Drosophila as a model for the *Anopheles* Malpighian tubule

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Gayle Overend

Integrative and Systems Biology
Faculty of Biomedical and Life Sciences
University of Glasgow
Glasgow
G11 6NU
UK

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The research reported within this thesis is my own work except where otherwise stated, and has not been submitted for any other degree.

Gayle Overend

Abstract

The insect Malpighian tubule is involved in osmoregulation, detoxification and immune function, physiological processes which are essential for insect development and survival. As the Malpighian tubules contain many ion channels and transporters, they could be an effective tissue for targeting with novel pesticides to control populations of Diptera. Many of the insecticide compounds used to control insect pest species are no longer suited to their task, and so new means of control must be found. The malarial mosquito, *Anopheles gambiae*, spreads the *Plasmodium* parasite which is responsible for over one million deaths each year, and is one of the species on which many current insecticides are no longer effective. *Anopheles* is notoriously difficult to study due to a lack of natural mutation stocks and transgenic capabilities, as well as the difficulties involved with maintaining a colony. The fruit-fly *Drosophila melanogaster* is a useful model organism for *Anopheles*, and previous studies suggest that the mechanisms of Malpighian tubule function are well conserved between the two species.

Following microarray investigations to identify genes which were highly enriched in both the *Anopheles* and *Drosophila* Malpighian tubules, four homologous gene-pairs were selected, *AGAP097752* and *CG15406, AGAP012251* and *Picot, AGAP009005* and *ZnT35C,* and *AGAP002587* and *CG8028*. Analysis of the *Anopheles* Malpighian tubule microarray data-set showed ion channels and transporters to be highly expressed in the tubules, although similarly to *Drosophila*, very few of the renal up-regulated genes have been characterised. The gene-pairs chosen were all novel, but putatively predicted to be involved in sugar transport, phosphate transport, zinc transport and monocarboxylate transport respectively. These are functions which are likely to be essential, but so far remain unstudied in the insect renal system. The gene-pairs were chosen with two main purposes; to determine how closely expression of the genes was conserved between *Anopheles* and *Drosophila*, and also to determine which of the genes were essential, and could therefore be effective insecticide targets.

The homologous gene-pair *AGAP007752* and *CG15406* have well-conserved expression in the Malpighian tubules, suggesting that they are functionally important genes. This was shown in *Drosophila*, where knockdown of *CG15406*

expression was lethal to the fly. A direct role in tubule fluid secretion was not found, and experiments to determine the sugars transported by *CG15406* were inconclusive, possibly due to an abundance of highly-expressed sugar transporters in the tubules. The inorganic phosphate co-transporters *AGAP012251* and *Picot* also show conservation of expression in the Malpighian tubules, and are likely to be involved in the transport of inorganic phosphate into the tubules for incorporation into metallo-organic concretions. In the *Anopheles* tubules the concretions are found in the main segment, in the *Drosophila* tubules they are located in the distal initial and transitional segments, where *AGAP012251* and *Picot* are expressed. *Picot* is essential for *Drosophila* development through to adulthood, and for survival as an adult, although the transporter does not appear to be directly involved in fluid secretion. Expression of neither *AGAP012251* nor *Picot* is confined to the tubules. The putative zinc transporters *AGAP009005* and *ZnT35C* show a highly conserved expression pattern, and appear to be involved in the secretion of excess zinc from the Malpighian tubules. *ZnT35C* is essential early-on in *Drosophila* development, and for survival in the adult fly. Similarly to *Picot* and *CG15406*, there is no direct role for *ZnT35C* in fluid secretion from the tubules under normal zinc conditions. The putative monocarboxylate transporters *AGAP002587* and *CG8028* are not as well conserved, as *AGAP002587* is highly up-regulated in the tubules of female mosquitoes both before and after a blood-feed, whereas *CG8028* has no sex-specific up-regulation. *CG8028* is not essential for *Drosophila* development or survival, and plays no discernable role in fluid secretion.

The data collected during this investigation suggests that in general there is a high level of conservation of expression between homologous transport genes in the *Anopheles* and *Drosophila* Malpighian tubules. The three gene-pairs which show the greatest conservation of expression are also essential for development and survival in *Drosophila*. This suggests that cross-species studies are an effective way of finding essential and important genes. The data collected also suggests that *Drosophila* is a reliable model for *Anopheles*, and could be used as a high-throughput system of finding genes which could be effective insecticide targets in Diptera.

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Abbreviations

ß-Gal	ß-galactosidase
µM	micromolar
ACS	anion/cation symport
ANOVA	analysis of variance
ATP	adenosine trisphosphate
Bl	bristle
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
cAMP	adenosine 3' -5' cyclic monophosphate
cDNA	complementary DNA
cGMP	guanosine 3' -5' cyclic monophosphate
Ci	Curies
CNS	central nervous system
cpm	counts per minute
cRNA	complete RNA
cT	threshold cycle
CyO	curly
Da	Dalton
DAPI	4,6-diamidine-2-phenylindole, dilactate
DDT	Dichloro-Diphenyl-Trichloroethane
DGRC	Drosophila Genome Research Centre
DIG	digoxigenin
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Drosophila 1	Drosophila Genome Array
Drosophila 2	Drosophila Genome Array 2.0
dsRNA	double stranded RNA
dATP	2' deoxyadenosine trisphosphate
dCTP	2' deoxycytosine trisphosphate
dGTP	2' deoxyguanosine trisphosphate
dNTP	2' deoxy(nucleotide) trisphosphate
dTTP	2' deoxythymidine trisphosphate

dUTP	2’ deoxyuridine trisphosphate
EDTA	ethylenediamine tetra acetic acid
EMS	ethyl methane sulphonate
EST	expressed sequence tag
EtBr	ethidium bromide
g	Acceleration equal to gravity
gDNA	genomic DNA
GFP	green fluorescence protein
GLUT	monosaccharide transporter
h	hours
HRP	horseradish peroxidase
HMIT	H^+ myo-inositol transporter
ICC	immunocytochemistry
kb	kilobases
M	molar
MAS5	microarray analysis software 5
MCS	multiple cloning site
MCT	monocarboxylate transporter
MFS	Major Facilitator Superfamily
min	minutes
mM	millimolar
MM	mismatch
mRNA	messenger RNA
MT	Malpighian tubules
MW	molecular weight
nM	nanomolar
NO	nitric oxide
OATP	organic anion transporter peptide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAT	PBS, Triton X-100, BSA
PBS	phosphate buffered saline
PBT	PBS, Triton X-100
PCR	polymerase chain reaction
P_i	inorganic phosphate
PiT	inorganic phosphate transporter

PM	perfect match
qPCR	quantitative PCR
RNA	ribonucleic acid
RNAi	RNA interference
RNAse	ribonuclease
rp49	ribosomal protein 49
RT	room temperature
s	seconds
SAGE	serial analysis of gene expression
SAM	statistical analysis of microarrays software
Sb	stubble
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
SHWFGF	Sir Henry Wellcome Functional Genomics Facility
SLC	solute carrier
SMCT	sodium-coupled monocarboxylate transporter
SPR	sex peptide receptor
SUT	sugar transporter
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethyenediamine
TM	transmembrane
TR	Texas Red
Tris	2-amino-2-(hydroxymethy)-1,3-propanediol
U	unit
UAS	upstream activating sequence
V	Volts
V-ATPase	vacuolar-type H^+ adenosine triphosphatase
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
ZnT	zinc transporter

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Chapter 1 - Introduction

1.1 Summary

The Dipterans are a highly important Class of insect, for both their beneficial impact on the environment, and their capacity to spread disease and destroy crops. Many insecticides which have previously proven useful for controlling populations of Diptera are no longer effective, and so new means of controlling pest species must be employed. This requires a greater understanding of insect behaviour and physiology, in combination with informative studies on the increase in insecticide resistance. Many of the most dangerous species, such as the malarial mosquito *Anopheles gambiae*, are difficult to study due to the intricacies of maintaining a colony and a lack of reliable transgenic techniques. A useful model organism for *Anopheles* is the fruit-fly *Drosophila melanogaster*. *Drosophila* has been studied for more than a century, largely due to the existence of stocks containing natural mutations, and the ease of making new *Drosophila* transgenic stocks. The insect renal system, the Malpighian tubules, is known to be involved in osmoregulation, detoxification and immunity. As disruption of these processes is lethal to the insect, the Malpighian tubules could be an effective tissue for insecticide targeting in Diptera. Microarray analysis is a valuable way of identifying important genes in a tissue, through enrichment of expression. By comparing microarray data-sets for the Malpighian tubules of *Anopheles* and *Drosophila* it is possible to identify homologous gene-pairs from the species which are enriched in the tubules. Conservation of these genes over hundreds of millions of years of evolution suggests that they are important for renal function in *Drosophila* and *Anopheles*, and could therefore be effective insecticide targets for controlling populations of Diptera.

1.2 Diptera as a Pest Species

Insecta is the largest and most diverse animal Class, containing more than half of the species described to date. The Diptera are perhaps the most important Order of Insecta for their impact on human health and economics. Almost 150,000 species of Diptera have been described (Evenhuis, 2005), including the insects commonly known as flies, midges, gnats and mosquitoes. They play a large part in almost all non-marine ecosystems, and haematophagous Dipterans are responsible for millions of deaths each year through the pathogens which they transmit. The main culprits are mosquitoes such as *Anopheles gambiae* and *Aedes aegypti*, which can spread the pathogens responsible for malaria, dengue, lymphatic filariasis and yellow fever whenever the female engages in a blood-meal. The tsetse fly (Diptera: Glossinidae) is also a major disease vector, able to spread the trypanosomes responsible for sleeping sickness in sub-Saharan Africa (Krafsur, 2009). *Culicoides* biting midges (Diptera: Ceratopogonidae) are able to pass both pathogens and viruses to man and animals, and are particularly important as they are able to spread arboviruses among livestock (Mellor, 2000). Dipteran flies can also have a massive effect on the economy, as they are responsible for the destruction of many grain and fruit crops (McPheron, 1996; Dhillon *et al.*, 2005). Historically populations of Diptera have been controlled by insecticide targeting, but increased resistance to many families of chemical insecticides means that this technique is no longer reliable.

1.2.1 Insecticide Resistance in Mosquitoes

As awareness has risen over the possible long-term effects of the organophosphate and organochlorine insecticides (Montgomery *et al.*, 2008; Rosas and Eskenazi, 2008), reliance has increased on non-toxic compounds found in nature and their synthetic derivatives. The Pyrethroid insecticides were developed from the chemical pyrethrin, found in plants of the *Chrysanthemum* genus (Page and Blackith, 1949). Like Dichloro-Diphenyl-Trichloroethane (DDT), the Pyrethroids act by activating voltage-sensitive sodium channels in the nervous system, which results in paralysis of the insect (Wouters and van den Bercken, 1978). In 1995 the Pyrethroids constituted approximately 23% of the worldwide insecticide market (Casida and Quistad, 1998), but recent studies

suggest that they have become ineffective in large areas of Africa and Asia (WHO, 2005).

Pyrethroid-based insecticides have been in use since the 1920s. Reliance on the pyrethroids as non-persistent and less acutely toxic insecticides has led to their overuse, and ultimately to large pockets of mosquitoes which are resistant to them. Figure 1.1 shows resistance to Permethrin, a pyrethroid-based insecticide, in *Anopheles gambiae* populated areas of West Africa. More areas tested showed resistance to Permethrin than susceptibility to it, with 25 – 30% of areas also showing resistance to two other pyrethroids, Deltamethrin and Lambdacyhalothrin (W.H.O., 2005).

Two modes of resistance to the pyrethroids have been found in insects; increased metabolic detoxification, and desensitisation of the nervous system. Enhanced metabolic detoxification is an increase in expression of enzymes such as the esterases and monoxygenases which break down the pyrethroids, rendering them ineffective (Ishaaya, 1993). Increased expression of a single P450 gene, *Cyp6g1*, can result in resistance to DDT in *Drosophila melanogaster* (Daborn *et al.*, 2002), and manipulation of *Cyp6g1* in a single tissue, the *Drosophila* Malpighian tubules, can alter survival of the insect after insecticide challenge (Yang *et al.*, 2007). Overexpression of *Cyp6g1* is usually associated with the insertion of an Accord transposable element into the 5' end of the gene. Enhanced metabolic detoxification can be counteracted by combining the insecticide with synergists which can inhibit their enzymatic activity. The second mode of resistance – desensitisation of the nervous system to the insecticide – is harder to counteract. First studied as early as the 1950s in the housefly *Musca domestica* (Busvine, 1951), knockdown resistance arises through selection of a population of insects which carry an allelic variant of the target sodium channel (Plapp and Hoyer, 1967). The insecticide is unable to interact effectively with the variant sodium channel, and therefore does not paralyse the insect. Ultimately this leads to a population of insects on which the insecticide is ineffective. Much time has been spent in the last three decades trying to understand the genetics and biochemical basis of resistance to the pyrethroids and DDT.

On the topic of malarial control, the World Health Organisation states in their 2008 Global Malarial Control and Elimination Review:

> "Malaria control relies heavily on a limited number of tools, in particular artemisinin derivatives and pyrethroids, which could be lost to resistance at any time. The future of global malaria control and elimination depends, therefore, on the ability of research and development to deliver a steady output of replacements for tools that are being lost to resistance and to supply new tools to make elimination of malaria possible in high transmission situations."

Understanding and controlling mosquitoes, and the diseases which they transmit, requires research at the molecular and physiological levels, as well as in-depth behavioural studies on their favoured life-style and habitat.

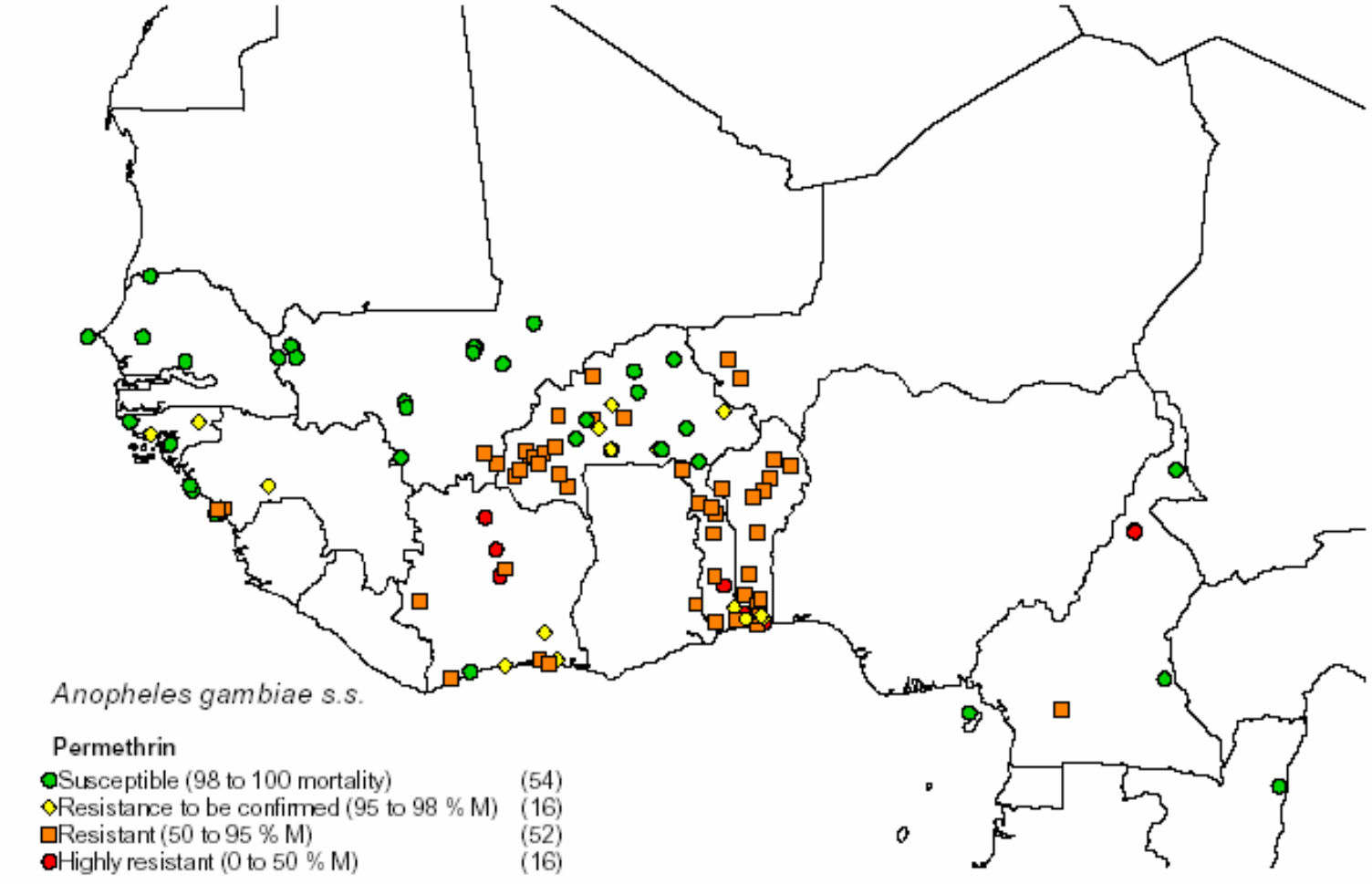


Figure 1.1 Distribution of Permethrin resistance in *Anopheles gambiae* in West Africa (W.H.O., 2005).

