of the larvae was observed over a 1 week period.

Results from the liposome experiments suggested there was no advantage to adding liposomes to dsRNA, and therefore further survival bioassays were performed without the addition of Lipofectamine 2000, using slightly different concentrations of dsRNA (0.5 μg/μl, 0.2 μg/μl, and 0.02 μg/μl) for each gene of interest. As with the liposome bioassays, larvae were soaked in 75μl of dsRNA for 2 hr, and then transferred to 48-well plates for observation. However, these larvae were observed for 2 weeks, and liver powder solution was added as a food source on a more regular basis (every 2 days).

3.2.3.2 Gene expression

As described above (section 3.2.3.1), first instar *A. aegypti* larvae were soaked for 2 hours in dsRNA treatments then transferred to Petri-dishes. However, only 0.5 µg/µl of dsRNA was used to treat the larvae, and rather than transferring to 48-well plates, larvae remained in Petri-dishes until collection for RNA extractions and determination of gene expression by qRT-PCR. Since the addition of liposomes did not appear to increase the effectiveness of dsRNA delivery (see results), they were not used in any further treatments.

Treated larvae were collected at 3, 5 and 7 days after the single 2 hour dsRNA treatment. All larvae still alive from each treatment were placed in a single 1.5 ml tube in *RNAlater* (Qiagen) and kept at -20°C until use. RNA was extracted from larvae using the Qiagen RNeasy kit and Qiagen QiaShedders to homogenize tissue samples (as done in Chapter 2, section 2.2.2). RNA concentrations were determined using a spectrophotometer, and cDNA was produced (as in Chapter 2, section 2.2.2) using random hexamers rather than oligo-dT primers.

The cDNA was then used to assess the extent of RNAi by measuring levels of *β-tubulin* present in the samples using qRT-PCR. These reactions were performed in triplicate on a BioRad iQ5 Real-Time PCR Detection System using 96-well plates with 20 µl as in Chapter 2, section 2.2.3. For this experiment, *S7rp* primers (see Chapter 2, section 2.2.3.1) were used to amplify an internal reference gene for comparing *β-tubulin* expression in *gus*- and *β-tubulin*-dsRNA treated larvae. Another set of primers were designed to target the *β-tubulin* gene itself (β-tubqPCRFwd: 5’ CGTCGTAGAACCGTACAAC, β-tubqPCRRev: 5’ CAGGCAGGTGGTAATCC, 187

bp). Analysis of gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) as in Chapter 2, section 2.2.3.1.

3.2.3.3 Tissue specific gene expression

Larvae were hatched as above (section 3.2.3.1), then collected in groups of 25 and soaked in 75 µl of either *gus*-, *β-tubL*-, or *β-tubS*-dsRNA at various concentrations (0.5 µg/µl, 0.25 µg/µl and 0.1 µg/µl) for 2 hours. Larvae were then transferred to small (60 mm) Petri-dishes with dechlorinated tap water and given some liver powder solution as a food source. After 3 days, live larvae were dissected to collect heads, guts, and the remaining carcass. Each tissue type was stored in 100 µl of *RNAlater* (Ambion) at -20°C until use. RNA extractions and cDNA synthesis were performed only on gut and carcass samples, not on collected head samples. RNA, cDNA, and qRT-PCR procedures were performed as in Chapter 2, section 2.2.2.

3.2.4 Delivery of *Aedes aegypti β-tubulin*-dsRNA to *Drosophila melanogaster*

3.2.4.1 Preparation and delivery of D. melanogaster β-tubulin dsRNA

To test the species-specificity of the *A. aegypti β-tubulin*-dsRNA, the mosquito-specific *βtubS*-dsRNA was delivered to another dipteran insect, the fruit fly *D. melanogaster*, to see if it would cross-silence the fruit fly's homologous *β-tubulin* gene. The 328 bp β-tubS fragment of the *A. aegypti β-tubulin* gene shares 85% identity with the homologous *D. melanogaster βTub56D* gene (FlyBase accession: FBgn0003887). However, within this region of the gene, no 19 to 21 nt length of identity can be found

between the two species' sequences, which suggests that no siRNA could be produced from either sequence that would effectively silence the other gene's expression.

RNA was extracted from 50 *D. melanogaster* first instar larvae and cDNA was prepared as previously described (Chapter 2, section 2.2.2). A 128 bp fragment of the *D. melanogaster βTub56D* gene, within the 328 bp sequence presumed to have no RNAi cross-silencing potential, was amplified from the *D. melanogaster* cDNA using the primers DmelβtubF: 5'AGGCGTCCGGTGGCAAGTACG and DmelβtubR: 5'CGACTGGCCGAACACAAAGTTG. This PCR product was first cloned into the PCR cloning vector pDrive (Qiagen), sequenced to confirm its identity, and subsequently subcloned into the dsRNA expression plasmid pL4440, as previously described (Chapter, section 2.2). DsRNA specific for this *D. melanogaster β-tubulin* gene fragment was synthesized as described previously (Chapter 2, section 2.2.5)

To deliver the dsRNAs to *D. melanogaster* larvae, adult *D. melanogaster* females were isolated and allowed to lay eggs on small Petri plates containing 1.5% agar and 50% grape juice. The eggs were allowed to hatch and the first instar larvae were collected and soaked in 0.5 μg/μl solutions of dsRNA for 2 hr. All dsRNA solutions used to treat *D. melanogaster* larvae were encapsulated in Lipofectamine 2000 (Invitrogen), since it was previously shown that liposomes increase the effectiveness of dsRNAs delivered to *D. melanogaster* larvae (Whyard *et al*., 2009). The larvae were exposed to the *β-tubulin* dsRNA specific to either *D. melanogaster* or *A. aegypti*, or to dsRNA specific to the *gus* gene from *E. coli*.

After treatment, larvae were placed in Petri dishes containing an agar-yeast-cornmeal medium for further development. Larvae were monitored for mortality, and a

subset was collected for determining knockdown levels of *β-tubulin* transcripts with qRT-PCR (as described in Chapter 2, section 2.2.3. Primer sets used to measure *D. melanogaster β-tubulin* expression are as follows: DmelβtubqPCRF: 5'AAGCTCACAACCCCAACCTA and DmelβtubqPCRR: 5'CAGGCATGAAGAAGTGCAGA, amplifying a 160 bp product.

3.2.5 *Chitin synthase* and *heat shock protein 83* dsRNA delivery

3.2.5.1 Preparation of chs and hsp83 dsRNA

Chitin synthase (*chs*, NCBI accession: AF223577), a gene necessary for proper moulting in insects, and heat shock protein 83 (*hsp83*, NCBI accession: AAEL011704), necessary for proper folding of proteins under stress, was PCR amplified from *A. aegypti* adults in the same manner as *β-tubulin* in section 3.2.1 using the following primers: ChsF: 5'CTGACGACGATGACGAAGAA, ChsR: 5'GATAATGATGGCCCACATCC, 344 bp and Hsp83F: 5'CTGCGTGAGTTGATCTCCAA , Hsp83R: 5'GGACGTGACGACGACCTTAT, 324 bp). The gene fragments were then cloned, sequenced and used for *in vitro* transcription of dsRNA as described in Chapter 2, sections 2.2.4 and 2.2.5.

3.2.5.2 Chs and hsp83 bioassays

First instar larvae were collected for bioassays and soaked in 0.5 μg/μl dsRNA solutions (with and without Lipofectamine 2000) as in section 3.2.3. Groups of larvae were then transferred to wells of 6-well tissue culture plates and monitored for survival.

For *chs*-dsRNA-treated larvae, mortality and length of larvae was recorded. For larvae treated with *hsp83*-dsRNA, half of the larvae were pre-treated by incubating at 32°C for 1 hr, allowed a 2 hr recovery at 25°C, and then treated to a 2 hr heat shock at 37°C. Larval mortality was recorded over time.

Subsets of treated larvae were collected 4 days after treatment to determine levels of gene knockdown. Larvae were stored in *RNAlater* until RNA extractions, cDNA synthesis and qRT-PCR (as done in Chapter 2, sections 2.2.2 and 2.2.3). qRT-PCR primer sets used to amplify *chs* and *hsp83* are as follows: ChsqPCRF: 5'TCCATCTACCATTGCCAACA and ChsqPCRR: 5'CGTACCGAACATCAACATGC, 118 bp; Hsp83qPCRF: 5'GTGTCGTCGATTCGGAAGAT and Hsp83qPCRR: 5'CCTTGTCTTCAGCGAGTTCC, 135 bp.