1.3 *Anopheles gambiae*

Malaria is spread by specific mosquito species of the genus *Anopheles*, a subfamily of the Culicidae. There are approximately 3500 species of Culicidae (Knight, 1977). Of the 430 species in the genus *Anopheles*, approximately 40 are able to transmit the malarial parasite (Service, 1993). Figure 1.2 shows the most prominent *Anopheles* strain capable of transmitting *Plasmodium* in each country worldwide. Although malaria is not currently found in many of these countries, the capability to spread the parasite should it be reintroduced is still present. *Anopheles gambiae sensu strictu* is the primary vector for the malarial parasite *Plasmodium falciparum* in Africa, where the vast majority of malarial deaths occur.

Several novel approaches to tackling mosquito insecticide resistance are being explored. The release of sterile mosquitoes (Atkinson *et al.*, 2007), the use of natural fungal insecticides (Thomas and Read, 2007), and the utilisation of naturally-occurring DNA elements as gene drive systems (Ruang-Areerate and Kittayapong, 2006) all have associated advantages and disadvantages. Whether there is a need to move focus from chemical insecticides to genetic control of mosquitoes is much debated. Sterile Insect Technique has been used successfully to control other insect species such as the screw-worm, *Cochliomyia hominovorrax*, the tsetse fly *Glossina morsitans* and the Mediterranean fruit-fly *Ceratitis capitata* (Robinson, 2002). Developing a similar control strategy for *Anopheles* is difficult due to the number of species involved in malaria transmission, the large number of mosquitoes which would have to be reared, and the reduced mating efficiency associated with transgenic mosquitoes. Sterile male mosquitoes must be able to compete in the wild for mates to reduce the size of the mosquito populations, but studies have shown that insertion of transgenics is often detrimental to mosquito fitness (Irvin *et al.*, 2004). Gene drive systems have similar difficulties, and it seems the most feasible approach is to better understand the processes essential to mosquitoes, and to target them with synthetic compounds or natural insecticides such as fungi.

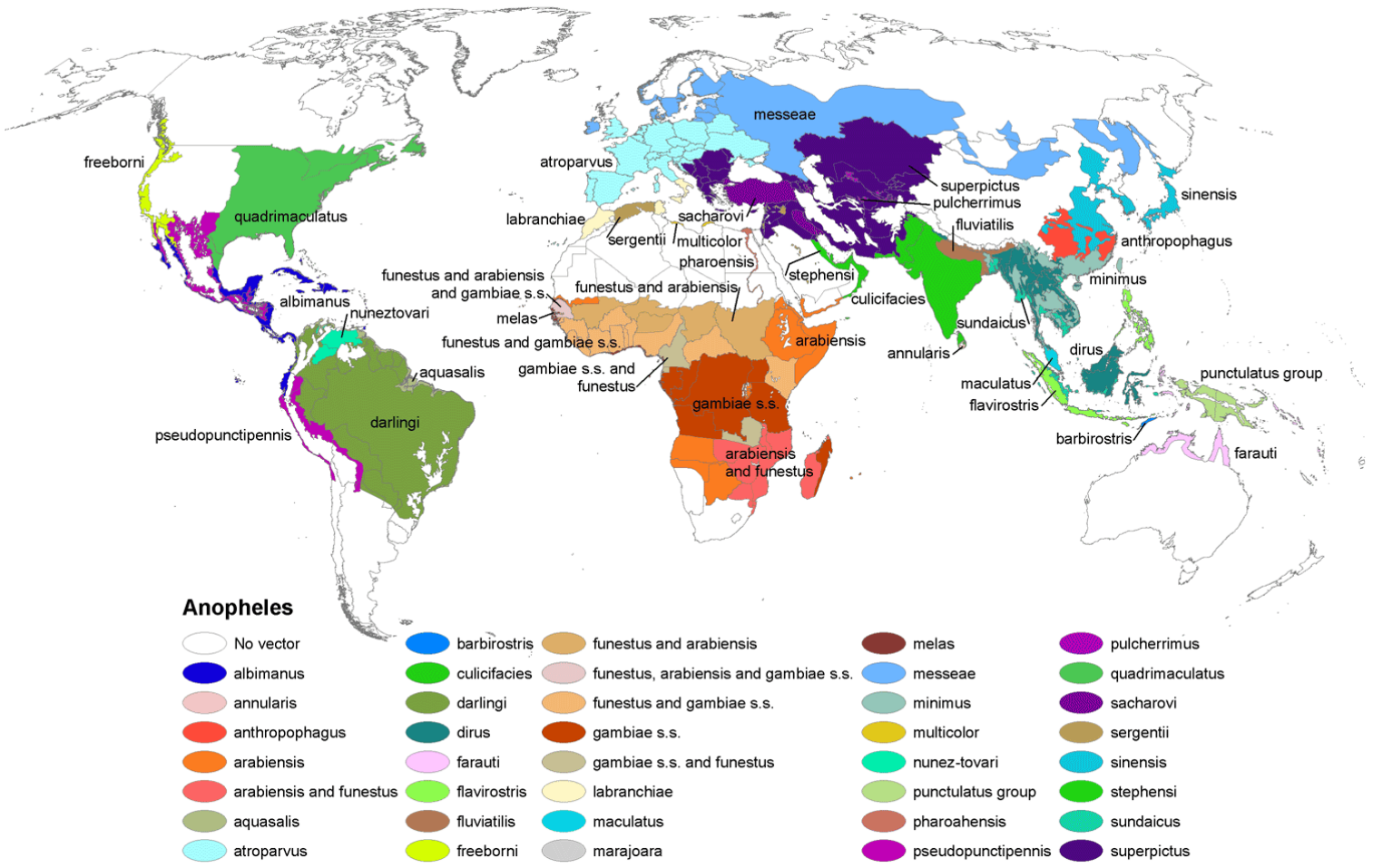


Figure 1.2 Map of the world showing the predominant malaria vector in each country (C.D.C., 2004).

1.3.1 The Search for Novel Insecticide Targets in Anopheles

The insect central nervous system (CNS) has been studied extensively as a target for insecticide compounds. As many putative CNS gene targets and compounds have already been investigated, alternative tissues are likely to become important pesticide targets. Current research in *Anopheles* is deciphering the complex pathways involved in processes such as immune function, oviposition and olfaction, and is likely to provide new tissues and genes which could be targeted to control mosquito populations.

Anopheles is a successful disease vector for several reasons; the female mosquito prefers human to animal blood, they take multiple blood-meals, and they are susceptible to parasite infection, which is not lethal to the insect. Much of the research on mosquitoes has been directed at understanding the mosquito immune response to parasite infection. These studies have found multiple levels of immune response, which are active from when the gametocytes enter the mosquito midgut, right through to when the sporozoites are transmitted back to a human, passing through multiple tissues on the way. The studies largely focus on the primary tissues that contact the parasite; the midgut, haemocoel and salivary glands (Levashina, 2004). The role of the mosquito Malpighian tubules in immune response is yet to be studied, but they are known to be an important immune tissue in *Drosophila melanogaster* (McGettigan *et al.*, 2005).

Field studies to determine the favoured feeding, mating and oviposition conditions for the many different species of *Anopheles* mosquitoes have been performed on populations throughout the world (Fontenille *et al.*, 2005). These studies aim to increase understanding of the inter- and intra-species differences in life-style and ability to transmit the malarial parasites. As it is unlikely that a single solution will wipe-out mosquito-borne disease in all areas in which they are endemic, a detailed understanding of the colonies local to each area is essential.

A growing area of interest in mosquito research is the sensory cells of the olfactory organs (Sutcliffe, 1994). The olfactory organs are likely to be involved in haematophagous host-seeking, mate-seeking for copulation, and locating a suitable site for oviposition. These are processes which affect the size of

mosquito populations and rate of malarial transmission, and could be used to influence mosquito behaviour if they were understood. These are just some of the areas of *Anopheles* research which are interesting and relevant to mosquito population control. This work focuses on the potential of an alternative tissue to the CNS for insecticide targeting, the Malpighian tubules.

1.3.2 Genetics

1.3.2.1 Spontaneous Mutations

Anopheles does not have the extensive array of stocks of spontaneous mutations which have been found and characterised in other species. The large number of *Anopheles* species and difficult nature of maintaining stocks means that chance mutations are rarely identified and studied. Mosquito stocks are expensive to maintain, and colonies will often reduce in number without explanation, resulting in stalled experiments and occasionally, loss of the entire colony. It is also difficult to obtain new mosquito stocks from labs which are not nearby or in the same country, as their high-maintenance life-cycle and potential to spread disease results in difficulty transporting them. The *Anopheles* mutant lines which have been characterized are mainly for obvious phenotypic traits, such as the white-eyed phenotype caused by the *white* eye-colour gene (Besansky *et al.*, 1995).

1.3.2.2 Directed Mutations

The motivating factors for the development of transgenics in *Anopheles* are different from the motivating factors in *Drosophila*. In *Drosophila* the techniques which support RNA inferference (RNAi) and gene over-expression have been developed to aid research into gene function. In mosquitoes the emphasis for improvement in transgenics is to use them as a tool to decrease the impact of mosquitoes as disease vectors. Gene expression systems are part of a strategy used to either decrease the ability of the malarial parasite to survive in the mosquito, or reduce the number of mosquitoes in the wild which are able to transmit the parasite. This has resulted in a range of transgenics which are more suitable for vector control than for deciphering gene function. The difficulty involved in creating transgenic mosquitoes is dependent on the species of

mosquito. Gene manipulation in *Aedes aegypti* and *Anopheles stephensi* is easier than in *Anopheles gambiae*, in which the use of transgenics is not yet prevalent (Lombardo *et al.*, 2005; Terenius *et al.*, 2008).

The stable transformation of several mosquito species with transposable elements such as Hermes and Minos has been possible for over a decade (Jasinskiene *et al.*, 1998; Catteruccia *et al.*, 2000). The method most often used to create transgenic mosquitoes is to inject embryos with a modified class II transposon plasmid containing the desired DNA sequence coupled to a phenotypic marker gene, usually coding for fluorescent eyes (Terenius *et al.*, 2008). Transgenic systems have been used to express proteins which interfere with parasite survival, and RNAi sequences which target specific disease viruses. Transgenic promoter sequences for specific life-stages and tissues have also been developed, to allow a targeted approach to peptide or RNAi expression (Lombardo *et al.*, 2005). As the construction of transgenic lines is still not routine in *Anopheles* the majority of RNAi studies are performed by injecting double-stranded mRNA into the mosquito, rather than expression of an RNAi construct inserted into the genome (Garver and Dimopoulos, 2007). Gamma radiation can also be used to cause random allelic mutations (Besansky *et al.*, 1995).

1.3.3 As an Experimental Organism

Laboratory colonies of *Anopheles* are difficult to rear, requiring daily maintenance and weekly blood-feeds, as well as carefully controlled heat and humidity. *Anopheles* is susceptible to the human malarial parasites, as well as the mouse malarial parasite *Plasmodium berghei*. Like most insects, the organs of *Anopheles*, such as the Malpighian tubules, gut and brain, are large enough for dissection and physiological experimentation. The *Anopheles gambiae* genome has been sequenced and published (Holt *et al.*, 2002), increasing the ease of gene and homology studies.

It is difficult to differentiate between the hundreds of strains of *Anopheles* which have been identified. Studies show that their susceptibility to the malarial parasite varies, as do their preferred feeding habits and ecological niches, with little understanding of the genetic and physiological reasons behind the

differences. Lab-based mosquito studies often use *Anopheles*, *Aedes aegypti* or *Culex pipiens*; field-based studies use whichever strains are native to the study area. *Anopheles* has been limited as a research organism by the difficulty of creating transgenics and lack of catalogued naturally occurring mutations. Although transgenic techniques are improving, the majority of immune studies have made use of injected dsRNA to knockdown gene expression, combined with tools such as GFP-tagged *Plasmodium* to track parasite survival. Field studies tend to examine behavioural traits such as the mosquito's feeding habits and oviposition preferences, to aid understanding of the mosquito life-style.

The sequencing of the *Anopheles* and *Aedes* genomes (Holt *et al.*, 2002; Nene *et al.*, 2007), followed by the development of an *Anopheles* microarray GeneChip (Affymetrix), has made new types of study possible. Microarrays provide a reliable perspective of how the mosquito transcriptome adapts to cope with challenges at both the tissue and whole organism level (See Chapter 3). The sequencing of the genomes has also resulted in greater proteomics capability, as demonstrated by Beyenbach *et al*, who utilised 2D gel-analysis and mass spectrometry to decipher the pathways involved in kinin-stimulated secretion from the *Aedes* Malpighian tubules (Beyenbach *et al.*, 2009).

1.3.4 Models for Anopheles

As previously discussed, *Anopheles gambiae* is not an easy organism to study. It is therefore important to utilise other species to infer function and to perform studies which are not yet possible in *Anopheles*. The ideal model for *Anopheles* would be another species of haematophagous mosquito, preferably one which is able to transmit malaria, but which has reliable transgenics and a thoroughly annotated genome. One such species, *Anopheles stephensi,* is primarily responsible for the spread of malaria in Asia, and can be stably transformed with transgenic vectors such as *pMinEGFP* (Catteruccia *et al.*, 2000). Unfortunately, *Anopheles stephensi* does not yet have an annotated genome, which makes homologous gene studies more difficult. Another obvious choice for multi-species studies is *Aedes aegypti*, which is susceptible to the avian malarial parasite *Plasmodium gallinaceum* (James, 2002), although *Aedes* is primarily important as a viral vector. *Aedes* has a fully sequenced genome (Nene *et al.*, 2007) and transgenic capability (Jasinskiene *et al.*, 1998), although vector transformation

is not yet routine in many laboratories. Despite the fact that *Aedes* has a much larger genome than *Anopheles* they have a similar number of genes (Waterhouse, 2008), and a commercially available microarray for *Aedes* would provide a powerful comparative tool. As transgenic capability improves it is likely that species such as *Aedes aegypti* will become model organisms for other mosquitoes, providing a reliable system in which to perform life-style, host-seeking and haematophagy studies, which may not be possible in other Dipterans. Although haematophagous Dipterans such as tsetse and midges could be useful as model systems, their transgenic and genomic resources have not yet progressed as far as the mosquitoes and fruit-fly. Organisms such as tsetse can also be difficult to culture, with long generation times and low reproductive rates, traits which are not desirable in a model organism (Krafsur, 2009). The other obvious system in which to study Dipterans is the fruit-fly, *Drosophila melanogaster*. The main disparity between *Drosophila* and *Anopheles* is that *Drosophila* is not haematophagous at any stage in its life-cycle. Unquestionably, there are important differences between fruit-fly and mosquito, and *Drosophila* may not be an appropriate model for many of the host-seeking and blood-feeding processes which are vital in *Anopheles*. *Drosophila* has the most thoroughly annotated genome of the Dipterans (Adams *et al.*, 2000), as well as an extensive range of transgenic and mutant fly stocks, and therefore could be used to study cellular processes which are common to both mosquito and fly.