3.3 Results

3.3.1 Oral delivery of *β-tubulin* dsRNA to *A. aegypti* larvae affects survival

β-tubulin (*β-tub*) is considered a housekeeping gene in eukaryotic organisms, as it encodes one of the subunit proteins of microtubules, which are vital components of a cell's cytoskeleton and are also involved in formation of spindle fibres during mitosis and meiosis. Disruption of this gene's expression would likely interfere with cell division and normal cellular functions, and would ultimately lead to cell death. Delivery of sufficient quantities of *β-tub* dsRNA to gut cells of *A. aegypti* larvae would be expected to result in death of the insects due to an inability of the gut cells to maintain their structure and function, and thereby failing to provide sufficient nutrients to sustain the insect during this growth phase of its life cycle.

First instar larvae were exposed to long and short fragments of *β-tub* dsRNA (*β-tubL*-dsRNA, 785 bp and *β-tubS*-dsRNA, 328 bp) in solution. The larvae were soaked once, for a 2 h period, in a range of concentrations of dsRNAs, and their survival after a two week period was recorded. After this single exposure to the dsRNA, it was observed that all larvae treated with three concentrations of the negative control *gus*-dsRNA (0.5 µg/µl, 0.2 µg/µl, and 0.02 µg/µl), and those treated with the lowest concentration of *β-tubS*- and *β-tubL*-dsRNAs (0.02 µg/µl) had similar survival rates between 84% and 97% (Table 5). However, when larvae were soaked in the second highest concentration of *β-tub* dsRNAs, survival decreased to around 50%, and then further decreased to around 12% when treated with the highest concentrations of *β-tub*-dsRNAs (Table 5).

Table 5. Survival of *A. aegypti* larvae two weeks after dsRNA treatment. Values represent the means and standard errors of 3 replicates.

Concentration (μg/μl)	dsRNA treatment	Survival (Mean % ± SEM)
0.5	*gus*-dsRNA	97.9 ± 2.1 [a]
	B-tubL-dsRNA	13.7 ± 3.9 [c]
	B-tubS-dsRNA	11.1 ± 5.9 [c]
0.2	*gus*-dsRNA	84.7 ± 9.0 [a]
	B-tubL-dsRNA	52.1 ± 8.3 [b]
	B-tubS-dsRNA	45.8 ± 4.2 [b]
0.02	*gus*-dsRNA	90.6 ± 2.5 [a]
	B-tubL-dsRNA	81.3 ± 0.0 [a]
	B-tubS-dsRNA	87.6 ± 5.3 [a]

[a] Survival of *gus*-dsRNA treated larvae were not statistically different from *β-tub*-dsRNA treatments at 0.02μg/μl after 14 days (ANOVA, $p>0.05$).
[b, c] Treatments of larvae with the same concentrations of either the S or L fragment of *β-tub*-dsRNA showed no significant differences in survival rates (ANOVA, $p>0.05$). Survival of *β-tub*-dsRNA treated larvae at 0.2 and 0.5 μg/μl were significantly different from one another, as well as all *gus*-dsRNA treated larvae, and *β-tub*-dsRNA treated larvae at 0.02μg/μl (ANOVA, $p<0.05$).

The same experiment was repeated with the addition of liposomes to the dsRNA. It is known that liposomes can increase the uptake of dsRNA and increase RNAi responses in *D. melanogaster* larvae (Whyard *et al*., 2009). However, liposome encapsulation of the dsRNA did not appear to give consistent results with *A. aegypti* larvae treated with *β-tub*-dsRNA (Fig. 18). The percent survival of larvae was observed 7 days after treatment with *gus*-, *β-tubL*-, and *β-tubS*-dsRNA with and without Lipofectamine 2000 (Invitrogen). For most doses tested, there was no observable difference in the survival rate of larvae treated with or without liposomes. Only two treatments showed significant differences in survival between liposome and non-liposome-encapsulated dsRNAs. Larvae treated with 0.05 μg/μl of *β-tubS*-dsRNA showed nearly 30% lower survival when treated with liposomes. In contrast, larvae treated with 0.2 μg/μl of *β-tubS*-dsRNA showed around 30% lower survival without the addition of liposomes. Aside from these two exceptions, which showed conflicting

responses to the presence or absence of liposomes, the majority of the treatments suggest that liposomes do not improve the efficiency of dsRNA in *A. aegypti* larvae.

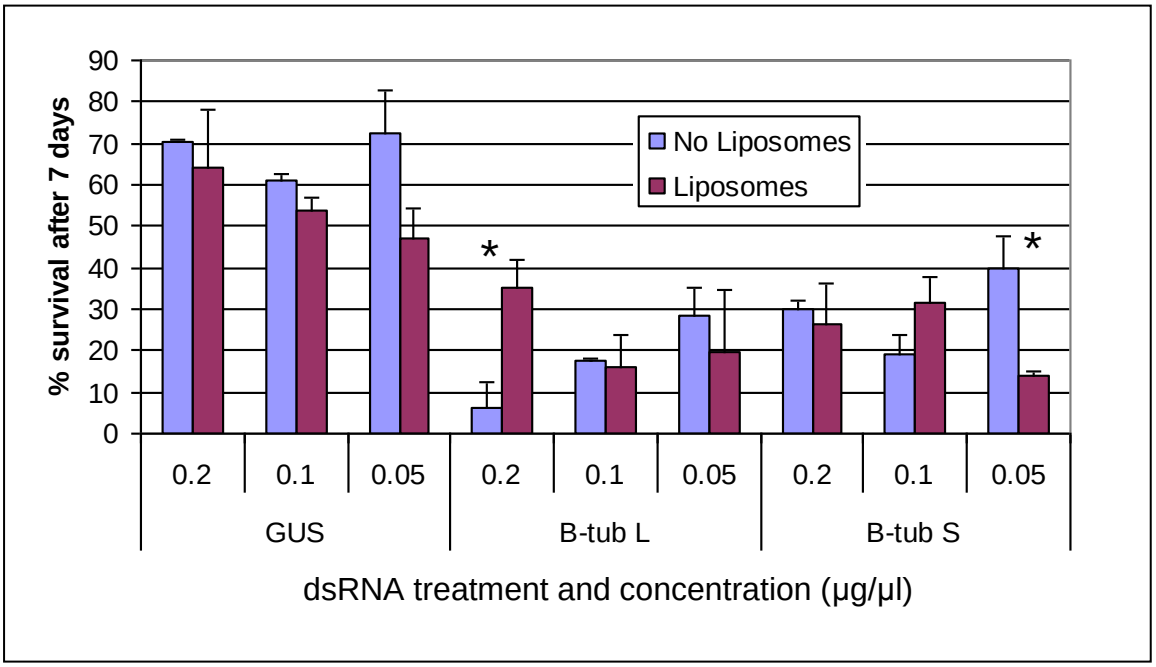

Figure 18. Comparison of liposome-encapsulated and non-encapsulated dsRNA on larval survival after 7 days. The values represent the means and standard errors for 3 replicate experiments.

* represent a significant difference in the survival of larvae treated with the liposome- and non-liposome-encapsulated dsRNAs (Student's t-test, $p<0.05$).

3.3.2 Oral delivery of *β-tubulin*-dsRNA can cause knockdown of *β-tubulin* expression

It is worth noting that the larval survival rate following exposure to the *β-tub*-dsRNAs in Figure 18 was not as low as in Table 5. This difference in survival between the two experiments is most likely due to the difference in the duration of each experiment; in the first experiment, survival was assessed after 14 days, whereas in the second experiment, survival was assessed after only 7 days. Presumably, the higher mortality rate over time is due to the latent effects of the dsRNA after the single treatment. To examine this latency of RNAi, the time course of dsRNA-induced mortality and its correlation with *β-tubulin* gene expression was examined.

By examining survival of dsRNA-treated larvae (Fig. 19) over the two week post-treatment period, two substantial drops in survival rates, around days six and nine, were observed in larvae treated with *β-tub*-dsRNA. While untreated and *gus*-dsRNA-treated larvae showed no significant decrease in survival during the two week period, larvae treated with 0.2 and 0.5 μg/μl *β-tub*-dsRNA showed significantly increased mortalities over the two week period. Interestingly, the single exposure to the dsRNA did not result in immediate mortality, but resulted in many of the larvae dying several days later before they could develop into pupae and adults.