By performing insecticide studies in multiple Dipterans it may be possible to tailor an insecticide to be as narrow or broad-ranging as required. By choosing targets which are highly homologous in haematophagous insects, but with low homology in non-haematophagous insects, it may be possible to target specific species without affecting the Dipterans which are beneficial to an ecosystem. Conversely, the opposite strategy could be used to produce wide-acting insecticides to control a range of Dipteran species.

1.4 Drosophila melanogaster

Drosophila has been used to study genetics since the early twentieth century. Fruit-flies are small, inexpensive, easy to maintain and breed, and produce hundreds of offspring in a short space of time, making them a useful model organism. The publication of the complete sequence of the *Drosophila* genome

(Adams *et al.*, 2000) has made *Drosophila* into a comprehensive model organism, with a well annotated genome, easily manipulated transgenics and a good pedigree for physiological studies. Fruit-flies are genetically very pliable; gene manipulation has become routine, and spontaneous and directed mutant lines are available from stock centres such as the Drosophila Genome Research Centre (DGRC). RNAi lines are available from stock centres such as the Vienna Drosophila RNAi Centre (VDRC) and the National Institute of Genetics (NIG-FLY) for a minimal fee.

1.4.1 Drosophila Physiology

Early *Drosophila* experiments utilised the phenotypic mutants found naturally in laboratory stocks to form theories of hereditary and control of development. In 1933 Thomas Hunt Morgan was awarded the Nobel Prize for his work on sex-linked gene transmission in *Drosophila*. In 1995 the Noble Prize in Medicine was awarded to Drs. Edward Lewis, Christiane Nuesslein-Volhard, and Eric Wieschaus, for fifty years of combined work on deciphering the genetic controls of development in *Drosophila*. Many of the genes they described have been found to have a good homologue in man, with an essential function in development. Genetic and developmental studies are still at the forefront of *Drosophila* research, and the scope for physiological studies has also become apparent. Current literature shows that *Drosophila* is being studied in diverse areas such as neuroscience (Crocker and Sehgal, 2008), immunity (Davies and Dow, 2008), sex determination and development (Telonis-Scott *et al.*, 2008), circadian cycle (Sofola *et al.*, 2008), insecticide resistance (Yang *et al.*, 2007) and renal function (Day *et al.*, 2008).

In recent years a reverse genetics approach has been used to assign function to genes and proteins. This involves mutating a gene, and assigning function from the resulting phenotype. Reverse genetics is being used to close the phenotype gap (Brown and Peters, 1996) – the discrepancy in knowledge between phenotypes, and the genes and proteins which are causing them. Gene function studies are feasible in *Drosophila* due to the transgenic systems which have been developed. Studies can be performed at a cellular, tissue, organ or whole organism level, an *in vivo* approach which would be performed in cultured cells for many other organisms.

1.4.2 Drosophila Genetics

Drosophila researchers worldwide have a philosophy of making their resources available to other research groups. Fly-lines and antibodies which have been published are usually available on request from the group which generated them. Many spontaneously mutated *Drosophila* lines have been isolated, and are maintained in stock centres around the world. A specialized set of tools have also been developed for the genetic manipulation of *Drosophila*, and are available for academic research.

1.4.2.1 Spontaneous Mutation

The availability of fly-lines containing spontaneous mutations was fundamental in attracting researchers to *Drosophila*. Aberrations in easily identifiable characteristics such as eye colour and mating behaviour helped to elucidate pathways and processes long before the fruit-fly genome was sequenced and annotated (Cline, 1978; Summers, 1982). Spontaneous mutations normally arise through chance, and can take the form of chromosome insertions, deletions or point mutations.

1.4.2.2 Directed Mutation

It is possible to perform both forward and reverse genetics using *Drosophila*. Gene mutation is easier than in other insects and mammals due to a range of naturally occurring genomic features, such as P-elements, which have been incorporated into an array of transgenic tools. Forward genetics relies on methods such as the Flp/FRT system (St Johnston, 2002) and ethyl methane sulphonate (EMS) to produce mutant fly-lines. Flies displaying a phenotype of interest can be identified, and the gene responsible for the phenotype located by determining the site of the mutation.

Since the sequencing of the *Drosophila* genome, the more popular approach is to utilise reverse genetics to determine gene function. Reverse genetic analysis is performed by altering the expression of a specific gene, and then assaying for a resultant phenotype. Reverse genetic analysis often utilises transgenic systems

developed from naturally occurring P-elements found in *Drosophila* (Sentry and Kaiser, 1992).

1.4.2.3 P-elements

Naturally occurring P-elements are short sequences of DNA which can relocate in the genome, disrupting the base sequence both where they jump out and where they reinsert. Their short DNA sequence contains a transposase gene which encodes the protein required to relocate the transposon, as well as repeat sequences on which the transposase acts. Transposition can be advantageous, inconsequential or lethal to the fly, and is usually prevented by repressor factors produced by the female (Lemaitre *et al.*, 1993).

Transgenic P-element constructs are derived from naturally occurring transposons and can mutate genes through insertional inactivation. They often contain additional DNA which alters the expression of a specific gene, by increasing or decreasing the amount of transcript (Ryder and Russell, 2003). P-element constructs can also contain markers such as *white*, which alter an easily identifiable physical trait, allowing identification of flies which have the construct inserted into their genome. Constructs are micro-injected into syncitial blastoderms where they are randomly incorporated into the genomic DNA of the embryo. If a P-element is incorporated into future somatic tissues, it can be passed on to the following generation and its presence tracked by the physical marker.

P-element constructs can also be adapted for use in enhancer trap gene studies, which aid the identification of genes with interesting or novel expression patterns (Rubin and Spradling, 1982; Bellen *et al.*, 1989). This method uses a P-element in which the transposase gene has been replaced with a reporter gene downstream of a weak promoter. When the P-element inserts into the genome near a genomic enhancer the introduced DNA takes on the expression pattern of a gene downstream of the enhancer. The reporter gene is often GAL4 (see section 1.4.2.4) or *LacZ* (Brand and Perrimon, 1993), both of which are used to visualise the expression pattern of the enhancer and the gene which it controls (Yang *et al.*, 1995).

1.4.2.4 The UAS/GAL4 system

P-elements are frequently used in conjunction with the UAS/GAL4 binary system (Brand and Perrimon, 1993). GAL4 is a yeast transcriptional activator, which activates transcription downstream of the yeast promoter Upstream Activating Sequence (UAS). GAL4 does not activate *Drosophila* promoter sequences, but is capable of driving transgene expression under the control of a UAS promoter. By cloning a transgene into a P-element construct downstream of a UAS promoter, it is possible to control transgene expression using a GAL4 driver fly-line (see Figure 1.3).

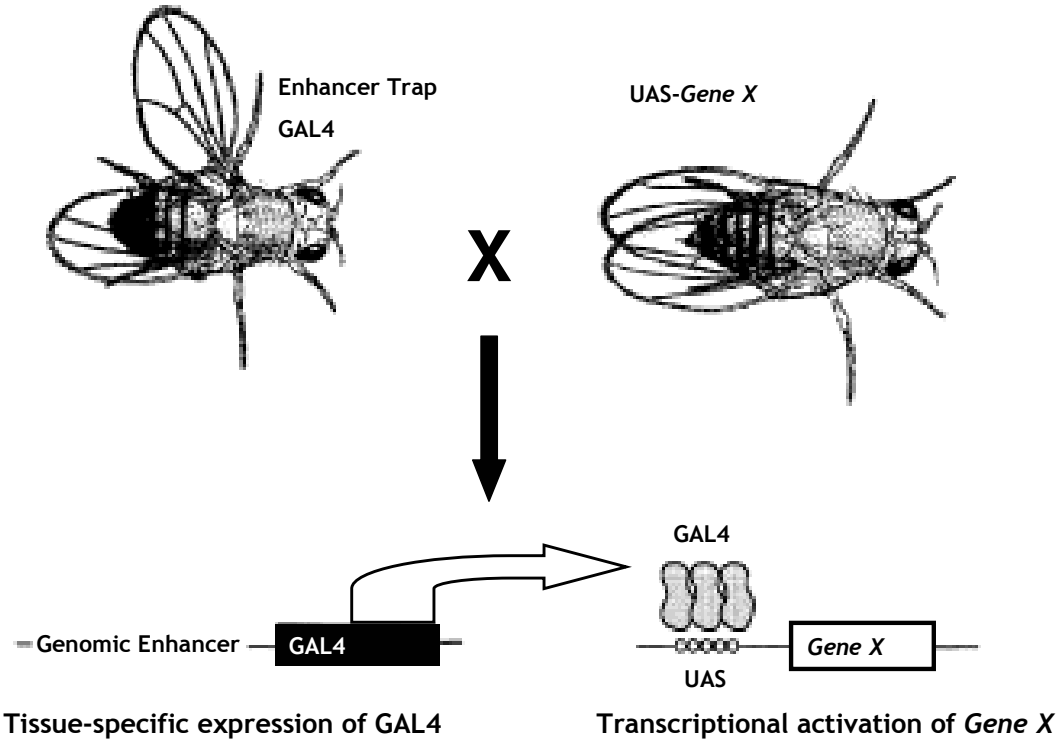


Figure 1.3 The UAS/GAL4 *Drosophila* expression system. Flies expressing GAL4 in a tissue-specific pattern are crossed with flies containing a UAS-transgene. The UAS-transgene is therefore only expressed in the chosen cell types (Brand and Perrimon, 1993).

The first GAL4 'driver' line was developed by Brand and Perrimon as an enhancer trap GAL4 construct (pGAWB) (Brand and Perrimon, 1993). In the last 15 years an impressive array of GAL4-driver lines has been constructed by modifying pGAWB. It is now possible to drive transgene expression in anything from a subset of neural cells to a whole organ, depending on your tissue of interest. There are many advantages to maintaining GAL4 lines with specific temporal and spatial

expression patterns. Driver lines can be maintained as a stable stock, and used to drive expression in any fly-line containing a transgene with a UAS promoter, reducing the complexity of cloning when making a new fly-line. Many UAS-transgenes are lethal or debilitating when expressed in *Drosophila*, and would be difficult or impossible to maintain if both the GAL4-driver and UAS-transgene elements were inserted in the same line. Maintaining them in separate parental fly-lines means they are much more likely to be viable. It also allows the experimenter to change the UAS-transgene expression pattern by crossing to different GAL4 parental lines, which is much easier than making and microinjecting a new construct with a different driver.

The UAS/GAL4 system can be used to express full gene sequences for protein over-expression, with or without additional tags. It can also be used for gene knockdown, through the action of a specific double-stranded RNA (dsRNA). RNAi lines can be made by inserting a double stranded hairpin loop sequence into a P-element, so that it expresses a dsRNA when driven (Kennerdell and Carthew, 2000). Dicer recognizes the dsRNA and breaks it down, along with any other RNA which contains the same sequence as the dsRNA. This decreases the amount of that particular transcript, effectively suppressing expression of that gene. The construct utilized in this study is the pWIZ construct (Lee and Carthew, 2003), which is species specific to *Drosophila* (See section 2.7.6). The transgene is constructed by inserting approximately 500-600 bases of specific gene sequence either side of an intron (*white*) in opposing directions. When expression of the construct is driven using a GAL4 driver, a dsRNA hairpin loop is transcribed which interferes with gene expression. Recent advances have resulted in new constructs such as pRISE (Kondo *et al.*, 2006), which, in theory, reduce the time it takes to make an RNAi construct from weeks to days.

1.5 Renal transport

Renal tissues are vital for maintaining homeostasis and removing potentially toxic compounds in both insects and mammals. Osmoregulation is performed by the kidneys in mammals and the Malpighian tubules in insects. The function and morphology of the Malpighian tubules have been studied extensively, and more than one thousand research papers focusing on the insect Malpighian tubules have been published. Studies have been performed on a large range of insects,

notably the stick insect *Dixippus morosus*, the blood-sucking bug *Rhodnius prolixus*, the tobacco hornworm *Manduca sexta*, the yellow fever mosquito *Aedes aegypti*, the desert locust *Schistocerca gregaria*, the house cricket *Acheta domesticus*, the red wood ant *Formica polycenta*, and the fruit-fly, *Drosophila melanogaster*. The Malpighian tubules are essential for the regulation of water, metabolites, ions and other substrates via membrane proteins such as active transporters and diffusion channels. In the vertebrate kidney these functions are carried out via ultra-filtration of a high blood pressure system. As insects operate at low blood pressure, ultra-filtration is not possible, and so ion transport is controlled by the action of hormonal peptides on epithelial tissues such as the tubules (Phillips, 1981; Coast, 1995). The tubules are also involved in the breakdown and excretion of toxins, such as insecticides (Yang *et al.*, 2007) and heavy metals (Yepiskoposyan *et al.*, 2006). Microarray studies show genes involved in detoxification such as the cytochrome P450s and glutathione-S-transferases are highly up-regulated in the tubules (Wang *et al.*, 2004). As well as osmoregulation and detoxification, the insect tubules have some unexpected roles, for example as an immune tissue. Insects employ an innate immune system (Hoffmann, 2003) to fight off microbial infection, and recent studies show that the tubule is capable of both sensing and fighting microbial infection independently of other immune tissues (McGettigan *et al.*, 2005).

The Malpighian tubules contain many transporters, channels and pores which are likely to be essential for insect survival. As the tubules are surrounded by the haemolymph, they are likely to contact compounds which are absorbed topically or ingested. They are therefore an ideal tissue for targeting insects with novel pesticide compounds.

1.6 The *Drosophila* Malpighian tubules

As with many other insects, the main excretory tissue in *Drosophila* is the Malpighian tubules, which perform a renal role analogous to the human kidney, alongside the hindgut, rectum and midgut. The tubules transport excess fluid and solutes from the haemolymph, and secrete them into the hindgut for excretion. They are a consummate model for studying transport in a live polarised epithelial tissue (Dow *et al.*, 1994; Dow and Davies, 2003), and have helped elucidate biological functions at both tissue and single cell level.

1.6.1 Morphology

The *Drosophila* tubules are simple, tubular, blind ended epithelia joined in pairs by a short common ureter to the alimentary canal, which float freely in the haemocoel (Wessing, 1978). Each fly possesses an anterior and posterior pair of tubules, which contribute equally to tubule function (O'Donnell and Maddrell, 1995). At ~2 mm in length, ~35 µm in diameter, and compromising approximately 150 cells, the *Drosophila* tubules are one of the smallest organs to be studied (Cabrero *et al.*, 2004).

The anterior and posterior tubules can be split morphologically into three segments; the enlarged initial segment, the transitional segment and the main segment, which joins to the common ureter. The anterior initial and transitional segments are larger and contain more cells than the equivalent segments in the posterior tubules (Figure 1.4). There is no obvious morphological difference between the male and female tubules.