In order to test whether the orally-delivered dsRNA could decrease expression of *β-tubulin*, larvae were soaked for 2 hr in 0.5 μg/μl of *β-tubL*-dsRNA. At days 3, 5 and 7 post-treatment, RNA was isolated from pooled groups of larvae and qRT-PCR was used to determine if decreases in *β-tubulin* expression were associated with the decreases in survival. At day 3, the level of *β-tub* expression following *β-tub*-dsRNA treatment

appeared to decrease slightly relative to the *gus*-dsRNA-treated controls, but the change was not statistically significant (Student's t-test, $p = 0.15$; Fig 20). Curiously, *β-tubulin* expression increased 3.5-fold by day 5, and then returned back down to control levels by day 7. Based on these results, it would appear that RNAi had not been adequately induced, and even more curiously, by day 5 post-treatment, *β-tubulin* expression had actually been up-regulated in response to the dsRNA treatment. It is worth noting however, that the *β-tubulin* gene expression measurements in this experiment were based on only the living larvae, and not on the larvae that had already died. It had been presumed that dead larvae would likely yield poor RNA recoveries as they would be undergoing necrosis, and therefore they should not be used in the gene expression measurements. It is possible therefore, that the live larvae had acquired a sublethal dose of dsRNA, and were able to increase *β-tubulin* expression to compensate for the initial knockdown of *β-tubulin* expression following the dsRNA treatment.

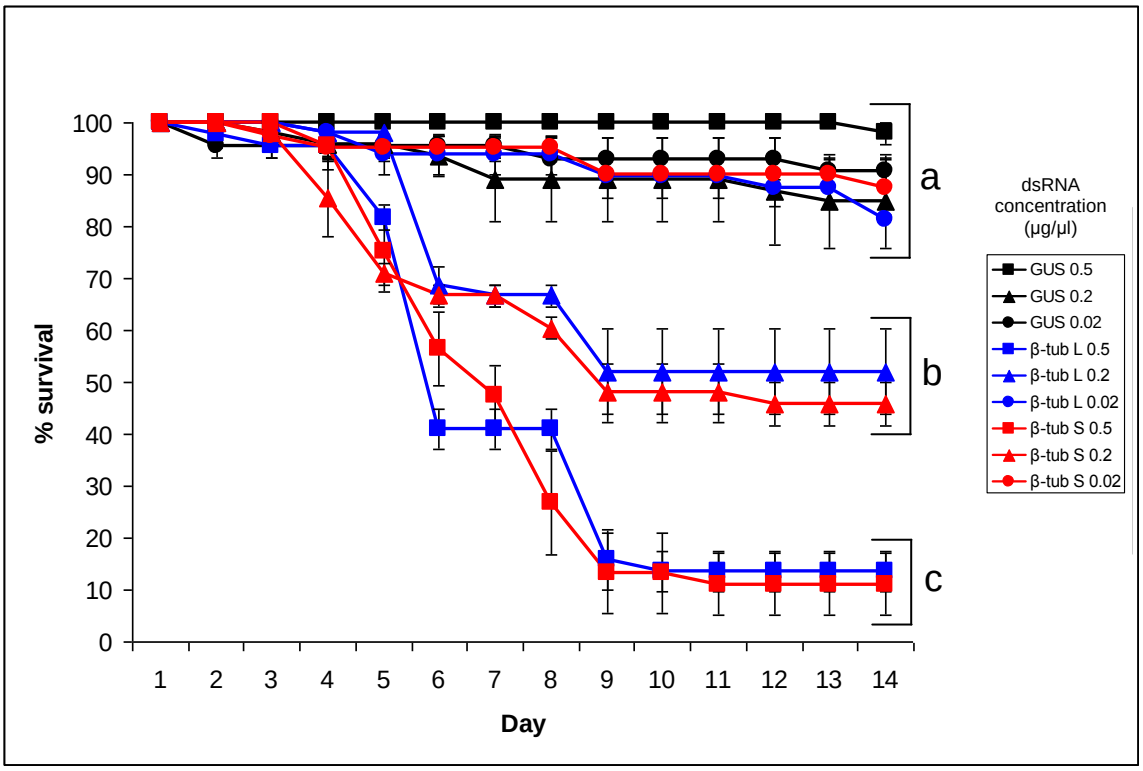

Figure 19. Survival of *A. aegypti* larvae over a two week period after dsRNA treatment. Three concentrations of dsRNA were used: 0.5 µg/µl, 0.2 µg/µl and 0.02 µg/µl.

a) Survival of *gus*-dsRNA treated larvae were not statistically different from *β-tub*-dsRNA treatments at 0.02 µg/µl after 14 days (ANOVA, $p>0.05$).

b and c) Survival of *β-tub*-dsRNA treated larvae at the same concentrations were not significantly different from one another (ANOVA, $p>0.05$). Survival of *β-tub*-dsRNA treated larvae at 0.2 and 0.5 µg/µl were significantly different from one another, as well as all *gus*-dsRNA treated larvae, and *β-tub*-dsRNA treated larvae at 0.02µg/µl (ANOVA, $p<0.05$).

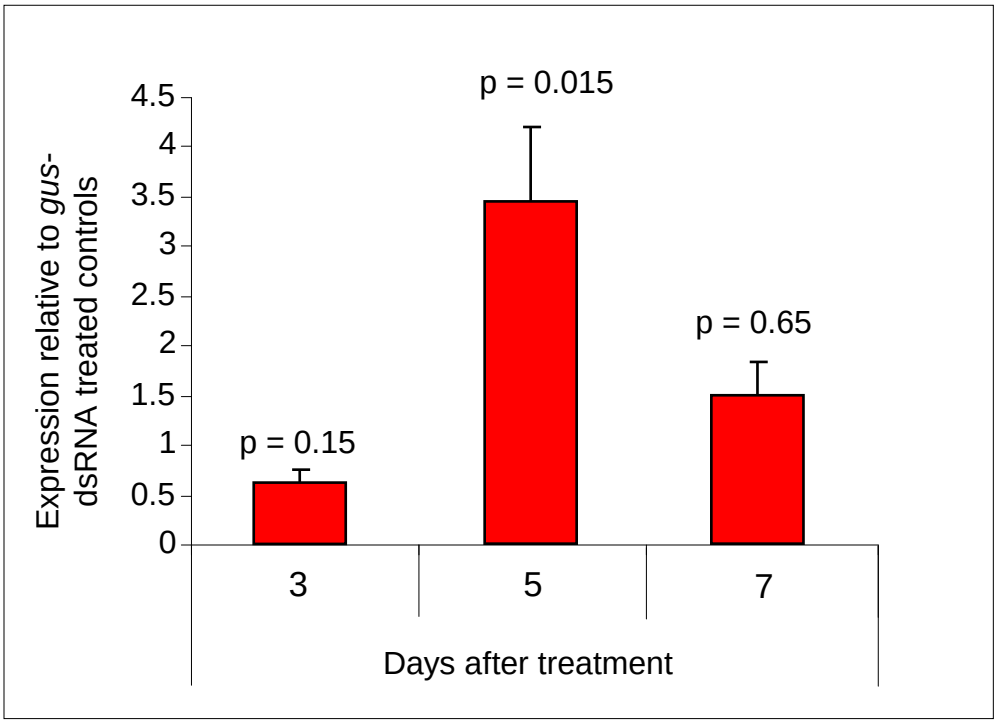

Figure 20. Gene expression of *β-tubulin* 3, 5 and 7 days after treatment with 0.5µg/µl *gus*-dsRNA or *β-tubL*-dsRNA. Changes in gene expression of *β-tub*-dsRNA treated larvae are relative to *gus*-dsRNA treated larvae at the same time period. Calculations were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Only relative expression between *gus*-dsRNA treated and *β-tub*-dsRNA treated larvae at day 5 is considered significantly different (Student's t-test, $p<0.05$).

3.3.3 Oral delivery of *β-tubulin*-dsRNA can cause knockdown of *β-tubulin* expression in the gut and other tissues

Previously, mosquito larvae had been soaked in food colouring solutions for 2 hr and it had been noted that only the midgut had contained the food dye (results not shown), suggesting that the gut is the main point of entry for the dsRNA when the larvae are soaking in a solution. By measuring changes in *β-tub* expression in the entire body of the treated larvae, the RNAi effect at the level of the gut tissue, where the dsRNA is

entering the body, may have been obscured. It is unknown whether dsRNA can travel beyond the gut, and since the gut itself represents only a small proportion of tissues present in a mosquito larvae, using whole bodies of larvae to measure the level of RNAi may have diluted any effect that dsRNA would have had on gut tissues.

To test if orally delivered *β-tub*-dsRNA could cause knockdown in *A. aegypti* guts, in addition to the possible induction of an RNAi response in other tissues, larvae were treated as before with different concentrations of *β-tubL*- and *β-tubS*-dsRNA (0.5 μg/μl, 0.25 μg/μl, and 0.1 μg/μl). However, three days after treatment with dsRNA, guts were dissected out of live larvae and pooled together for qRT-PCR. The remaining carcass (minus heads) was also collected and pooled for qRT-PCR.

Larvae treated with the highest concentration of *β-tubL*-dsRNA (0.5 μg/μl) showed around 50% decrease in *β-tubulin* expression in the gut compared to *gus*-dsRNA treated larvae. In addition, there was also, approximately, a 40% decrease in *β-tubulin* expression in the remaining carcass tissues (Fig. 21). *β-tubS*-dsRNA gave varying results, which will be discussed further in the Discussion.

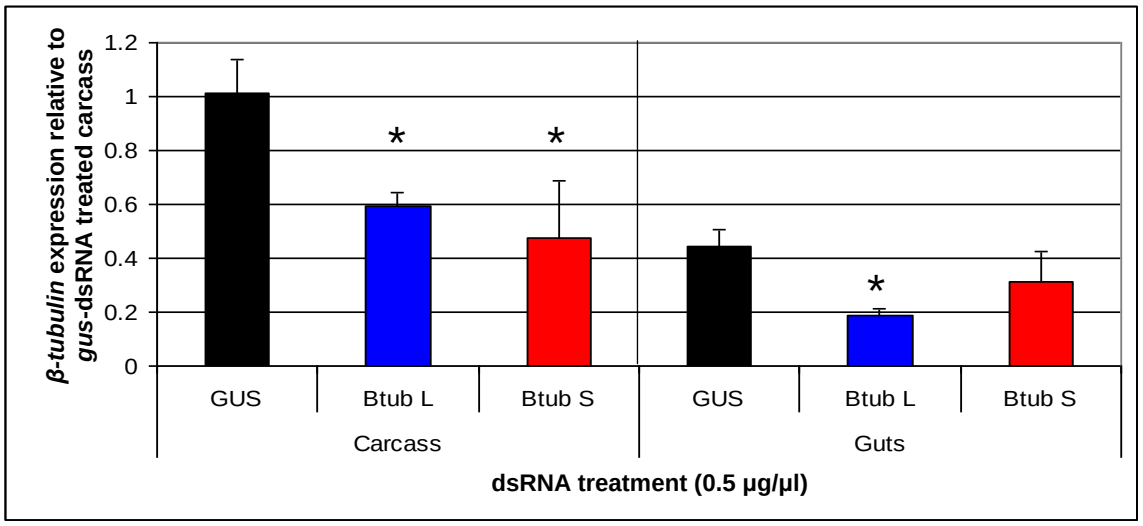

Figure 21. Gene expression of *β-tubulin* in guts and carcasses 3 days after dsRNA treatment with 0.5µg/µl concentrations. Larvae were separated into gut tissues and remaining carcass for qRT-PCR. Changes in gene expression are relative to *β-tubulin* expression in *gus*-treated carcass samples. Comparisons were made using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**β-tubulin* expression is significantly lower in carcasses of *β-tub*-dsRNA treated larvae compared to *gus*-dsRNA treated larvae (ANOVA, $p<0.05$). Guts of *gus*-dsRNA and *β-tubS*-treated larvae did not have significantly different levels of *β-tubulin* expression, however, expression in *β-tubL*-dsRNA treated larvae was significantly lower (ANOVA).

Larvae treated with lower concentrations of dsRNA did not show significantly lower expression of *β-tubulin* (Table 6).

Table 6. Decrease in *β-tubulin* expression in guts and remaining carcass of *A. aegypti* larvae 3 days after treatment with dsRNA. The values represent the means and standard errors of three replicate experiments.

Concentration (μg/μl)	dsRNA treatment	% decrease in *β-tubulin* expression (Mean ± SEM)	
		Guts	Carcass
0.5	*gus*	0.00 ± 9.10	0.00 ± 7.53
	β-tubL	50.1 ± 5.21[a,c]	40.3 ± 6.00[a,c]
	β-tubS	18.2 ± 14.5[b,c]	49.3 ± 16.1[a,c]
0.25	*gus*	0.00 ± 7.00	0.00 ± 6.85
	β-tubL	24.7 ± 13.0[b,d]	-20.4 ± 13.7[b,c]
	β-tubS	-53.4 ± 28.0[b,d]	-42.8 ± 27.2[b,c]
0.1	*gus*	0.00 ± 21.4	0.00 ± 19.2
	β-tubL	-37.3 ± 19.4[b,c]	-50.3 ± 20.8[b,c]
	β-tubS	-34.7 ± 17.4[b,c]	-15.1 ± 11.7[b,c]

[a] Values are significantly different from *gus*-dsRNA treatments of the same tissues and concentration (ANOVA, $p<0.05$).
[b] Values are not significantly different from *gus*-dsRNA treatments of the same tissues and concentration (ANOVA, $p>0.05$).
[c] Values are significantly different from corresponding *β-tub*-dsRNA treatments of the same tissues and concentration (ANOVA, $p<0.05$).
[d] Values are not significantly different from corresponding *β-tub*-dsRNA treatments of the same tissues and concentration (ANOVA, $p>0.05$).

3.3.4 Oral delivery of *chitin synthase* and *heat shock protein 83* dsRNAs to *A. aegypti* larvae

First instar larvae were soaked for 2 hr in 100 μl of 0.5 μg/μl dsRNA specific to either the chitin synthase (*chs*) or the heat shock protein 83 (*hsp83*) genes with or without the addition of Lipofectamine 2000. Chitin synthase is an enzyme required for the synthesis of chitin, the main component of an insect's exoskeleton and the gut's peritrophic membrane (reviewed in Merzendorfer, 2006), while heat shock proteins are a family of chaperone proteins that aid in folding and stabilizing other proteins under different conditions. Heat shock protein 83 is specifically involved in folding and unfolding of proteins under heat stress. In some insect species, including the African migratory locust, *Locusta migratoria* (Whyard *et al*., 1986), the light brown applemoth, *Epiphyas postvittana* (Lester and Greenwood, 1997), the flesh fly, *Sarcophaga*

crassipalpis (Chen *et al*., 1991), and the model insect, *D. melanogaster* (Krebs and Feder, 1998), pretreatment at a sublethal heat shock temperature can induce production of heat shock proteins, which can subsequently confer protection to the insects if later subjected to a brief lethal temperature.

All mosquito larvae were observed for 1 week after treatment and mortality was recorded. qRT-PCR was performed to determine knockdown levels of target genes. Additionally, for *chs*-dsRNA treated larvae, visible differences in larval lengths were observed and measured (Table 7).

Table 7. Knockdown, mortality and length of larvae after treatment with dsRNAs. *A. aegypti* larvae were treated with (+L) and without the addition of liposomes for *chs* and *hsp83* dsRNAs. The values represent the means and standard errors for three replicate experiments.