The main segment of the tubules is composed of two cell types; the columnar epithelial principal cells and the star-shaped stellate cells. The principal cells have deep basal infoldings and long apical microvilli, and are more abundant than the stellate cells (Cabrero *et al.*, 2004). The stellate cells are comparatively small and thin, with shallow basal infoldings and short apical microvilli (Wessing, 1978). Enhancer trapping has been used to investigate the morphology of the tubule with great success. As well as identifying a distinct 'lower' segment in both the anterior and posterior tubules, Sozen *et al* discovered bar-shaped cells and tiny cells in the tubule (Sozen *et al.*, 1997). The bar-shaped cells are thought to be the equivalent of stellate cells in the initial segment, and the tiny cells may be stem cells, or neuroendocrine in function (Sozen *et al.*, 1997; Singh *et al.*, 2007).

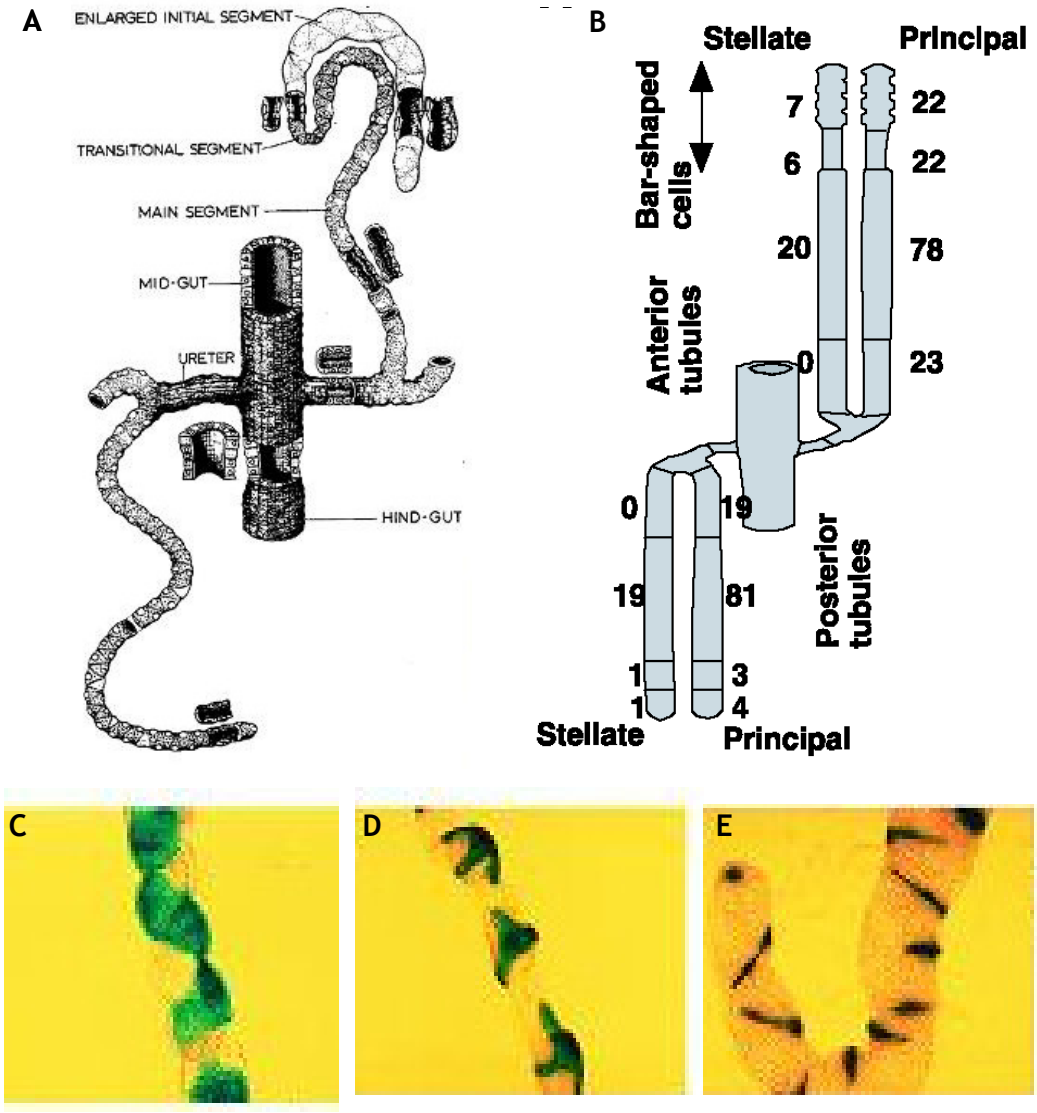


Figure 1.4 *Drosophila* Malpighian tubule morphology. A: classical morphology (Wessing, 1978). B: detailed morphology and cell counts. C: stained principal cells, D: stained stellate cells, and E: stained bar-shaped cells in the initial segment, from enhancer trap investigations (Sozen *et al.*, 1997).

1.6.2 Physiology

The Malpighian tubules perform a multitude of physiological roles in *Drosophila*, from the expected renal and hepatic roles, to a surprising role in immune response (McGettigan *et al.*, 2005). Osmoregulation and ion homeostasis are arguably the housekeeping functions of the tubules, as they are performed continually throughout the life-cycle of the fly.

Active cation transport by the tubules is described by the Wieczorek model (Wieczorek *et al.*, 1991; Wieczorek, 1999; 2000). The model suggests that the principal cells use the apical proton-pumping activity of the Vacuolar ATPase (V-ATPase) to build a favourable transport gradient. Apical alkali metal-proton exchangers are then able to drive potassium into the tubule lumen, with water following the potassium due to the osmotic gradient. The Wieczorek model seems to fit the tubule experimental data in *Drosophila*, as V-ATPase inhibitors abolish fluid secretion (Dow *et al.*, 1994), and the V-ATPase subunits are highly enriched and expressed in the principal cells (Wang *et al.*, 2004; Allan *et al.*, 2005). The principal cells are also involved in the active transport of cations and anions (O'Donnell *et al.*, 1996; Torrie *et al.*, 2004).

The stellate cells appear to be the site of transcellular chloride shunt and therefore water movement (Dow and Davies, 2003). Chloride shunt is controlled by the hormone Drosokinin and the second messenger intracellular Ca^{2+}, which increase transcellular conductance through chloride channels (O'Donnell *et al.*, 1998). Other hormones involved in osmoregulation include serotonin, corticotrophin-releasing factor (CRF) and calcitonin (Coast, 2007), which activate second messengers such as nitric oxide (NO), guanosine 3',5'-cyclic monophosphate (cGMP), 3'-5'-cyclic adenosine monophosphate (cAMP) and intracellular Ca^{2+}. (Davies *et al.*, 1995; Broderick *et al.*, 2003; MacPherson *et al.*, 2004; Pollock *et al.*, 2004)

The function of the bar-shaped cells in the initial and transitional segments is not well understood, although they may be a functionally distinct set of stellate cells. The tiny cells found in the ureter are potentially neural (Sozen *et al.*, 1997), or multipotent stem cells (Singh *et al.*, 2007). Despite great headway being made in understanding Malpighian tubule function, there are many classes of gene which are highly up-regulated in the transcriptome but remain uncharacterised (see Section 1.9).

1.7 The *Anopheles* Malpighian tubules

The Malpighian tubules are the primary renal tissue in *Anopheles* throughout the life-cycle. The tubules secrete excess fluid and solutes into the hindgut for excretion, altering the composition of the urine in response to the environment

and life-stage of the mosquito. The morphology and physiology of the *Anopheles* tubules are not yet as well understood as the *Drosophila* tubules, although the closely-related *Aedes aegypti* tubules have been studied in great detail.

1.7.1 Morphology

The *Anopheles* Malpighian tubules take the form of simple, tubular, blind-ended epithelia which are joined to the alimentary canal and float freely in the haemocoel. In this aspect they are similar to the *Drosophila* tubules. They differ in the respect that there are five tubules instead of four. The five tubules do not appear to join at a common ureter, although they attach to the alimentary canal at the same junction, where the midgut meets the hindgut. The five tubules are not equally sized; two of the tubules are ~5 mm in length, the other three tubules are ~3 mm in length (Cabrero *et al.*, 2004). As with *Aedes aegypti* (Plawner, 1991), the female tubules are much larger than the male tubules, most likely to cope with the massive natriuresis and diuresis which follows a blood-meal (Williams, 1983; Wheelock, 1988).

The segmentation of the *Anopheles* tubules is not as well understood as in *Drosophila*. Staining for alkaline phosphatase activity delineates the lower 10% of the tubule as it does in *Drosophila* (Cabrero *et al.*, 2004). The visually distinctive enlarged initial and transitional segments which can be seen in the *Drosophila* anterior tubule are not obvious in *Anopheles*, although it seems likely that if they exist functionally, they would be found in the two lengthier tubules. As in the other Dipterans, the *Anopheles* tubules are composed of both principal and stellate cells (Cabrero *et al.*, 2004).

1.7.2 Physiology

Malpighian tubule function has been investigated in both *Aedes* and *Anopheles*. Although headway is being made, the depth of knowledge does not yet equal that of the *Drosophila* tubules, probably due to the difficulty of mosquito transgenics. The experimental data collected so far suggests that the renal function of *Anopheles* tubules is likely to rely on the same types of transporters and channels other Dipterans, such as V-ATPases, Na^+/K^+-ATPases, $Na^+/K^+/2Cl^-$ co-transporters, aquaporins and sodium/proton exchangers (NHEs) (Pullikuth *et*

al., 2003). At two stages in the mosquito life-cycle the tubules have to quickly adjust their purpose. The first challenge is the transition from aquatic larvae/pupae to terrestrial adult. Freshwater mosquito larvae rely on the tubules and anal papillae (Bradley, 1987) to maintain haemolymph volume and concentration in a highly hypo-osmotic environment. After eclosion the salivary gland, midgut and hindgut also become important, as the focus switches quickly from water excretion to conservation. The second major stress which affects the tubules is the female taking on a blood-meal which is more than twice the mass of her unfed body (Roitberg, 2003). The mosquito takes on the nutrients, vitamins, minerals and electrolytes required for egg maturation, but she also acquires unwanted salts and water which must be rapidly transported from the haemolymph and excreted (Petzel *et al.*, 1987). The tubules and renal organs must quickly alter function, from conserving water and vital ions to producing copious amounts of Na^+ rich urine. The speed of this switch is reflected by the fact that the mosquito begins natriuresis while she is still feeding.

Both of these changes in tubule purpose are likely to be stimulated by peptide hormones released by the brain. Numerous peptide hormones which activate diuresis have been identified, such as the calcitonin-like Mosquito Natriuretic Peptide (MNP) (Petzel *et al.*, 1987; Coast *et al.*, 2005) and the *Anopheles* leucokinin peptides and receptor (Radford *et al.*, 2004).

1.8 Drosophila as a Model for *Anopheles*

The applications of *Drosophila* as a model organism are wide and varied, and recent studies include modelling human diseases (Chang *et al.*, 2008), exploring life-span and ageing (Broughton *et al.*, 2008), and deciphering the neural networks involved in olfactory memory (Keene and Waddell, 2007). For studies of Dipteran insects *Drosophila* is a useful model organism, due to its extensively studied development and ease of transgenics.

1.8.1 As a Physiological Model

There are certain behavioural aspects of *Anopheles* for which *Drosophila* is unlikely to be an appropriate model. Host-seeking for blood-feeding and haematophagous behaviour itself are processes without parallel in *Drosophila,*

although many other physiological processes correlate well. *Drosophila* was of great importance in developing transgenic techniques for use in mosquitoes. The marker used in the first transposable element-mediated germline transformation of a mosquito was the *Drosophila* gene *cinnabar* (Cornel *et al.*, 1997; Jasinskiene *et al.*, 1998). The promoters used in early mosquito transgenics also originated from *Drosophila*, and were not only successful, but retained the tissue specific expression patterns which were established in *Drosophila (*Atkinson and Michel, *2002)*.

The feasibility of performing high-throughput RNAi screens in *Drosophila* also makes it an attractive model for *Anopheles*. Yapici *et al* used comprehensive *Drosophila* RNAi screens to identify the *sex peptide receptor* (*SPR*) which signals for post-mating behaviour to commence in female Diptera (Yapici *et al.*, 2008). Although *Drosophila* sex peptide did not activate the homologous *Anopheles SPR*, the close conservation between species suggests a similar role is likely.

Drosophila has also been used with great success as an immune model for *Anopheles*. In their recent paper, Brandt *et al* used *Drosophila* as a model to identify factors required for the growth of the avian malarial parasite *Plasmodium gallinaceum* (Brandt *et al.,* 2008). The screen found 18 genes not previously known to interact with *Plasmodium*. Five genes were knocked-down in *Anopheles* infected with *Plasmodium berghei*, and four of these genes were found to effect *Plasmodium* growth. Comparison of the *Anopheles* and *Drosophila* genomes has also shown extensive similarities between immune signalling genes, such as in the Toll pathway (Christophides *et al.*, 2002).

These are just a few of the studies in which *Drosophila* has been used as a model for *Anopheles*, but they demonstrate the wide range of ways in which the fruit-fly can be utilised.

1.8.2 As a Malpighian Tubule Model

The range of molecular tools available to *Drosophila* biologists makes *Drosophila* a valuable model for investigating Dipteran Malpighian tubule function, and to date the majority of studies performed dually in *Drosophila* and mosquito have shown a high level of similarity between the two renal systems. In a study comparing the Malpighian tubules across insects, Cabrero *et al* showed

conservation of alkaline phosphatase expression in the lower 10% of the tubules in the four Dipterans assayed (Cabrero *et al.*, 2004). In *Drosophila* alkaline phosphatase expression delineates the absorptive region of the tubule, and the same expression pattern can be seen in the lower 10% of *Anopheles stephensii*, *Aedes aegypti* and *Glossina mortisans* tubules, but not in the Orthopteran *Schistocerca gregaria* (Cabrero *et al.*, 2004). Radford *et al* also demonstrated that leucokinin signalling is closely preserved between *Drosophila* and *Anopheles* (Radford *et al.*, 2004*)*. Sequence similarity allowed the identification of *Anopheles* leucokinin I-III and the *Anopheles* leucokinin receptor, which is conserved enough to be activated by *Drosophila* leucokinin. They also determined that in *Anopheles*, like *Drosophila*, the leucokinin receptor is localised to the stellate cells. In their 2004 paper on nitric oxide (NO) signalling in Diptera, Pollock *et al* showed that *Drosophila*, *Anopheles stephensi* and *Aedes aegypti* all express nitric oxide synthase (NOS) in the principal cells of the tubules. They determined that capa peptide stimulation of NO and guanosine 3', 5'-cyclic monophosphate (cGMP) signalling increases fluid secretion from the tubules across Diptera, but not in Orthoptera (Pollock *et al.*, 2004). These studies all suggest that the main functions of the tubules such as osmoregulation, and the physiological processes which control them, have been conserved in Diptera. The most significant difference identified between fly and mosquito tubules is in the secretion of chloride. Chloride secretion is via the stellate cells in the *Drosophila* Malpighian tubules (O'Donnell *et al.*, 1996). Under resting conditions the route of chloride secretion in the *Aedes aegypti* tubules also appears to be via the stellate cells (O'Connor and Beyenbach, 2001). This changes after kinin stimulation, when the route of secretion in *Aedes* becomes extracellular, between the epithelial cells (Pannabecker *et al.*, 1993; Yu and Beyenbach, 2001; Beyenbach, 2003; Yu and Beyenbach, 2004). It is not yet known whether the basal or stimulated route of chloride secretion in the *Anopheles* Malpighian tubules differs from that of the *Drosophila* tubules.

1.9 Microarray Analysis of *Anopheles* and *Drosophila*

Microarrays allow a snapshot, or as many snapshots as required, of the transcriptome of an organism, tissue or cell-type under specific conditions of interest. When designed from a genome which is complete and well annotated microarrays can provide a wealth of information, from time-series results, to

gene expression changes after a stimulus. The *Drosophila* Malpighian tubules have been the subject of some particularly powerful studies, and the combined *Anopheles*/*Plasmodium* Affymetrix chip is also becoming the industry standard for *Anopheles* gene expression experiments.