Species Treated	dsRNA treatment	Target gene	% knockdown of target gene	% mortality	Mean length of larvae (mm)
A. aegypti	*gus*	*chs*	0.0 ± 9.0	14.4 ± 1.1	5.28 ± 0.04
	gus+L	*chs*	0.0 ± 13.4	13.3 ± 1.9	5.27 ± 0.04
	chs	*chs*	31.5 ± 4.7 [a]	45.6 ± 2.9 [a]	3.61 ± 0.09 [a]
	chs+L	*chs*	35.4 ± 3.6 [a]	46.7 ± 5.8 [a]	3.43 ± 0.09 [a]
No heat shock prior to dsRNA treatment	*gus*	*hsp83*	0.0 ± 12.7	10 ± 1.9	-
	gus+L	*hsp83*	0.0 ± 10.6	11.1 ± 1.1	-
	hsp83	*hsp83*	30.2 ± 9.6 [*]	30 ± 1.9 [a]	-
	hsp83+L	*hsp83*	23.6 ± 13.3 [**]	27.8 ± 2.9 [a]	-
32°C heat shock prior to dsRNA treatment	*gus*	*hsp83*	-	13.3 ± 1.9	-
	gus +L	*hsp83*	-	15.6 ± 1.1	-
	hsp83	*hsp83*	-	58.9 ± 1.1 [a]	-
	hsp83 +L	*hsp83*	-	60 ± 1.9 [a]	-

[a]Gene specific dsRNA treatments were statistically different from corresponding *gus*-dsRNA treatments (ANOVA, $p<0.05$).
Gus-dsRNA treatments of the same experiment with and without liposomes were not statistically different from one another (ANOVA, $p>0.05$).
Gene specific dsRNA treatments of the same experiment with and without liposomes were not statistically different (ANOVA, $p>0.05$).
**gus* vs *hsp83* - Student's t-test, $p = 0.1032$
***gus*+L vs *hsp83*+L - Student's t-test, $p = 0.2693$

Larvae treated with dsRNA specific to *chs* (*chs*-dsRNA) showed both decreased growth and increased mortality after 1 week compared to larvae treated with *gus*-dsRNA

(Table 7). Mortality was approximately 30% higher in larvae treated with *chs*-dsRNA, both with and without the addition of liposomes, compared to larvae treated with control *gus*-dsRNA with and without liposomes (Table 7), suggesting that, like the experiments with *β-tub*-dsRNA, liposomes had no effect on efficacy of RNAi. qRT-PCR performed on whole bodies of larvae revealed 31-35% knockdown of *chs* expression a week after dsRNA exposure, compared to *gus*-dsRNA treated larvae (Table 7). In addition to mortality and gene knockdown, larvae treated with *chs*-dsRNA appeared to have significantly slower and/or stunted growth, being nearly one-third shorter than the control larvae (Table 7).

Larvae that were treated with dsRNA specific to *hsp83* (*hsp83*-dsRNA) also showed higher mortality than control larvae. Those that were treated with dsRNA then pretreated at 32°C for 1 hr prior to being heat shocked at 37°C for 2 hr showed approximately 45% higher levels of mortality, compared to only 20% higher mortality in larvae that were not pretreated (Table 7). qRT-PCR performed on whole larvae showed some knockdown of *hsp83* in *hsp83*-dsRNA treated larvae, however, the differences in gene expression were not found to be statistically significant from *gus*-dsRNA treated larvae.

Similar to the *β-tub*- and *chs*-dsRNA experiments, mortality of larvae in the *hsp83*-dsRNA experiments was not significantly different between larvae treated with and without liposomes of the same dsRNA treatments, again suggesting that liposomes do not enhance the ability of dsRNAs to cause gene silencing.

3.3.5 Effect of *A. aegypti β-tubulin*-dsRNA on *D. melanogaster* larvae

To test the species-specificity of *A. aegypti β-tubulin*-dsRNA, first instar *D. melanogaster* larvae were also soaked in *gus*-dsRNA, *β-tubulin*-dsRNA specific for *A. aegypti* (*Ae-tub*, 328 bp), or dsRNA specific for the *β-tubulin* gene of *D. melanogaster* (*Dm-tub*, 170 bp). Since it is known that liposomes can enhance the gene knockdown effects of dsRNA in *D. melanogaster* (Whyard *et al*., 2009), larvae for this experiment were soaked for 2 hr in dsRNAs combined with Lipofectamine 2000 (Invitrogen). After treatment, larvae were placed in standard *Drosophila* medium, observed for mortality, and a subset were collected for qRT-PCR to determine the levels of *β-tubulin* knockdown.

Mortality between *D. melanogaster* larvae treated with *gus*- and *Ae-tub*-dsRNAs was not significantly different, however there was around 25% higher mortality in *D. melanogaster* larvae treated with dsRNA specific to their own species (Table 8).

Table 8. Mortality and qRT-PCR data for *D. melanogaster* larvae treated with *β-tubulin* dsRNAs. The values represent the means and standard errors for three replicate experiments.

Species Treated	DsRNA treatment	Target gene	% mortality	% knockdown of target gene
D. melanogaster	*gus*	*Dm-tub*	4 ± 1.6	0 ± 1.3
	Ae-tub	*Dm-tub*	6 ± 2.6	4.2 ± 1.5
	Dm-tub	*Dm-tub*	31.0 ± 4.1 [a]	50.6 ± 3.7 [a]

[a]Mortality and *Dm-tub* expression in *Dm-tub*-dsRNA treated larvae is significantly different from that in *gus*-dsRNA and *Ae-tub*-dsRNA treated larvae (ANOVA, $p<0.05$).

3.4 Discussion

Delivery of dsRNA to aquatic insects by a simple soaking or feeding method has many applications, including rapid functional screening of newly identified genes, and potential use in the development of species-specific insecticides. Previously, feeding of dsRNA has been shown to effectively induce gene silencing in many terrestrial pest insects, particularly Lepidoptera larvae (Bautista *et al*., 2009; Mao *et al*., 2007; Turner *et al*., 2006), but also Coleoptera (Baum *et al*., 2007; Whyard *et al*., 2009), Hymenoptera (Maori *et al*., 2009), Diptera (Walshe *et al*., 2009; Whyard *et al*., 2009), Hemiptera (Araujo *et al*., 2006; Chen *et al*., 2010; Shakesby *et al*., 2009; Whyard *et al*., 2009), crickets (Meyering-Vos and Müller, 2007) termites (Zhou *et al*., 2008) and ticks (Soares *et al*., 2005), but not in any insects with an aquatic life stage. I have shown that an RNAi response can be induced by soaking first instar *A. aegypti* larvae in moderate concentrations of dsRNA as a method for oral delivery.

Although direct soaking is probably the easiest method of orally delivering dsRNA to an organism, it is not the only way. Several studies have shown that droplet feeding (Bautista *et al*., 2009; Turner *et al*., 2006; Whyard *et al*., 2009), adding dsRNA to dry diets (Shakesby *et al*., 2009; Whyard *et al*., 2009) and also feeding bacteria that express the dsRNA (Gurley *et al*., 2008; Newmark *et al*., 2003; Reddien *et al*., 2005; Timmons *et al*., 2001) can effectively induce gene silencing in several organisms. Adding dsRNA to diets, whether liquid or dry, usually requires that the organisms be starved prior to treatment to ensure that they can find and receive a considerable dose of the dsRNA. With bacterial feeding, strains of *E. coli* that lack RNAse III are transformed with plasmids containing genes of interest between convergent T7 promoters. DsRNA

synthesis is induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) which induces the production of a T7 RNA polymerase enzyme, a gene also transformed within the cell, to express either a hairpin RNA structure, or both sense and anti-sense RNA strands. The first study to demonstrate bacterial oral delivery of dsRNA was done by Timmons *et al*. (2001) using *C. elegans*. The worms were added to plates containing the dsRNA-producing bacteria as their sole source of food. RNAi phenotypes were usually observed within one to five days, depending on the target gene. This method has also worked in the planarian *Schmidtea mediterranea*, where the bacteria were mixed with the normal diet of homogenized liver, and made into solidified chunks using agarose. This feeding method has been used for wide-scale screening of regeneration and other uncharacterized genes in that species (Gurley *et al*., 2008; Newmark *et al*., 2003; Reddien *et al*., 2005). More recently, bacterial feeding of dsRNA has been successful in some insect species like honeybees (Maori *et al*., 2009) and *Spodoptera exigua* (Tian *et al*., 2009), and even in the protozoan *Entamoeba histolytica* (Solis *et al*., 2009) which causes amoebic dysentery in humans.

Soaking organisms in dsRNA has also successfully delivered dsRNA to nematodes like *C. elegans* (Tabara *et al*., 2008), *Meloidogyne incognita* and *Globodera pallida* (Dalzell *et al*., 2009), platyhelminthes *Dugesia japonica* (Orii *et al*., 2003), *Schistosoma mansoni* (Ndegwa *et al*., 2007), and *S. mediterranea* (Whyard, unpublished), but not in any insect species besides *D. melanogaster* (Whyard *et al*., 2009). It was shown that the addition of liposomes to dsRNA solutions used to soak *D. melanogaster* larvae, particularly Lipofectamine 2000 (Invitrogen), can increase RNAi responses by nearly 50% (Whyard *et al.*, 2009). This, however, was not the case in *A. aegypti* larvae.

Liposomes are vesicles composed of lipid bilayers which can more readily fuse with cell membranes within an organism to deliver dsRNAs, or other substances encapsulated by them, to cells. All dsRNA treatments in this study (dsRNA specific to *β-tubulin*, *chitin synthase* and *hsp83*) using Lipofectamine 2000 to encapsulate the dsRNA showed either no significant difference in the transcript levels compared to larvae treated with naked dsRNA, or conflicting results, as with *β-tub*-dsRNA experiments, suggesting that liposomes do not increase the uptake of dsRNA into cells of *A. aegypti* larvae to allow higher levels of gene silencing.

It is beneficial to know that liposomes are not required to treat mosquito larvae with dsRNA, as it appears that different species react differently to the addition of liposomes, and to dsRNAs themselves. Rajagopal *et al*. (2002) found that feeding dsRNA to larvae of the lepidopteran, *Spodoptera litura*, did not reduce transcript levels of targeted genes, whereas direct injection of the dsRNAs did. It is possible that, as in *D. melanogaster* larvae, dsRNA delivery to *S. litura* larvae could be enhanced by the addition of liposomes. However, species that do not require liposomes to induce significant RNAi responses could be more easily and cheaply treated with dsRNAs on their own.

This study has shown that gene transcripts can be reduced in *A. aegypti* by soaking first instar larvae in dsRNA solutions for 2 hours. Depending on the gene being targeted, the RNAi induced by dsRNA can cause mortality over time, as was shown with *β-tub*-dsRNA treatments, where larval mortality was observed over two weeks. Also, it is known that dsRNA may persist within an organism for several days. For example, in adult honeybees (*Apis mellifera*) dsRNA template to target *vitellogenin* in females was

observed in the abdomen as many as 15 days after intra-abdominal injection, and it was also suggested that RNAi could be activated as many as 21 days after dsRNA injection (Amdam *et al*., 2003). In addition, Turner *et al*. (2006) droplet fed third instar larvae of the light brown apple moth, *Epiphyas postvittana*, dsRNA to target a gene expressed in antennae (*EposPBP1*). Knockdown of the gene was observed in adults, suggesting that the dsRNA persisted throughout the molting process, an average of 18 days.

It is interesting to note that a one-time soaking of larvae in dsRNA was enough to cause gene knockdown, and mortality, whereas with many experiments done on worms, soaking in dsRNA was done on a continual basis (Orii *et al*., 2003; Tabara *et al*., 1998). In my experiments, larvae were collected three days after treatment with *β-tub*-dsRNA, and four days after treatment with *chs*- and *hsp83*-dsRNA, and most showed significant knockdown of the respective gene transcripts compared to larvae treated with *gus*-dsRNA.

Although *hsp83*-dsRNA treated larvae showed lower transcript levels than *gus*-treated larvae, the values were not statistically significant. However, significant mortality was observed in *hsp83*-dsRNA treated larvae. Heat shock proteins are normally required to aid in folding and unfolding of proteins under stress. One of the most well known heat shock proteins is *hsp70*. This protein is know to be expressed at low levels in *D. melanogaster*, and then rapidly increases by at least two orders of magnitude if the organism is subjected to heat shock. *Hsp83*, however, is expressed at high levels during normal development, and increases only a few-fold when subjected to heat shock (Xiao and Lis, 1989). Naturally high levels of *hsp83* transcript in developing larvae could have

masked the significance of gene knockdown levels, yet this reduction in expression may have been enough to affect development and cause mortality.

Larvae treated with *chs*-dsRNA showed significant transcript knockdown levels, around 30%. Chitin synthase is an enzyme that catalyzes the synthesis of chitin, which is produced by fungi, nematodes and arthropods. It is one of the most abundant amino polysaccharides found in nature, formed by linear polymers of β-(1-4)-linked *N*-acetyl glucosamines, and makes up the exoskeleton of insects. It is also found in tracheal cuticles and the peritrophic matrix (reviewed in Merzendorfer, 2006). Chitin synthase is known to be encoded by two genes, *chs-A* and *chs-B*. *Chs-A* is expressed in the epidermis and other ectodermal cells like tracheal cells, while *chs-B* is expressed in gut cells that produce the peritrophic matrix (Merzendorfer, 2006). The chitin synthase fragment used for our experiment is homologous to *chs-A* from *D. melanogaster* (NCBI Accession: NM_001144525). As *chs* is required for exoskeleton development, and is critical for proper moulting, and it has been suggested that insect development is dependent on the precisely tuned expression pattern of *chs* genes. According to Merzendorfer (2006), fifth instar tobacco hornworms (*M. sexta*) injected with *chs*-dsRNA showed severe head deformities and subsequent death during pupal stages. However, ingestion of *chs*-dsRNA by first instar mosquito larvae may not have provided a high enough dose to trigger RNAi throughout all chitin synthesizing tissues and cause high mortality rates, but was potent enough to stunt growth by affecting the synthesis of a new exoskeleton.

β-tubL-dsRNA treated larvae yielded the highest levels of knockdown in whole bodies, as well as separated guts and carcasses, ranging from 40 to 50%. Together, α and β-tubulin subunit proteins form heterodimers which make up microtubules. Microtubules

are essential for normal functioning of cells, and are involved in the composition of mitotic and meiotic spindles, elongated neural processes, cilia, flagella, and cell shape. In *D. melanogaster*, there are different forms of *β-tubulin* genes, some of which are generally expressed, while others are expressed specifically during embryogenesis (Bialojan *et al*., 1984). The targeted *β-tubulin* gene from *A. aegypti* is homologous to *βtub56D* in *D. melanogaster*, which is expressed in all cells and is suggested to be responsible for microtubule structure and cell division (Bialojan *et al*., 1984).

There are a few reasons why higher knockdown levels of this important *β-tubulin* gene were not observed in *A. aegypti* larvae after dsRNA soaking. First, whole bodies of larvae samples were collected at three, five and seven days after the one-time treatments in dsRNA. Had samples been collected sooner than three days after treatment, higher knockdown levels may have been observed if gene transcripts were initially knocked down without having the opportunity to re-synthesize depleted transcripts. At five days after treatment, the tubulin transcripts appear to increase, which could be explained by the cells' attempts to rapidly replenish lost *β-tubulin* transcripts necessary for development, which resulted in overcompensation within the organism. Then, by seven days after treatment, the larvae were able to return *β-tubulin* levels to normal. In 2004, Gatehouse *et al*. showed reductions of hypopharyngeal amylase enzyme activity from 80% one day after injection with dsRNA specific to that gene, compared to only 27% three days after injection in *A. mellifera*. They suggested that the relatively rapid decrease in enzyme activity after three days implies a high turnover rate for that enzyme. This could also be the case for *β-tubulin*, which is an important gene for growth, development, and structural upkeep of cells, particularly in gut cells which are constantly being

regenerated. This is especially true in growing larvae, where gut cells are growing and dividing rapidly. For example, in *M. sexta*, epithelial cells in the midgut increase in number by 200-fold by the end of their larval phase (Baldwin and Hakim, 1991). In *A. aegypti*, it is known that the midgut can grow at least 5-fold between first and third instar stages (Ray *et al*., 2009). If cells are rapidly dividing, the demand for genes such as *β-tubulin*, and other genes critical for mitosis and composition of cell structure could be so great that they are constantly being expressed, and so RNAi knockdown levels for those genes wouldn't be as great as levels seen for genes that aren't as abundantly expressed.

Another item to note is that all larvae collected to determine changes in gene expression were all live larvae, while it is the larvae that died due to dsRNA treatment that, potentially, have the lowest gene expression levels. Sampling from only living larvae may have skewed results to represent individuals which may have received a smaller, less potent dose of dsRNA which was enough to somewhat reduce transcript levels, but not enough to cause mortality, or those individuals with reduced transcripts which were already replenished by the time they were collected for analysis.

When guts of treated larvae were dissected and gene expression was compared to that of the rest of the body, it seems that the effects of dsRNA was able to travel beyond the initial point of entry (i.e. gut cells), since there was approximately 50% knockdown of *β-tubulin* transcripts in the guts of *β-tubL*-dsRNA treated larvae, compared to *gus*-dsRNA treated larvae, while there was also around 40% knockdown of *β-tubulin* in the remaining carcass (Fig. 21, section 3.3.3). The fact that *β-tubS*-dsRNA did not induce as much knockdown in guts compared to carcasses could be explained by rapid turnover rates for *β-tubulin* in dividing gut cells (as mentioned earlier), as well as large variation in gene

expression among individuals. However, the data still implies that there is a mechanism that enables dsRNA to travel beyond the tissues to which the dsRNA was first delivered.