1.9.1 Microarrays in Drosophila

A microarray comparing the transcriptome of the Malpighian tubules of 7-day old *Drosophila* with matched whole adult *Drosophila* was performed by Wang *et al* (2004). Analysis of the microarray produced a list of 307 genes which were statistically enriched (at least twice the expression) in tubule in comparison to the average whole-fly signal. Of the ten genes with the largest expression signals in the tubule only five had a predicted function, suggesting the existence of physiological processes in the tubule which are yet to be characterised. If the data-set is viewed by transcript enrichment in the tubule, the list becomes dominated by organic and inorganic solute transporters. Almost every class of transporter is represented by at least one gene; broad-specificity transporters for organic cations, anions, monocarboxylates, amino acids and multivitamins, as well as more specific sugar, copper and zinc transporters (Wang *et al.*, 2004). Several of these categories, such as the zinc, monocarboxylate and sugar transporters have little-to-no published research concerning their function in fly.

With the advent of FlyAtlas (Chintapalli *et al.*, 2007), it is possible to check transcript expression of a *Drosophila* gene-of-interest in twenty tissues, including adult brain, Malpighian tubules, midgut, hindgut, salivary gland, ovary and testis, larval fat body and the larval Malpighian tubules. Although the microarray data has been collected at a specific time-point in the life-cycle and therefore is a snapshot of gene expression, it is an incredibly powerful snapshot. The data shows the specific transcriptome of each organ, hinting at the factors which they have in common, and those which make each tissue unique.

1.9.2 Microarrays in Anopheles

Microarray studies have become routine in *Anopheles* since the sequencing and annotation of the genome (Holt *et al.*, 2002). Their uses include tracking the changes in the transcriptome which coincide with the mosquito life-cycle,

determining the immune genes and pathways which are activated after plasmodium infection, and revealing the array of detoxification enzymes which are transcribed in response to insecticide challenge.

In 2006, Marinotti *et al* investigated sex-specific gene expression in whole adult mosquitoes, as well as the effect of haematophagous feeding on the transcriptome (Dissanayake *et al.*, 2006; Marinotti *et al.*, 2006). They determined that >3,000 genes have sex-specific or preferential expression, which corroborates the previous studies on the *Anopheles* transcriptome (Hahn and Lanzaro, 2005). Marinotti *et al* also found extensive variation in gene expression after a blood-meal, with 126 genes enriched >100-fold in whole-mosquito after haematophagy. The majority of genes which were enriched after a blood-meal showed increased expression of ~2-3-fold. The microarray data also determined that the transcriptome continues to vary for up to 96 h after a blood-meal, coinciding with the gonotrophic cycle (Marinotti *et al.*, 2006).

Recent publications have used microarrays to assess the transcriptome in compartments of the alimentary canal and midgut (Warr *et al.*, 2007; Neira Oviedo *et al.*, 2008), as well as insecticide resistance in specific populations of mosquitoes (Awolola *et al.*, 2008; Djouaka *et al.*, 2008). The microarray experiments which have been performed have provided an almost unanswerably large list of ideas and points of interest which require further investigation.

1.10 Aims of this Project

The *Anopheles* Malpighian tubules are relatively untouched in terms of physiology and function. The Malpighian tubule versus whole-mosquito microarray introduced in this thesis allows the opportunity to identify genes which are highly up-regulated in the tubules at different stages in the life-cycle. These genes are ideal candidates for developing new insecticides against if they also prove to be essential for mosquito survival. By choosing candidate genes which have a good homologue in *Drosophila* which is also up-regulated in the tubules, it is possible to do genetic and physiological manipulations which would be difficult in *Anopheles*. Choosing genes which are tubule enriched and specific in *Drosophila* and *Anopheles* favours genes which are conserved in Diptera and are therefore more likely to be essential. The aim of this project was thus to identify four genes which are highly enriched in the *Anopheles* tubules, with homologues which are enriched in the *Drosophila* tubules. The expression and importance of the genes both within the tubules and the whole organism was investigated, with a view to understanding whether they would make good insecticide gene targets. Whether *Drosophila* is an effective model for *Anopheles* is also discussed throughout this thesis.

Chapter 2 - Methods and Materials

This chapter describes the experimental protocols used during this thesis and summarises the rearing conditions for *Anopheles gambiae* and *Drosophila melanogaster*. Relevant references for methods used are listed where applicable.

2.1 Drosophila melanogaster

Drosophila melanogaster was the organism primarily studied during this investigation. Wild-type Oregon R and transgenically modified *Drosophila* were both used, and their origin and maintenance are described in this section.

2.1.1 Drosophila Stocks

Drosophila melanogaster was used for the majority of the experiments described in this thesis. Listed in Table 2.1 are the *Drosophila* lab-strains used in this study and their genotype and applications within the project. The UAS/GAL4 driver lines and Oregon R wild-type flies were stocks maintained within our lab. The w^{1118} fly-line was maintained by BestGene for microinjection of transgene constructs. Listed in Table 2.2 are the transgenic fly-lines created and used during this study.

Table 2.1 – *Drosophila* stocks used during this study.

Strain	Genotype	Purpose
Oregon R	*Wild type*	Genomic DNA, cDNA, protein, immunocytochemistry, *in situ* hybridisation, secretion assays, transport assays
w^{1118} (Hazelrigg, 1984)	w^{1118}; +/+; +/+	Microinjection
c42-GAL4 (Sozen *et al.*, 1997)	*w*; +/+; *c42-GAL4/c42-GAL4*	GAL4 crosses (expression in the main and lower segment principal cells and bar shaped cells)
Actin-GAL4 (Stergiopoulos et al., 2009)	*w*; *Act5c-GAL4/CyO*; +/+	GAL4 crosses (ubiquitous expression in *Drosophila*)
Uro-GAL4 (Terhzaz et al., 2010)	*Uro-GAL4*; +/+; +/+	GAL4 crosses (expression in the main segment principal cells)
c724-GAL4 (Sozen *et al.*, 1997)	*w*; *c724-GAL4/c724-GAL4*; +/+	GAL4 crosses (expression in the main segment stellate cells and bar-shaped cells)
ELAV-GAL4 (Bloomington Stock Centre)	*w*; +/+; $elav^{c155}$-*GAL4*/$elav^{c155}$-*GAL4*	GAL4 crosses (expression in the nervous system)

Table 2.2 – *Drosophila* transgenic fly-lines created and used during this study.

Fly-line	*Genotype*	Purpose
CG15406.c1.l1	*w; +/+; CG15406.c1.l1/TM3,sb*	Knocks down *CG15406* expression by RNAi
CG15406.c1.l4	*w; +/+; CG15406.c1.l4/TM3,sb*	Knocks down *CG15406* expression by RNAi
CG15406.c1.l9	*w; CG15406.c1.l9/CyO; +/+*	Knocks down *CG15406* expression by RNAi
CG15406.c2.l4	*w; +/+; CG15406.c2.l4/TM3,sb*	Knocks down *CG15406* expression by RNAi
CG15406.c2.l7	*w; CG15406.c2.l7/CyO; +/+*	Knocks down *CG15406* expression by RNAi
CG15406.c2.l8	*w; CG15406.c2.l8/CyO; +/+*	Knocks down *CG15406* expression by RNAi
CG15406.c3.l3	*w; CG15406.c3.l3/CyO; +/+*	Knocks down *CG15406* expression by RNAi
CG15406.c3.l4	*w; +/+; CG15406.c3.l4/TM3,sb*	Knocks down *CG15406* expression by RNAi
CG15406.c3.l5	*w; CG15406.c3.l5/CyO; +/+*	Knocks down *CG15406* expression by RNAi
Picot.c1.l3	*w; Picot.c1.l3/CyO; +/+*	Knocks down *Picot* expression by RNAi
Picot.c1.l5	*w; +/+; Picot.c1.l5/TM3,sb*	Knocks down *Picot* expression by RNAi
Picot.c1.l6	*w; +/+; Picot.c1.l6/TM3,sb*	Knocks down *Picot* expression by RNAi
Picot.c2.l3	*w; Picot.c2.l3/CyO; +/+*	Knocks down *Picot* expression by RNAi
Picot.c2.l5	*w; Picot.c2.l5/CyO; +/+*	Knocks down *Picot* expression by RNAi
Picot.c2.l6	*w; +/+; Picot.c2.l6/TM3,sb*	Knocks down *Picot* expression by RNAi
Picot.c3.l2	*w; +/+; Picot.c3.l2/TM3,sb*	Knocks down *Picot* expression by RNAi
Picot.c3.l3	*w; Picot.c3.l3/CyO; +/+*	Knocks down *Picot* expression by RNAi
Picot.c3.l5	*w; +/+; Picot.c3.l5/TM3,sb*	Knocks down *Picot* expression by RNAi
CG3994.c1.l1	*w; +/+; CG3994.c1.l1/TM3,sb*	Knocks down *CG3994* expression by RNAi
CG3994.c1.l3	*w; CG3994.c1.l3/CyO; +/+*	Knocks down *CG3994* expression by RNAi
CG3994.c1.l4	*w; CG3994.c1.l4/CyO; +/+*	Knocks down *CG3994* expression by RNAi
CG3994.c2.l1	*w; CG3994.c2.l1/CyO; +/+*	Knocks down *CG3994* expression by RNAi
CG3994.c2.l2	*w; CG3994.c2.l2/CyO; +/+*	Knocks down *CG3994* expression by RNAi
CG3994.c2.l8	*w; +/+; CG3994.c2.l8/TM3,sb*	Knocks down *CG3994* expression by RNAi
CG3994.c3.l1	*w; CG3994.c3.l1/CyO; +/+*	Knocks down *CG3994* expression by RNAi
CG3994.c3.l2	*w; CG3994.c3.l2/CyO; +/+*	Knocks down *CG3994* expression by RNAi
CG3994.c3.l4	*w; +/+; CG3994.c3.l4/TM3,sb*	Knocks down *CG3994* expression by RNAi
CG8028.c1.l1	*w; +/+; CG8028.c1.l1/TM3,sb*	Knocks down *CG8028* expression by RNAi
CG8028.c1.l3	*w; +/+; CG8028.c1.l3/TM3,sb*	Knocks down *CG8028* expression by RNAi
CG8028.c1.l4	*w; CG8028.c1.l4/CyO; +/+*	Knocks down *CG8028* expression by RNAi
CG8028.c2.l1	*w; CG8028.c2.l1/CyO; +/+*	Knocks down *CG8028* expression by RNAi
CG8028.c2.l2	*w; CG8028.c2.l2/CyO; +/+*	Knocks down *CG8028* expression by RNAi
CG8028.c2.l4	*w; +/+; CG8028.c2.l4/TM3,sb*	Knocks down *CG8028* expression by RNAi
CG8028.c3.l1	*w; +/+; CG8028.c3.l1/TM3,sb*	Knocks down *CG8028* expression by RNAi
CG8028.c3.l4	*w; CG8028.c3.l4/CyO; +/+*	Knocks down *CG8028* expression by RNAi
CG8028.c3.l6	*w; CG8028.c3.l6/CyO; +/+*	Knocks down *CG8028* expression by RNAi

2.1.2 Drosophila Rearing

Flies were maintained in vials on standard *Drosophila* medium (Section 4.1.9) at 20-22°C in a 12: 12, light: dark cycle. When large quantities of flies were required they were reared in large bottles under identical conditions. Adult flies were separated from eggs and pupae on a daily basis to ensure *Drosophila* were aged 7 days old for all experiments, unless otherwise stated.

2.2 Anopheles gambiae

Anopheles mosquitoes were used for microarray experiments, quantitative PCR, *in situ* hybridisations and Western blots experiments. They were kindly provided by Dr Lisa Ranford-Cartwright (Division of Infection and Immunity, University of Glasgow).

2.2.1 Anopheles stocks

Mosquitoes from an *Anopheles gambiae* G3 strain colony were used throughout this thesis. The colony was reared from eggs obtained from Imperial College, London, and maintained in the Department of Infection and Immunity, University of Glasgow.

2.2.2 Anopheles rearing

Anopheles mosquitoes were reared in an insectary room maintained at 28ºC and ~80% humidity with a 12 h day: night cycle. Adult mosquitoes were given access to 5% glucose in 0.05% para-aminobenzoic acid water, and larvae fed with ground-up TetraMin tropical fish food (Tetra, USA). Adult mosquitoes were offered a human blood-meal through an artificial membrane once or twice every week depending on stock requirements. Eggs and larvae were reared in water tanks separate from the adult mosquito cages. Pupae were collected on a daily basis, and placed in mosquito cages to hatch and to age to 3-5 days for experimental use. Mosquitoes were removed from the temperature and humidity controlled chamber on the morning of each experiment.

2.3 Affymetrix Microarrays

Two microarray data-sets provided the foundation for this work. The first microarray was published by Wang *et al*, and compares the transcriptome of whole *Drosophila* to the transcriptome of the Malpighian tubules (Wang *et al.*, 2004). The second is the corresponding *Anopheles* study, which compares the transcriptome of whole *Anopheles* to the transcriptome of the Malpighian tubules at different stages in the mosquito life-cycle (unpublished data).The methods used to prepare the samples and analyse the data-sets are described here.

2.3.1 Sample Preparation

Four biological samples, each containing ~2000 Malpighian tubules, were dissected from uninfected *Anopheles* larvae, adult males, adult females and adult females 3 h after a blood-meal. Four whole-mosquito samples, each containing 30 adult *Anopheles*, were also prepared. RNA extraction and hybridisation to the GeneChip was performed by the Sir Henry Wellcome Functional Genomic Facility (SHWFGF, University of Glasgow). Briefly, RNA was extracted from the *Anopheles* samples using RNeasy columns (Qiagen, UK), in accordance with the manufacturer's protocol. Each cRNA sample was prepared independently using the Affymetrix standard protocol, and the samples were hybridised to the Affymetrix GeneChip *Plasmodium*/*Anopheles* Genome Array for analysis of the transcriptome.

For the *Drosophila* microarray six samples containing ~2000 tubules each, and six whole-fly samples containing 30 flies each, were prepared (Wang *et al.*, 2004). RNA extraction and hybridisation to the GeneChip was performed by the SHWFGF. Briefly, RNA was extracted using RNeasy columns (Qiagen, UK) in accordance with the manufacturer's protocol. Each cRNA sample was prepared independently using the Affymetrix standard protocol, and the samples hybridised to the Affymetrix *Drosophila*1 GeneChip, for analysis of the transcriptome.

2.3.2 Data Analysis

The Affymetrix GeneChips were analysed using the Affymetrix MAS5 software, in which the average expression was set to 100. Annotation of the probe sets was obtained from NetAffx (Affymetrix, USA). Subsequent microarray analysis was performed using GeneSpring 6 (Agilent Technologies, USA). GeneSpring 6 analysis was performed by Dr Pawel Herzyk (SHWFGF, University of Glasgow).

2.4 Nucleic Acid Isolation and Quantification

Deoxyribonucleic acid (DNA) and messenger ribonucleic acid (mRNA) were used to provide the templates necessary for PCR, and the initial sequences for RNA interference (RNAi) constructs and *in situ* hybridisation probe constructs. The isolation of DNA and mRNA from biological tissues is described in this section.

2.4.1 Genomic DNA Isolation

Genomic DNA (gDNA) was prepared using twenty flies per sample. The flies were homogenised in 200 µl of Buffer A [100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% (w/v) SDS, in H_2O], using a disposable tissue grinder (Konets, Germany). An additional 200 µl of Buffer A was then added and the sample homogenised until only cuticles remained. The suspension was incubated at 65ºC for 30 min, and 88 µl of LiCl/KAc solution [1 part 5 M K-Acetate : 2.5 parts 6 M LiCl mixed immediately prior to use] added to the suspension and incubated on ice for 10 – 20 min. The sample was centrifuged for 15 min at 19,000 *g*. Approximately 1ml of the supernatant was transferred to a new tube, and 600 µl isopropanol added. The sample was again centrifuged at 19,000 *g* for 15 min. The supernatant was removed and the DNA washed with 70% (v/v) ethanol in nuclease-free H_2O and air-dried. Finally, the DNA was resuspended in 150 µl of nuclease-free H_2O, quantified by spectrophotometry and stored at -20ºC.