Many organisms, including mammals, have putative transmembrane proteins that specifically transport dsRNAs across cell membranes (Gordon and Waterhouse, 2007). These SID proteins were first discovered in *C. elegans* by Winston *et al*. (2002), who named them for systemic RNA interference-deficient mutants that failed to show spreading of an RNAi response from the pharynx of the worm to the body wall, as would be seen in normal worms. The SID-1 protein in *C. elegans* is widely expressed and concentrated at cell membranes (Winston *et al*., 2002). It is suggested to have a long (> 400 amino acids), extracellular N-terminus, a cytosolic C-terminus, and 11 transmembrane domains, where five of the first six domains span the entire cell membrane (Feinberg and Hunter, 2003). The membrane spanning portions of the gene seem to be particularly important, as a change in one amino acid in the fourth transmembrane domain results in loss of function of the protein (Feinberg and Hunter, 2003). It has also been suggested that SID-1 acts as a channel to transport dsRNAs by passive diffusion, as opposed to active transport, phagocytosis or endocytosis. This was determined when Feinberg and Hunter (2003) used oligomycin to deplete cells of ATP (which is required for active transport, phagocytosis and endocytosis) for 30 minutes prior to adding dsRNA to the cells. They observed that 300-fold more dsRNA was taken up by *Drosophila* S2 cells that were transgenically modified to express functional SID-1 proteins, compared to cells expressing non-functional SID-1. This indicated that ATP is not required for dsRNA uptake in cells that possess SID-1.

As mentioned, putative homologs of SID proteins have been found in virtually all taxa, with the exception of plants, and curiously dipteran insects, whereas most other arthropod groups possess some gene similar to that of *sid-1* (Gordon and Waterhouse, 2007). Plants are able to transport dsRNA across intercellular bridges or plasmodesmata, and can also use phloem to transport RNAs long distances (Voinnet *et al*., 1998). The mechanism in dipterans, however, is still being investigated. It is known that *Drosophila* S2 cells have the ability for cell-autonomous RNAi (where dsRNA enters a cell, and the RNAi machinery will cause gene silencing only within that cell), but they do not show systemic RNAi. However, when S2 cells ectopically express *sid-1*, they do show a systemic RNAi response (Feinberg and Hunter, 2003). *A. aegypti* is a dipteran species, and as such, no genes similar to *sid-1* have been found in its genome. Therefore, there must be some other mechanism to allow uptake of dsRNA within the organism, and movement of that dsRNA from cell to cell. Feinberg and Hunter (2003), in addition to finding that uptake by SID-1 can be a passive process, found that uptake of dsRNA in normal *Drosophila* S2 cells (which lack SID-1) uses some ATP-dependent process. This was determined when mock-ATP-depleted S2 cells expressing *sid-1* showed 30-fold more internalized dsRNA than S2 cells expressing a non-functional *sid-1*, as opposed to ATP-depleted cells expressing *sid-1* showing 300-fold more internalized dsRNA than cells with the non-functional *sid-1* (Feinberg and Hunter, 2003). This means that the natural dsRNA uptake mechanism may not be as efficient as SID-1, and it requires ATP.

Some have speculated that the uptake mechanism in *Drosophila* involves receptor-mediated endocytosis. Saleh *et al*. (2006) performed a genome-wide screen to identify genes required for dsRNA uptake and discovered that many components of the

endocytic pathway were involved, such as clathrin heavy chain and its adapter protein AP-50. Clathrin proteins form a structural scaffold for newly forming vesicles in a cell, like those that would be formed during receptor-mediated endocytosis. Three heavy chains and three light chains compose the clathrin molecule, which form a three-legged structure called a triskelion. When the triskelions come together they create lattices that can change from flattened to curved, depending on the stage of the forming vesicle. Adaptor proteins are required to bind the clathrin scaffolding to the surface of the forming vesicle. These proteins are involved in early endocytic uptake.

Other genes found in *Drosophila* S2 cells that are required for dsRNA uptake included several genes involved in vesicle trafficking and protein sorting, members of the Golgi complex family, and genes involved in protein transport and cytoskeleton organization. Therefore, it was concluded that dsRNA must enter the RNAi pathway through the intracellular vesicle network, specifically receptor-mediated endocytosis (Saleh *et al*., 2006).

Ulvila *et al*. (2006) did a similar screen of *Drosophila* S2 genes to identify components of the RNAi machinery and identified two endocytotic receptors that may be involved in dsRNA uptake, the scavenger receptors SC-R1 and EATER. Scavenger receptors are normally involved in phagocytocis of bacterial pathogens and display specificity for multiple ligands, and it is suggested that dsRNA may be a previously unrecognized ligand for these receptors. It was also found that expression of SC-R1 in mammalian cell lines allowed for higher rates of internalization of long dsRNA (Ulvila *et al*., 2006), further supporting their role in receptor-mediated endocytosis of dsRNA uptake.

As *Drosophila* and *A. aegypti* are closely related insects, it is likely that they both utilize the same mechanism for dsRNA uptake, however, according to my results, it appears that *A. aegypti* may display some systemic RNAi response whereas *D. melanogaster* does not. Since dipterans do not express the dsRNA transport channel *sid-1*, there must be some other method for dsRNA to travel from cell to cell. Previously, Winston *et al*. (2002) had observed that neurons in *C. elegans* did not express *sid-1*, but were still able to export dsRNA from those cells. Jose *et al*. (2009) have found that, although *sid-1* is required for importing dsRNA, it may not be required for export. To test this, they used *C. elegans* expressing green fluorescent protein (*gfp*) in the pharynx, gut and body wall muscle cells, and expressed hairpin RNA specific to *gfp* (gfp-hpRNA) only in the pharynx. These same worms also lacked expression of *sid-1* in all cells of the body, except for the gut. It was found that after expression of gfp-hpRNA in the pharynx, there was detectable silencing of *gfp* in the gut as well. This meant that the RNAi silencing trigger was able to be transported out of cells of the pharynx and reach gut cells without SID-1 proteins. They also did experiments using ingested *gfp*-dsRNA, which was given to worms expressing *sid-1* only in body wall muscle cells. It was observed that dsRNA that entered the gut was able to silence *gfp* expression in the body wall. Jose *et al*. (2009) suggest that transport of silencing triggers from the gut lumen may involve transcytosis, where vesicles carry dsRNA across the interior of the cell where they can then release their contents on the other side. This same method of transcytosis could occur in mosquito larvae to allow *β-tub*-dsRNA to reach other tissues within the organism, and not just gut cells that would be the initial point of entry for the dsRNA.

If dsRNA is to be a potential method of controlling pest insects, it is important to know that it can target a specific organism without harming other pests. Using *A. aegypti* and *D. melanogaster*, we have shown that it is possible to design species-specific dsRNAs to target one species without affecting the other. It appears that as long as dsRNA fragments for two species do not contain identical sequences of 19-21 nucleotides long (the same size as siRNAs), species-specific dsRNAs can easily be designed. Several studies have also shown species specificity of dsRNAs. For example, Baum *et al*. (2007) created transgenic plants expressing RNA hairpins to target *β-tubulin*, *V-ATPase A* and *V-ATPase E* in the western corn rootworm (*Diabrotica virgifera virgifera*), without affecting both the southern corn rootworm (*Diabrotica undecimpuncta howardii*) and the Colorado potato beetle (*Leptinotarsa decemlineata*). Similar dsRNA design was done by Whyard *et al*. (2009) who were able to create dsRNAs specific to eight insect species, including four closely related *Drosophila* species.

From this study, it appears that RNAi does have the potential to be used as an insecticide against aquatic stages of mosquito species. There is the possibility of delivering dsRNAs as a solution that can be ingested by developing larvae, perhaps by simply spraying into ponds, ditches, or other known habitats of these pest insects. For dsRNAs to be deployed as pesticides, it will be necessary to develop cost-effective production systems. Further research on delivery methods (discussed further in Chapter 4) and methods for mass production of dsRNAs is necessary if they are to be used as pesticides.

Of course, more research must be done to determine optimal dsRNA concentrations and sequence lengths for delivery to the desired species. More

importantly, appropriate gene targets must be investigated. Possible housekeeping genes could be targeted to affect a particular life stage where there are high rates of mortality, or perhaps there is a gene that, if knocked down, affects all life stages equally. Genes that affect reproduction of terrestrial adults, but can be targeted in the aquatic juvenile stages, are also good candidates for an RNAi pesticide. For example, there are several sex differentiation genes that have been identified in *D. melanogaster* that have homologues in mosquitoes. There are also genes that are involved in egg and sperm production which could be good candidates for dsRNA treatment. Targeting these genes could skew sex ratios or prevent fertility in specific sexes, which is not only an alternative to current chemical pesticides, but also an alternative to the widely used practice of irradiating males of a species for sterile insect technique.

Chapter 4: Conclusions and Future Directions

From the two studies conducted in Chapters 2 and 3, it is evident that RNAi can be used as a molecular biology tool to assess the functions of genes, and has potential applications as a species-specific pesticide in mosquitoes. Although the phenotypic data collected from knocking down the *Aacyp* gene in *A. aegypti* via dsRNA injections did not reveal a specific function for this gene, likely functions, such as facilitating moulting, have been ruled out. Due to the diverse functional nature of the CYP gene family, there are many more possible functions to test for *Aacyp*, whereas, if targeting a gene with a more specific predicted function, changes in phenotype would be more readily observed following RNAi knockdown. However, the relative ease and quickness of delivering dsRNA to mosquitoes enhances its use as a high-throughput screening tool. The same RNAi knockdown experiments for *Aacyp* could be repeated, but rather than assess changes in moulting or reproductive viability, changes to mating behaviours or even tolerance to chemical insecticides such as permethrin could be measured.

A. aegypti, in particular, may be soaked in dsRNA at young larval stages for high-throughput screening, or injected at any life stage in order to knock down a gene and assess its function. Although 100% knockdown of the genes tested was not readily observed, partial knockdown of a gene may still be enough to cause a phenotypic change, particularly for those genes that are not highly expressed or perhaps have low turnover rates. Genes with high turnover rates (such as housekeeping genes) may be more likely to out-compete the RNAi machinery and replenish their transcripts equally as fast as RISC can degrade them.

Using RNAi, it is feasible to quickly screen mosquito genes that could be involved in specific processes that are detrimental to humans, such as blood-feeding and virus transmission. Information on genes involved in blood-feeding and blood-meal digestion, or even host-seeking behaviours, could be used to impede these processes, preventing females from finding hosts, blood feeding, or digesting a blood-meal properly, thereby rendering their offspring inviable. This could prevent the spread of several viruses transmitted by the mosquito, particularly Dengue, but also Yellow Fever and West Nile Virus, a disease that has recently begun to affect North America. RNAi screens of viral genes and their interactions within the mosquito could be tested by knocking down various viral genes and observing changes in viral replication and transmission within hosts.

The use for RNAi as a biological, species-specific pesticide is quite promising, as according to my data, significant knockdown of the housekeeping *β-tubulin* gene, and the *chitin synthase* gene were achieved simply by soaking first instar larvae in dsRNA solutions for short periods of time. According to a recent review paper by Huvenne and Smagghe (2010), there are several things to consider when optimizing dsRNA for oral delivery to an organism, and my study provides support to many of their conclusions. They suggest that the concentration, length and sequence of dsRNA can be critical for inducing significant RNAi responses in an organism. According to my study, there did appear to be a dose dependent response to *β-tubulin*-specific dsRNA, where higher concentrations of the dsRNA caused higher mortality in the larvae. It has been suggested that an optimal concentration of dsRNA must be found to elicit maximal gene silencing, and using dsRNA concentrations above that optimum no longer increases the amount of

observable gene silencing. This optimal dsRNA concentration, however, is specific to the organism, and most likely to the life stage that is being treated as well. For example, Meyering-Vos and Müller (2007) found that concentrations of 10 μg/μl were no more effective at silencing the neuropeptide gene *sulfakinin* than concentrations of 2 μg/μl in adult female Mediterranean field crickets (*Gryllus bimaculatus*). In the pea aphid (*Acyrthosiphon pisum*), dsRNA concentrations above 1 μg/μl for a putative aquaporin gene did not increase gene silencing any further in 6-day-old aphids (Shakesby *et al*., 2009). In my study, dose dependency seemed to affect mortality of *A. aegypti* larvae up to 0.5 μg/μl. However, it is definitely possible that the optimal dsRNA concentration for first instar larvae is higher than what was used, and even more gene silencing could have been seen using concentrations above 0.5 μg/μl. Knowing the optimum dsRNA concentration for an insect is important, especially in the production of dsRNA insecticides, to prevent waste of reagents and an unnecessary addition of chemicals to the environment.

The length of the dsRNA fragment being used is also important, according to Huvenne and Smagghe (2010), with longer dsRNAs typically being more effective than shorter dsRNAs. Shih *et al*. (2009) noted that while shorter (100 bp) dsRNAs appear to enter cells through transport channels such as SID-1 faster than longer (500 bp) dsRNAs, the longer fragments are better initiators of the gene silencing mechanism. The results from my *β-tub*-dsRNA experiments confirmed that the longer (728 bp) dsRNAs can cause a more potent RNAi effect than the shorter (385 bp) dsRNA (based on the qRT-PCR data), although both dsRNAs were equally effective at killing mosquito larvae,

perhaps because the extent of RNAi from either treatment was sufficient to disrupt normal gut functioning.

Finally, Huvenne and Smagghe (2010) note that the actual sequence being used to target a specific gene with RNAi is critical to preventing off-target effects. Depending on the application, it is important to prevent other species from being affected, as well as other genes within the organism. In the case of RNAi screening to investigate gene function, dsRNAs should be specific enough to target one gene only. However, for the development of a species-specific insecticide, the nucleotide sequence used may be from a conserved gene to target many different insects. For example, genes involved in insect specific pathways such as those involved in moulting or feeding could be targeted with dsRNAs. In the case of highly conserved genes that may have homologues in a broad range of species, Whyard *et al.* (2009) found that the 3' untranslated region (3'UTR) of a gene could be used for dsRNA design. For example, in four *Drosophila* species (*D. melanogaster*, *D. sechellia*, *D. yakuba* and *D. pseudoobscura*) they were able to use the 3'UTR to design dsRNA sequences specific to each individual's *γ-tubulin* gene (*γTub23C*). Among these four species, the sequence identity of this gene ranges from 79 to 96%, which makes it nearly impossible to find parts of the gene without overlapping sections of 19-21 nt long. Therefore, they used siRNAs specific to the 3'UTRs to induce species-specific gene knockdown and mortality.

The method of dsRNA delivery for RNAi is also very important, and may be dependent on the gene being targeted. Tissues beyond the gut may be more difficult to reach in a simple feeding or soaking method. Even though my results suggest that the RNAi effect spread beyond the gut in the feeding mosquito larvae, the extent of silencing

beyond the gut may not be sufficient to silence genes effectively in distant tissues. Nevertheless, using RNAi to target gut genes required for nutrient uptake could cause enough damage to kill the insect as a chemical pesticide would, but still be species-specific. RNAi seems to be a good candidate for the development of novel insecticides, particularly in lepidopterans, and recently a review paper has been published on the viability of RNAi as a novel pesticide in this family of insects (Terenius *et al*., 2010). However, these are terrestrial insects, and only terrestrial stages of insects have been tested for feeding and soaking as a method to deliver dsRNA. In my study, I have shown that it is possible to knock down a gene using RNAi in an insect with aquatic larval stages. The possibility to simply treat an aquatic habitat with a solution of dsRNA is fairly intriguing as a method to eliminate an aquatic pest-species.

Currently, our method of producing dsRNA involves only very small-scale *in vitro* synthesis reactions, and clearly, the scale of dsRNA production would have to be addressed if the dsRNA was to be developed as a pesticide. To make the process cost-effective, it might be possible to use genetically modified bacteria or yeast to mass produce dsRNAs, which can then be either extracted from the microorganisms and mixed into artificial diets or baited food, or perhaps the microorganisms can simply be killed and mixed into oral formulations that are attractive to the grazing insects in the environment. Certainly, another aspect of the research would have to assess the stability of the dsRNA in any of these novel formulations.

Naturally, there are limitations to using RNAi to examine gene functions or use it as a species-specific pesticide. As mentioned, the nature of the gene will greatly affect the RNAi response within an insect. In the case of pesticide development, genes should be

screened for prominent phenotypic affects to determine which genes will do the greatest damage to the insect when silenced. With the rapid increase of genome sequencing data available for many more insect species, RNAi will undoubtedly play an important role in identifying the functions of newly sequenced genes and identify new candidate genes for RNAi pesticide applications.

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