2.4.2 Messenger RNA Extraction

Messenger RNA (mRNA) was prepared using the Qiagen RNA extraction kit according to the manufacturer's instructions (Qiagen, UK). RNA was extracted from either ten whole-insects, or the Malpighian tubules of 25 insects. Each

sample was placed in 100 µl RLT buffer containing 1% ß-Mercaptoethanol (Sigma-Alrdich, UK), homogenised by sonication, and stored at -80ºC until the mRNA was extracted (a maximum of 7 days). The mRNA was eluted in 30 µl of nuclease-free H_20 or TE buffer, quantified by spectrophotometry, and stored immediately at -80ºC.

2.4.3 Complementary DNA Preparation

Complementary DNA (cDNA) was prepared from mRNA using the Superscript II kit (Invitrogen, UK) in accordance with the manufacturer's protocol. The total volume of each Superscript II reaction was 20 µl. After preparation, each sample was quantified and checked for degradation or impurities before being stored at -20ºC.

2.4.4 Plasmid DNA Isolation

Small scale plasmid DNA preparation was carried out using the Qiaprep Spin Miniprep kit (Qiagen, UK) in accordance with the manufacturer's instructions. The plasmid DNA was eluted in 30 µl of nuclease-free H_2O and quantified using spectrophotometry, before being stored at -20ºC.

Large scale preparation of plasmid DNA for germline transformation and cloning was performed using the Qiagen Plasmid Maxi kit or Endofree Maxi kit in accordance with the manufacturer's instructions (Qiagen, UK). The plasmid DNA was resuspended in 200 µl – 1 ml nuclease-free H_2O depending on the concentration required, quantified using spectrophotometry, and stored at -20ºC.

2.4.5 Quantification of Nucleic Acids

Nucleic acid concentrations were estimated by spectrophotometry using measured absorption levels at 260 nm and 280 nm on a Thermo Scientific NanoDrop™ 1000 Spectrophotometer. It was assumed that an OD of 1 at 260 nm corresponds to 50 $\mu g/ml^{-1}$ of double-stranded DNA and 40 $\mu g/ml^{-1}$ of single-stranded DNA and RNA. Readings were zeroed with the solution in which the samples had been eluted or diluted. The ratio of A260/A280 provided an estimate of nucleic acid purity. Values of 1.8 for DNA and 2.0 for RNA indicated

pure preparations. Double-stranded linear DNA was also semi-quantified by comparison with specific bands from a 1kb ladder standard (Invitrogen, UK) on a 1% agarose gel.

2.5 Oligonucleotide Synthesis

Oligonucleotides were synthesised by the MWG Biotech custom primer service on a 0.01 µmol scale, purified by High Purity Salt Free (HPSF®) technology, and their quality assessed by Matrix Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF) analysis. Oligonucleotides were received as a lyophilised pellet, which was resuspended in nuclease-free H_2O to a stock concentration of 100 µM, and later diluted with nuclease-free H_2O to a working concentration of 6.6 µM. All primer stocks were stored at -20°C. A list of all the primers used during this study is provided in Appendix 1.

2.6 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify specific regions from a DNA template. The area of amplification was designated using specific DNA primers 17 – 22 nucleotides in length. The DNA template was derived from gDNA, mRNA or plasmid DNA. The purpose of amplification was to assess the presence of a specific region in the template, or to amplify a specific region of the template for future applications.

2.6.1 Standard PCR

The standard PCR protocol was used in the everyday amplification of DNAs. The amount of template DNA varied, with ~100 ng of genomic template DNA used per reaction and ~10 ng or less of plasmid template used per reaction. For reactions using *Taq* DNA polymerase (Invitrogen, UK), dNTPs (Invitrogen, UK) were added at 200 µM each to single strength PCR buffer. Left and right primers at a concentration of 150 nM, and 0.25 U of *Taq* polymerase, were also added to each reaction. When Thermoprime With Reddymix™ PCR Buffer (ABgene, UK) was used, template and primers at the same concentrations as above were added to the pre-aliquotted mix.

Cycling was performed in thin walled 0.2 ml PCR tubes in a Hybaid OmnE, Hybaid PCR Sprint or Hybaid PCR Express-Gradient thermocycler. A typical cycling procedure is described in Table 2.3.

Table 2.3 Typical cycling procedure for standard DNA amplification using *Taq* DNA polymerase.

Step	Temperature	Time	
Initial template denaturation	94°C	3 min	
Denaturation	94°C	15 s	25 – 35 cycles
Annealing	50-60°C	30 s	
Extension	72°C	30 s - 5 min	
Final product extension	72°C	5 min	

The annealing temperature used depended on the melting temperature of the primers, and was typically ~5°C lower than the melting temperature. The extension time at 72°C was altered depending on the expected length of the product, and was usually 30 s for every 500 bp of DNA to be amplified. When multiple PCR reactions were run at the same time or the same samples run using different annealing temperatures, the Hybaid Gradient 96-well PCR machine was used, and a gradient imposed for the annealing temperatures across the block.

2.6.2 Pfu PCR

Pfu DNA polymerase (Promega, UK) is a thermostable enzyme from *Pyrococcus furiosus* which catalyses DNA dependent polymerisation of nucleotides into duplex DNA in the 5'→3' direction and exhibits 3'→5' exonuclease (proofreading) activity. It was used for PCR reactions requiring high fidelity DNA amplification, such as the preparation of RNAi expression constructs.

The *Pfu* reaction mix was set up as follows: 5 µl of 10x *Pfu* DNA polymerase buffer, 1 µl dNTP mix (final concentration of 200 µM for each dNTP), 2 µl of the forward and reverse primers (final concentration of 260 nM for each primer), 1 µl of DNA template (concentrations as before) or nuclease-free H_2O (control), 0.5 µl *Pfu* DNA polymerase (1.25 Units), made up to a final volume of 50 µl with nuclease-free H_2O. The reaction was cycled as described in Table 2.3, however

the extension time was increased to 1 min per 500 bp as *Pfu* exhibits a lower extension rate than *Taq* DNA polymerase.

2.6.3 Quantitative PCR

Quantitative PCR (qPCR) was used to compare the relative amount of a specific transcript between cDNA samples, for both *Drosophila* and *Anopheles*. Variations in the amount of transcript observed between samples were used to identify differences in gene expression.

The Malpighian tubules were dissected from 25 flies or 25 mosquitoes for each sample, and mRNA extracted and transcribed into cDNA as previously described (Sections 2.4.2 and 2.4.3). Specific primers were designed for each gene to generate an optimal product of 150 – 300 bp (for primer sequences see Appendix 1). The primer pairs were designed to span an intron/exon boundary where possible, to control for possible gDNA contamination.

To quantify the amount of expression of genes-of-interest, qPCR reactions were performed using the fluorescent double-stranded DNA dye DyNAmo™ SYBR® Green (Finnzymes, Finland). For each cDNA sample, the reaction was set up in triplicate to contain 12.5 µl 2x SYBR Green Master Mix (*Tbr* DNA polymerase, SYBR Green I, optimised PCR buffer, 5mM $MgCl_2$, dNTP mix), 1 µl of each primer (0.3 µM final concentration) and 1 µl of template cDNA (~500 ng), made up to 25 µl with nuclease-free H_2O. Additionally, to allow quantification of each gene of interest relative to a standard reference gene, reactions were also set up in triplicate for each cDNA sample containing primers specific for a reference gene. The ribosomal genes *rp49* and *s7* were used as reference genes in *Drosophila* and *Anopheles* experiments respectively.

Reactions were prepared on ice using optical grade 0.2 ml PCR strips (Starlab, UK), alongside two blank samples (1 x SYBR Green Master Mix), primer-only control samples for each set of primers, and a range of external standards for each gene containing 10^{-1} – 10^{-7} ng of template DNA (obtained from PCR amplification). PCR cycling was performed using an Opticon™ 3 thermal cycler (Bio-Rad, UK) according to the protocol described in Table 2.4.

Table 2.4 Typical cycling conditions for qPCR.

Step	Temperature	Time	
Initial denaturation	95°C	10 min	
Denaturation	94°C	30 s	36 cycles
Annealing	50-60°C	30 s	
Extension	72°C	45 s	
Absorption reading	76°C	10 s	
Incubation	72°C	5 min	
Melting curve	63 - 90°C	Read every 0.2°C, hold 1 s	

After amplification, each set of qPCR reactions was analysed using the Opticon™ 3 software in accordance with the manufacturer's instructions. Absolute quantification of gene expression was calculated using a standard curve whereby the threshold cycle C(t) values of each unknown sample were compared to the C(t) values of gene standards of known DNA concentrations. The C(t) value is the number of PCR cycles after which the SybrGreen fluorescence was detectable above the background fluorescence. The estimated amount of each gene of interest was adjusted to take into account variation in the original quantity of cDNA. This was done using the reference genes *rp49* and *s7*, which are expressed stably in every tissue of *Drosophila* and *Anopheles*. The specificity of each primer pair was analysed using melting curve data.

In the qPCR validation of the *Anopheles* microarray, data is presented as the ratio of expression between the whole mosquito, and the tubules of larvae, male adults, female sugar-fed adults and female blood-fed adults (± standard error of the mean [S.E.M]).In the qPCR validation of the *Drosophila* microarray experiment data is presented as the ratio of gene expression between the whole-fly and the Malpighian tubules (± S.E.M.). In the qPCR experiment comparing gene expression in the tubules of adult *Drosophila* males and females the data is presented as the ratio of expression between the male adult tubules and the female adult tubules (± S.E.M.). Results were plotted using GraphPad Prism 4.0 software (GraphPad, USA), and the statistical significance of the data determined using one-way ANOVA or Student's *t*-tests where appropriate.

2.6.4 Direct Colony Screening by PCR

PCR was used to identify recombinant bacterial colonies which contained a vector with a DNA insert, without setting-up overnight growth cultures. PCR was performed using one primer which bound to the insert and one primer which hybridized to the vector (facing into the cloning site), to identify clones which contained the insert in a particular direction. The chosen colonies were marked and numbered, and a sample of the colony transferred to the PCR reaction mix with a sterile tip. The standard PCR protocol was followed, as described in Section 2.6.1.

2.7 Cloning Procedures

The cloning techniques described in this section were used to insert specific sequences of DNA into an expression plasmid. This includes preparation of the insert, preparation of the plasmid, ligation of the insert to the vector, and the transformation of competent cells for plasmid selection.

2.7.1 Restriction Digests

DNA sequences were digested for 2-4 h at 37°C in the buffer appropriate to the restriction enzyme or enzymes being used (New England Biolabs, USA). A typical digest consisted of 1 µl of enzyme added to 1 µg of purified DNA in a final volume of 50 µl nuclease-free H_2O, containing the appropriate 1X NEBuffer and 1X BSA, if required. The quantity of DNA digested ranged from 200 ng to 4 µg of DNA or PCR product, depending on the downstream application. When digesting with multiple enzymes which required different buffers, the two digests were performed sequentially. After completion, the first digest was purified using the Qiagen PCR purification kit, and then the second digest was performed.

2.7.2 Agarose Gel Electrophoresis of DNA

DNAs were separated in an agarose gel, consisting of 1 % agarose in 0.5X TBE [90 mM Tris, 90 mM boric acid (pH 8.3), 2 mM EDTA] containing 0.1 µg ml^{-1} ethidium bromide, using 0.5X TBE as the electrophoresis buffer. If required, prior to sample loading, 6X loading dye [0.25 % (w/v) bromophenol blue, 0.25 % (w/v)

xylene cyanol, 30 % (v/v) glycerol in H_2O] was added to the samples to a final 1X concentration. DNA bands were visualised using a UV light transilluminator, and the band sizes compared to a standard 1 kb ladder (Invitrogen, UK).

2.7.3 Purification of DNA from Agarose Gels

DNA bands of interest were excised from an agarose gel using a clean scalpel blade. DNA was extracted using the Qiagen Gel Extraction Kit, in accordance with the manufacturer's instructions (Qiagen, UK). DNA was typically eluted in 30 µl nuclease-free H_2O (pH 7.0 - 8.5), quantified using spectrophotometry, and stored immediately at -20°C.

2.7.4 DNA Ligations into pUAST Plasmids

To clone an insert into a vector, the vector DNA and the insert were first digested with appropriate restriction enzymes. When a non-directional ligation was performed both the vector and the insert were digested with a single restriction enzyme. The vector was also dephosphorylated prior to the ligation reaction, to prevent it from re-annealing to itself. Dephosphorylation was performed during the last 15 min of the restriction digest, by the addition of 1 U of intestinal alkaline phosphatase (Promega, USA). After digestion, the plasmid vector was purified using the Qiagen PCR purification kit in accordance with the manufacturer's instructions, and the DNA eluted in 30 µl nuclease-free H_2O. If the insert was a digested PCR product it was purified in the same manner as the plasmid vector.

For the ligation reaction, a molecular ratio of 2:1 insert: vector was used, typically with 50-100 ng of vector. Ligations were performed using the Rapid DNA Ligation Kit (Roche, USA). The ligation mixture was prepared in accordance with the manufacturer's instructions, and the reaction incubated for 20 min at room temperature before transformation.

2.7.5 Cloning PCR Products into TOPO Vectors

Two vectors from the TOPO cloning range (Invitrogen, UK) were utilised. TOPO-pCRII is a dual promoter vector with a T7 promoter at the 5' end and a Sp6 promoter at the 3' end of the multiple cloning site (MCS). It was used for *in situ*

hybridisation RNA probe preparation for both *Anopheles* and *Drosophila*. The second vector, pCR2.1, contains a T7 promoter at the 5' end of the MCS and was used for general cloning.

PCR products were cloned directly into the appropriate TOPO® vector in accordance with the manufacturer's instructions, and transformed into TOP10 cells (Invitrogen, UK). Ligations were performed by the addition of 1 µl of purified PCR product to 0.5 µl of TOPO® linearised vector and 0.5 µl of 6X salt solution (1.2 M NaCl, 0.06 M $MgCl_2$). The reaction volume was made up to 3 µl with nuclease-free H_2O, and the reaction incubated at room temperature for 5 min.

2.7.6 Plasmids

A range of DNA plasmids were used during this project (Table 2.5).

Table 2.5 - Plasmids used during this study

Plasmid	Purpose
pWIZ	For germline transformation of cloned RNAi sequences, under the control of a UAS enhancer sequence
pCR®2.1	For the cloning of PCR products according to the TOPO TA cloning kit protocol (Invitrogen).
pCR®II	For the cloning of PCR products to make RNA *in situ* probes, according to the TOPO TA cloning kit protocol (Invitrogen).
LD22509	*Drosophila* EST clone containing the cDNA sequence of *Picot*
RE54080	*Drosophila* EST clone containing the cDNA sequence of *CG3994*
AT22075	*Drosophila* EST clone containing the cDNA sequence of *CG15406*
RE57622	*Drosophila* EST clone containing the cDNA sequence of *CG8028*

The pWIZ construct (Lee and Carthew, 2003) was used to produce transgenic vectors for the knockdown of specific transcripts in *Drosophila*. The pWIZ construct contains a UAS promoter region which allows regulated expression in transgenic *Drosophila* through crossing with GAL4 driver fly-lines (Figure 2.1). The *Drosophila* EST clones containing the cDNA sequence of *CG15406, Picot, CG3994* and *CG8028* were acquired from the *Drosophila* Genome Research

Centre (DGRC). The EST plasmid was provided on Whatman paper, and was transformed into competent cells using the methods suggested by the DGRC.

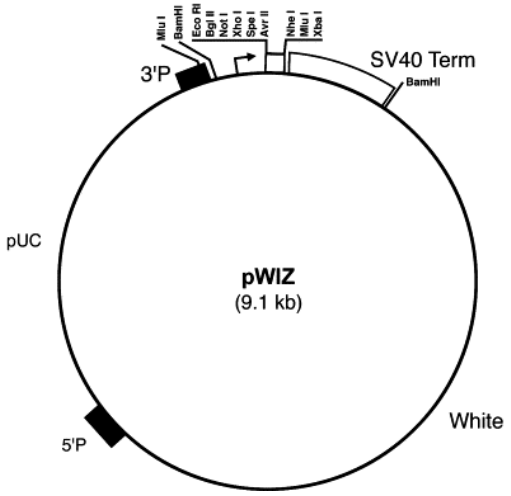


Figure 2.1 The *Drosophila* pWIZ UAS-RNAi vector. The pWIZ vector contains an intron from the *white* gene, flanked by two multiple-cloning sites. Insertion of an RNAi inverted repeat sequence of ~600 bp on each side of the *white* intron allows expression of a loopless hairpin RNA after splicing. Expression is activated by a GAL4 driver (Lee and Carthew, 2003).

2.7.7 Competent Cell Strains

Constructs were transformed into either TOP10 or DH5α ™ competent cells (Table 2.6) during cloning procedures. TOP10 cells were used for the cloning and sub-cloning of the TOPO vectors, the DH5α ™ cells were used during cloning of the pUAST plasmid.

Table 2.6 – Competent cell strains used during this study

Strain	Genotype	Purpose
TOP10 competent cells (Invitrogen, UK)	(F^- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*Z ΔM15, Δ*lac*X74, *rec*A1, *deo*R, *ara*D139, Δ(*ara-leu*)7697,*gal*U, *gal*K, *rps*L, (Str^R), *end*A1,*nup*G)	RNAi and *in situ* hybridisation constructs, cloning of large vectors
DH5α ™ subcloning efficiency competent cells (Invitrogen, UK)	(F^- ϕ80d*lac*Z ΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 (r_K^-,m^K+), *pho*A, *sup*E44,λ^-, *thi*-1, *gyr*A96, *rel*A1).	General sub-cloning

2.7.8 Transformation of DH5α Competent Cells

Plasmids were transformed into DH5α™ subcloning efficiency chemically competent cells by the addition of 50-100 ng of plasmid to 50 µl of cells on ice, followed by a 15 min incubation on ice. The cells were heat shocked at 37°C for 30 s, left on ice for a further 2 min, and 300 µl of L-broth (10 g Bacto-tryptone, 5 g dried yeast, 5 g NaCl made up to 1 l in water) added. This was followed by a 1 h incubation at 37°C to allow expression of the ampicillin resistance gene. 100 µl of the transformation was then spread onto pre-warmed L-Agar plates (10 g Bacto-tryptone, 5 g dried yeast, 5 g NaCl made up to 1 l in water) containing 100 µg ml^{-1} ampicillin.

2.7.9 Transformation of TOP10 Competent Cells

Plasmids were transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, UK) by the addition of 1 µl ligation mixture or plasmid into 25 µl of cells. The cells were heat shocked at 42°C for 30 s and left on ice for a further 2 min. 250 µl of S.O.C. medium (20 g Bacto-tryptone, 5 g dried yeast, 0.5 g NaCl, 2.5 mM KCl made up to 1 l in water) was added and the sample shaken at 250 rpm for 1 h at 37°C to allow expression of the antibiotic resistance gene.

100 µl of the transformation was spread onto pre-warmed L-Agar plates containing the appropriate antibiotic (either 100 µg ml^{-1} ampicillin or 50 µg ml^{-1} kanamycin) and 160 mg X-gal, and incubated overnight at 37°C. After PCR colony screening (Section 2.6.4), the appropriate colonies were grown separately overnight (with shaking) at 37°C in 5 ml L-broth containing 100 µg ml^{-1} ampicillin. The plasmids were isolated using Qiaprep Spin Miniprep columns in accordance with the manufacturer's instructions (Qiagen, UK).

2.7.10 Plasmid Selection

The DNA plasmids utilised during this study contained either the ampicillin or kanamycin resistance genes, which were used as a transformation selection factor. Colonies transformed with the plasmid were selected by the addition of ampicillin (100 µg ml^{-1}) or kanamycin (50 µg ml^{-1}) to the L-Agar or L-Broth growth medium. Ampicillin was stored as a 100 mg ml^{-1} stock solution (w/v) in

50% H_2O, 50% ethanol, at -20 °C. Kanamycin was purchased in a 50 mg ml^{-1} solution (Sigma-Alrdich, UK) and stored at 4 °C.

2.7.11 Storage of Bacterial Cultures

1 ml of bacterial culture was added to 1 ml of 2 % (w/v) peptone, 40 % (v/v) glycerol solution (in H_2O), and frozen in liquid nitrogen. Frozen bacterial stocks were stored at -70°C.

2.7.12 Electrophoresis of RNA

RNA was denatured by the addition of ; 3.5 µl formaldehyde, 10 µl formamide, 2 µl 5X MOPS (0.1 M MOPS (pH 7), 40 mM sodium acetate, 5 mM EDTA (pH 8)) and heating to 70ºC for 10 min, then snap-chilling on ice prior to the addition of 2.5 µl loading dye. The RNA was electrophoresed in a denaturing gel (20 % (v/v) 5X MOPS, 18 % (v/v) formaldehyde, 1 % agarose in DEPC-treated water) in 1X MOPS, containing 0.01 µg ml^{-1} EtBr, as the electrophoresis buffer. Sizes were compared to the Gibco BRL 0.24-9.5 kb RNA ladder (Gibco, UK).

2.7.13 Details of DNA Constructs

Table 2.7 contains the constructs which were produced during this study. The sequence of the primer pairs used can be found in Appendix 1.

Table 2.7 - Generation of constructs used in this study

Construct	Method of cloning	PCR primer pairs
pWIZ Picot.c1	AvrII, NheI (both compatible with XbaI)	Picot.c1iF, Picot.c1iR
pWIZ Picot.c2	AvrII, NheI (both compatible with XbaI)	Picot.c2iF, Picot.c2iR
pWIZ Picot.c3	AvrII, NheI (both compatible with XbaI)	Picot.c3iF, Picot.c3iR
pWIZ CG3994.c1	AvrII, NheI (both compatible with XbaI)	CG3994.c1iF, CG3994.c1iR
pWIZ CG3994.c2	AvrII, NheI (both compatible with XbaI)	CG3994.c2iF, CG3994.c2iR
pWIZ CG3994.c3	AvrII, NheI (both compatible with XbaI)	CG3994.c3iF, CG3994.c3iR
pWIZ CG15406.c1	AvrII, NheI (both compatible with XbaI)	CG15406.c1iF, CG15406.c1iR
pWIZ CG15406.c2	AvrII, NheI (both compatible with XbaI)	CG15406.c2iF, CG15406.c2iR
pWIZ CG15406.c3	AvrII, NheI (both compatible with XbaI)	CG15406.c3iF, CG15406.c3iR
pWIZ CG8028.c1	AvrII, NheI (both compatible with XbaI)	CG8028.c1iF, CG8028.c1iR
pWIZ CG8028.c2	AvrII, NheI (both compatible with XbaI)	CG8028.c2iF, CG8028.c2iR
pWIZ CG8028.c3	AvrII, NheI (both compatible with XbaI)	CG8028.c3iF, CG8028.c3iR
pCR2.0 Picot InSitu	TOPO©	PicotqF, PicotqR
pCR2.0 CG3994 InSitu	TOPO©	CG3994sF, CG3994sR
pCR2.0 CG15406 InSitu	TOPO©	CG15406sF, CG15406sR
pCR2.0 CG8028 InSitu	TOPO©	CG8028sF, CG8028sR
pCR2.0 AG12251 InSitu	TOPO©	AG12251sF, AG12251sR
pCR2.0 AG9005 InSitu	TOPO©	AG9005sF, AG9005sR
pCR2.0 AG7752 InSitu	TOPO©	AG7752sF, AG7752sR
pCR2.0 AG2587 InSitu	TOPO©	AG2587sF, AG2587sR

2.8 Automated DNA Sequencing

Automated sequencing was performed by the Sir Henry Wellcome Functional Genomics Facility (SHWFGF, University of Glasgow).

Automated sequencing at the SHWFGF was performed as a single-stranded reaction with template and primer supplied at 1 µg and 3.2 pmol, respectively, with a PCR mix containing fluorescently labelled dideoxynucleotides. Samples were run on an agarose gel, and the nucleotides detected on an ABI automated DNA sequencer. Analysis was performed using an Applied Biosystems automated sequence analysis programme and the sequences were down-loaded from the server onto MacVector (Version 7.0, from MacVector, Inc.) and further analysed.

2.9 Germline Transformation

Germline transformation of w^{1118} flies with transgene plasmids was performed by BestGene *Drosophila* Injection Services, USA. A maxi-prep of each purified plasmid containing at least 50 µg of plasmid in DEPC-treated water was supplied to BestGene. The microinjection helper plasmid was supplied by BestGene.

2.9.1 Determining the Chromosome of Insertion

Homozygous lines for each construct were crossed to a balancer line (w^-; Bl/CyO; $TM2e^-/TM6Tb^-$). The Bl marker chromosome confers a bristle phenotype on the hairs of the fly, and the CyO chromosome confers a curly wing phenotype. The $TM6Tb^-$ chromosome confers a tubby pupal phenotype, whereas the $TM2e^-$ chromosome in combination with the $TM6Tb^-$ chromosome confers an ebony colour on the cuticle. The red-eyed F1 progeny of the balancer line cross were backcrossed to the balancer line, and the red-eyed F2 progeny analysed for phenotypic markers. In red-eyed F2 progeny with ebony bodies, curly wings and bristles, the insertion was then known to be on the X (1^{st}) chromosome. In red-eyed F2 flies with ebony bodies, but only curly wings or bristles, the insertion was known to be on the 2^{nd} chromosome. In red-eyed F2 progeny without ebony bodies, but with curly wings and bristles, the insertion was known to be on the 3^{rd} chromosome.

2.10 Transgenic *Drosophila* crosses

UAS-RNAi fly-lines were crossed to various GAL4 drivers to determine whether gene knockdown in the fly, or specific tissues in the fly, had any effect on fly development and survival through to adulthood.

2.10.1 Crossing Strategy

Female virgin flies of the required genotype were collected daily and maintained in fresh vials containing no males for 3-4 days, and discarded if viable eggs were laid. Male flies used for crosses were 3-5 days old and removed from stock vials as required. Crosses set up in vials contained 3 females and 3-5 males, crosses set up in bottles contained 9 females and 9-12 males.

2.10.2 Emergence Counts of Drosophila Crosses

GAL4-driven UAS-RNAi crosses were set up in vials in triplicate and reared in standard conditions. After 7 days the parental flies were removed from the vial, and progeny flies began to eclose approximately 5 days later. They were collected each day and separated into those which contained the GAL4-driven UAS-RNAi construct, and those which didn't. The number of flies in each category was counted, and compared to the expected ratio from that cross.

For example: $\frac{\textit{Picot}.c1.l3}{CyO} \times \frac{\textit{Actin-GAL4}}{CyO\text{-}GFP}$

is a cross of *Picot* construct 1 (c1) line 3 (l3) on the second chromosome balanced over CyO, to *Actin-GAL4* on the second chromosome, balanced over CyO-GFP. All other chromosomes are wildtype. The resulting adult flies from this cross could have the following chromosome 2 genotypes:

(1) $\frac{\textit{Picot}.c1.l3}{\textit{Actin-GAL4}}$ (2) $\frac{\textit{Picot}.c1.l3}{Cyo\text{-}GFP}$ (3) $\frac{CyO}{\textit{Actin-GAL4}}$ (4) $\frac{CyO}{CyO\text{-}GFP}$

As genotype (4) is lethal as it contains CyO on both copies of chromosome 2, we would expect 1/3 of the eclosed flies to be straight-winged and contain the UAS-RNAi construct driven by *Actin-GAL4*, and 2/3 to have curly wings (from CyO), and not contain the construct driven by *Actin-GAL4*. The number of flies of each genotype was counted every day, until all the flies from the original cross had emerged. The ratio of observed flies of each genotype was then compared to the ratio of expected flies of each genotype, and Chi-squared analysis performed.

Flies which contained the GAL4-driven UAS-RNAi construct were maintained in fresh vials and aged to 7 days for tubule dissection and RNAi knockdown quantification using qPCR.

2.11 In Situ Hybridization of the Malpighian Tubules

In situ hybridisation was used to determine the expression pattern of specific genes in both the *Drosophila* and *Anopheles* Malpighian tubules.

2.11.1 Probe Preparation

Primers were designed to produce *in situ* hybridisation probes of approximately 200 bp for each gene of interest in *Anopheles* and *Drosophila*. Areas of the gene with low sequence homology to the rest of the genome were chosen. The probes were cloned into pCR®II-TOPO vector (Invitrogen, UK), and the direction of the insert determined. The vector was linearised by separate restriction digests at both sides of the MCS. This produced a copy of the insert which could be transcribed by the SP6 promoter and one which could be transcribed by the T7 promoter, to produce sense and anti-sense probes. The digests were cleaned-up using the QIAquick PCR Purification Kit (Qiagen, UK), linearization checked by gel analysis, and the plasmid quantified using spectrophotometric analysis.

The Ambion MEGAscript® T7 and SP6 kits were used to produce the antisense and sense probes via *in vitro* RNA transcription (Ambion, UK). A typical 10 µl reaction contained 1 µl DIG RNA Labelling Mix (Roche, UK), 1 µl 10X buffer, 1 µg linearised plasmid DNA and 1 µl enzyme mix. The reaction was incubated at 37°C for 2 h, followed by a 15 min incubation with 1 µl DNase1 (Sigma-Aldrich, UK). Ethanol precipitation was performed to clean-up the RNA probe. 2.5 µl 4M LiCl and 75 µl pre-chilled ethanol (-20°C) were added to the reaction, mixed, and stored at -80°C for 1-2 h. The samples were centrifuged at 13 000 *g* for 15 min at 4°C and the supernatant removed. The pellet was washed with 50 µl cold (-20°C) ethanol, 70% (v/v), and centrifuged at 13 000 *g* for 5 min. The ethanol was decanted and the pellet briefly air-dried. The pellet was dissolved in 30 µl nuclease-free water by incubating at 37 °C for 30 min.

The RNA was quantified using spectrophotometry and an aliquot run on a TAE gel to verify the size and integrity of the probe. The probe was stored at -80°C.

2.11.2 Drosophila In Situ Hybridisation

Tubules were dissected in batches of 10 with hindgut still attached and placed in eppendorfs in Schneider's medium. The Schneider's medium was carefully removed with a 200 µl pipette and postfix solution added to each eppendorf for 20 min. The tubules were washed 3 x 2 min with PBT, and then incubated for 3 min with proteinase K (Sigma-Aldrich, UK) in PBT (4 µg ml^{-1}). The proteinase reaction was stopped with 2 x 5 min washes of PBT containing 2 mg ml^{-1} glycine, washed 2 x 2 min with PBT, and then incubated with postfix for a further 20 min at RT. The tissue was washed 5 x 2 min with PBT, followed by one wash with hybridisation buffer diluted 1:2 with PBT. The tissue was washed once with hybridisation buffer, prior to a 1 h pre-incubation with hybridisation buffer at 55°C. The samples were bathed in 100 µl hybridisation buffer containing 40-50 ng of the sense or antisense riboprobe, sealed with Parafilm-M (American National Can, USA) and incubated for 43 h at 55°C. Following hybridisation, the samples were washed 7 x 1 h with hybridisation buffer at 55°C followed by a final wash overnight with hybridisation buffer at 55°C. The samples were then washed once with 50% hybridisation buffer and 50% PBT, followed by 4 x 10 min washes with PBT. The samples were then incubated for 2 h at RT with 200 µl of pre-absorbed alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Roche, UK) diluted 1:2000 in PBT. The antibody was pre-absorbed by dissecting the guts and tubules from 10 flies, adding to the diluted antibody and incubating at 4°C on a rotating apparatus for 1-2 h. The tissue samples were washed 6 x 20 min with PBT to remove any unbound antibody, and then incubated 2 x 5 min with DIG3 buffer. The colour reaction was started by the addition of DIG3 buffer containing BCIP (188 µg ml^{-1}) and NBT (375 µg ml^{-1}) and incubated in darkness at RT for 15 min to 1 h. Colour development was stopped with extensive washing with 50 mM EDTA in PBT. The tubules were mounted on slides in 70% glycerol, after 30 min washes with 30% glycerol and 50% glycerol. The slides were sealed with glycerol/gelatin (Sigma-Aldrich, UK).

Samples were viewed through a Zeiss Axiophot microscope using the brightfield and x5 and x10 magnifications. Images were captured using a Zeiss Axiocam HRC system and processed using Axiovision 3.0.6 software.

In situ hybridisation Solutions:

100 mM phosphate buffer, pH 7.0

61.5 ml 1M K_2HPO_4
38.5 ml 1M KH_2PO_4

Hybridisation buffer

5 ml 20 X SSC
10 ml formamide
2ml 100 mM phosphate buffer pH 7.0
2 ml 1.4 M NaCl
20 mg glycogen
20 µl Tween-20
1 ml PBT

PBT buffer

10 ml 100 mM phosphate buffer
10 ml 1.4 M NaCl
100 µl Tween 20
80 ml H_2O

Postfix buffer

1 ml formaldehyde
19 ml PBT

20 X SSC, pH 7.0

3.0 M NaCl
0.3 M sodium citrate

2.11.3 Anopheles In Situ Hybridisation

Tubules were dissected in batches of 10 with hindgut still attached and placed in eppendorfs in PBS buffer containing 50 mM ethylene glycol tetra-acetic acid (EGTA) and 4% (v/v) formaldehyde for 1 h. Tissues were rinsed briefly with methanol at RT and washed 5 x 5 min with agitation in 100% ethanol. Tissues were fixed for 30 min using PBT. Tissues were washed with PBT 5 x 2 min with agitation and incubated with Proteinase K (4 µg ml^{-1}) in PBT for 3 min. Tissues were placed on ice briefly, then washed 4 x 2 min with PBT at RT. Tissues were fixed in PBT + 5% formaldehyde and washed 5 x 5 min in PBT with agitation at RT. Tissues were washed in 50% PBT: 50% hybridization solution for 10 min with agitation at RT, followed by a wash with hybridisation solution (50% formamide, 25% 20 X SSC, 1.5 mg Heparin in nuclease-free water). Tissues were washed again in hybridisation solution and incubated at 55°C for 2 h in hybridisation solution. The hybridisation solution was replaced with hybridisation solution containing 50 - 100 ng of the specific sense or anti-sense probe for that gene,

sealed with Parafilm-M, and maintained at 55°C overnight. Tissues were washed in hybridisation solution 7 x 1 h at 55°C followed by one wash with 50% hybridisation solution: 50% PBT, and then 4 washes with PBT. Tissues were incubated overnight at 4°C with pre-absorbed alkaline phosphatase-conjugated anti-digoxigenin Fab fragments diluted 1:1000 in PBT. To pre-absorb the anti-digoxigenin Fab fragments the diluted antibody was added to 10 dissected guts and tubules and incubated at 4°C on rotation apparatus for 2 h. The unbound antibody was removed from the tissue with extensive washing with PBT. The samples were then incubated with DIG3 buffer for 2 x 5 min. The colour reaction was started by the addition of DIG3 buffer containing BCIP (188 µg ml^{-1}) and NBT (375 µg ml^{-1}). The tissues were incubated in the dark at RT until colour had developed (usually 15 min – 2 h). Colour development was stopped with extensive washing with PBT containing 50 mM EDTA. Slides were mounted in 70% glycerol after 30 min washes with 30% glycerol and 50% glycerol. Slides were sealed with glycerol/gelatin (Sigma).

Samples were viewed through a Zeiss Axiophot microscope using the brightfield and x5 and x10 magnifications. Images were captured using a Zeiss Axiocam HRC system and processed using Axiovision 3.0.6 software.

2.12 Antibodies

This section describes the design and purification of the polyclonal antibodies which were used during this study. The antibodies were used for Western blotting and immunocytochemistry (ICC) studies, in both *Anopheles* and *Drosophila* (Sections 2.14 and 2.15). Commercially obtained secondary antibodies were also used, and these are listed alongside the primary polyclonal antibodies (Table 2.8), with the concentration at which they were used during each experiment.

Table 2.8 Antibodies used during this study

Antibody and Source	Dilution and Purpose
Anti-Picot, affinity purified (rabbit polyclonal, this study, Genosphere biotechnologies)	1:600 ICC 1:1000 Western
Anti-CG15406, affinity purified (rabbit polyclonal, this study, Genosphere biotechnologies)	1:500 ICC 1:500 Western
Anti-AGAP007752, affinity purified (rabbit polyclonal, this study, Genosphere biotechnologies)	1:500 ICC 1:1000 Western
Anti-AGAP012251, affinity purified (rabbit polyclonal, this study, Genosphere biotechnologies)	1:750 ICC 1:500 Western
HRP labelled anti-rabbit IgG H&L (donkey polyclonal, Diagnostics Scotland	1:5000 Western
FITC labelled anti-rabbit IgG H&L (donkey polyclonal, Diagnostics Scotland)	1:1000 ICC

2.12.1 Design of Peptide Sequences

A fourteen amino acid antigenic sequence was selected from *AGAP007752, AGAP012251, CG15406* and *Picot*. The antigenicity (hydrophilicity) and protein structure of each gene was predicted, and a region of the gene picked which would be available for antibody binding. MacVector was used for protein modeling. The chosen peptides were screened using basic local alignment search tool (BLAST) for low sequence similarity to other proteins in the *Anopheles* and *Drosophila* ENSEMBL databases (Benson *et al.*, 2009; Hubbard *et al.*, 2009). Any peptide which had >6 consecutive amino acids in common with another protein was rejected. A Cysteine amino acid was added to the 5' end of the peptide sequence to allow purification using affinity columns. The antigenic sequences used for antibody preparation can be found in Table 2.9. The antigenic peptide and immune serum were generated by Genosphere biotechnologies (France).

Table 2.9 Antigenic sequences used for antibody production in rabbit.

Antibody	Peptide sequence
Anti-AGAP007752	C Q N S R V T L A D F K S R E
Anti-AGAP012251	C P D N D E P Q K P V S I E A
Anti-CG15406	C K S L R Y Y R R C D G P N V
Anti-Picot	C N H T A I K S G E A E E Y D

2.12.2 Isolation of IgG Fraction from Immune Serum

The following protocol was used to isolate the IgG fraction from rabbit immune serum. A 'HiTrap Protein A HP' column (Amersham Pharmacia, UK) was equilibrated by passing 30 ml of phosphate buffer saline (PBS)(14 mM NaCl, 0.2 mM KCl, 1 mM Na_2HPO_4, 0.2 mM KH_2PO_4 made up in H_2O and buffered to pH 7.4 using HCl) through it at a rate of ~2 ml per min. 5 ml of immune-serum was filtered through a 0.22 µM filter and syringed through the column to bind. The column was washed with 30 ml of PBS and the IgG fraction eluted with 17 ml of 100 mM glycine (pH 3.0). The first 2 ml of elution was discarded and the remaining 15 ml of IgG collected in a 50 ml Falcon tube containing 1.5 ml 1 M Tris-HCl (pH 8.0). The absorbance at 280 nm was measured to determine the yield using a spectrophotometer. The IgG fraction was dialysed overnight against PBS using a dialysis tube (size 9, Medicell International Ltd, UK) which had been pre-treated in PBS with 0.05% (w/v) sodium azide.

2.12.3 Preparation of Affinity Columns

Affinity columns were prepared using the specific antigen for each antibody. The bottom cap was fitted to a 10 ml polypropylene column (Pierce, UK) and the column filled with deionised water. A frit was pushed to the bottom of the column. The water was drained through the frit by removing the end cap, and 5 ml of Sulfolink slurry (Pierce, UK) was added and allowed to sediment for 30 min. The slurry buffer was removed down to the surface of the gel and 25 ml of column buffer (50 mM Tris-HCl, 5 mM Na-EDTA, pH 8.5) was added to the column. The buffer was allowed to run through the column and a further 25 ml of buffer added and allowed to run through to the top of the gel. 1 mg of

antibody-specific peptide was dissolved in 4 ml of column buffer and added to the column, which was then sealed and mixed on a rotator for 15 min. The column was set upright and left to settle for 45 min. Both end caps were removed and the solution drained to the top of the gel. 15 ml of 50 mM cysteine in column buffer was added to the column. The column was sealed again, before being placed on a rotator for a further 15 min. The column was set upright and allowed to settle for 45 min and another frit fitted just above the level of the gel. The end cap was removed and the solution allowed to drain. The column was washed by passing 60 ml of 1 M NaCl through it, followed by 50 ml of PBS and then 40 ml of 0.05 % (w/v) sodium azide in PBS, retaining some liquid above the gel at all times. The end caps were fitted and the column stored at 4ºC until further use.

2.12.4 Affinity Purification of Antibodies

Before use, each affinity column was brought to room temperature and the sodium azide in PBS drained. The column was washed with 30 ml of PBS before the IgG fraction was passed through it. This was followed by a further wash with 30 ml of PBS. Finally the antibody was eluted with 15 ml of 0.1 M glycine (pH 3.0). Twelve 1 ml fractions were collected into 1.5 ml eppendorfs containing 100 µl of Tris-HCl (pH 8.0). At this point, the yield was determined by measuring the absorption at 280 nm for each fraction. Fractions with readings greater than 1 were pooled and dialysed overnight against PBS containing 0.01% (w/v) sodium azide. The absorption at 280 nm was measured again in order to ascertain the final yield using the equation:

Antibody concentration (mg ml^{-1}) = O.D. (at 280 nm) x 1.35 mg ml^{-1}

The antibodies were aliquotted into 1.5 ml eppendorfs and stored at -20ºC.

2.13 Protein Extraction

Protein was extracted from 6 whole mosquitoes/flies, 30 heads or 300 tubules for use in Western blotting. The tissue was dissected into 100 µl of Tris-Lysis buffer (2% (w/v) SDS, 70 mM Tris, pH 6.8) containing 1 µl of protease inhibitor cocktail (Sigma-Aldrich, UK), in a 1.5 ml eppendorf tube. It was homogenised

using a hand-held pestle followed by 3 x 1 sec homogenisations with a Microson Ultrasonic Cell Disrupter. The sample was then centrifuged at 14, 000 *g* for 10 min to remove debris and the supernatant transferred to a new tube. Samples were stored at -80ºC until the Bradford assay and Western blot were performed, usually within one week.

2.13.1 Bradford Assays

Bradford assays were used to determine the protein content of each sample before Western blotting. Assays were performed on a 96-well plate for compatability with a spectrophotometer. Twenty-four standards containing 0 - 5 µg of BSA were each made up to 50 µl in water, in sets of three standards for each BSA amount. Around 2 µl of each protein sample (usually approximately 2 µg of protein) was also made up to a final volume of 50 µl in triplicate. 200 µl of 1X Bradford reagent (Bio-Rad, USA) was added to each well. The absorption of each sample at 590 nm was measured using a spectrophotometer. A calibration curve was derived from the twenty-four standard measurements and used to estimate the protein concentrations of each mosquito or fly sample.

2.14 Electrophoresis and Western Blotting

Electrophoresis was used to separate proteins in a sample on the basis of their size. The proteins were hybridised to a membrane during Western blotting, and presence of specific proteins assessed by antibody binding.

2.14.1 Protein Electrophoresis

Protein electrophoresis was performed using either the Bio-Rad Ready Gel Mini PROTEAN II Cell Kit, or the Bio-Rad Mini-PROTEAN III Cell Kit system. SDS-Polyacrylamide gels were made using resolving gel (1.7 ml 10% acrylamide mix, 1.3 ml 1.5 M tris (pH 8.8), 50 µl 10% SDS, 50 µl 10% ammonium persulfate, 5 µl TEMED, made up to 5 ml with water) for the lower 80%, and stacking gel (170 µl 10% acrylamide mix, 130 µl 1.5 M tris (pH 6.8), 10 µl 10% SDS, 10 µl 10% ammonium persulfate, 2 µl TEMED, made up to 1 ml with water) for the remainder of the gel where the comb was inserted. The apparatus was filled with running buffer (7.2 g glycine, 1.5 g Tris base, 6 ml 10% (w/v) SDS made up

to 500 ml with water). The samples were prepared by adding 6X SDS-PAGE loading buffer (0.35 M Tris HCl (pH 6.8), 10% (w/v) SDS, 36% (v/v) glycerol) to the protein sample. They were then briefly vortexed, heated to 95ºC for 5 min in a boiling water bath, pulse spun and vortexed again before loading into the gel wells. Pre-stained Rainbow marker (Amersham Pharmacia, UK) was used to determine the size of the protein bands in the gel. Ice packs were used to minimise heating of the blotting apparatus and gels. A constant 50 V was applied for 30 min and then 100 V applied for ~1 h until the loading dye was close to the bottom of the gel.

2.14.2 Western Blotting

Proteins in the SDS-PAGE gel were transferred onto a Hybond ECL membrane (Amersham Pharmacia, UK) to enable antibody recognition of specific proteins. The transfer was performed using a Bio-Rad Mini-gel Blotting Kit, by placing the gel and the wet membrane between pieces of Whatmann 3 MM paper and placing it into the blotting apparatus. The apparatus was filled with transfer buffer (20% (v/v) methanol, 14.4 g glycine, 3 g Tris base made up to 1 l with water). Ice packs were used to minimise heating of the blotting apparatus and gels, and a constant 50 V was applied for 1 h.

2.14.3 Western Hybridisation

After the blots were removed from the transfer apparatus, blocking was performed overnight in PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) Marvel milk at 4ºC.

The blots were washed 6X 5 min in PBS containing 0.1% (v/v) Tween 20. The primary antibody was mixed with blocking solution (PBS containing 0.1% (v/v) Tween 20 and 1% (w/v) Marvel milk) to the appropriate concentration and the blot incubated in 5 ml of the solution for 2 h at RT. This was followed by washes in PBS containing 0.1% (v/v) Tween 20; four washes were carried out for 5 min and then 4 further washes for 10 min on a horizontal shaker at RT. The secondary antibody was diluted in blocking solution and incubated with the blot for 1 h. The blots were washed similarly to after primary antibody blotting, with

the final wash in PBS without Tween 20. Signal detection was performed immediately after the PBS wash.

2.14.4 Western Signal Detection

Horseradish peroxidise (HRP) conjugated secondary antibodies were used to detect primary antibody hybridisation on Western blots. HRP activity was detected by chemiluminescence, using the ECL™ Western Blot analysis system (Amersham Pharmacia, UK). It was performed by adding equal volumes of 'reagent 1' and 'reagent 2' to the blot and incubating at room temperature for 1 min. The blot was then wrapped in Saran Wrap and exposed to ECL film (Amersham Pharmacia, UK) for between 30 s and 2 min before development using an X-OMAT film processor.

To confirm that efficient transfer had occurred, blots were stained using Coomassie Brilliant Blue for 2 min on a horizontal shaker after the development reaction. The blot was then de-stained using de-staining solution (10% (v/v) acetic acid, 45% (v/v) methanol, 45% (v/v) water). The de-staining solution was changed frequently until the bands on the protein membrane appeared sharp and the background white.

2.15 Immunocytochemistry of Malpighian Tubules

Immunocytochemistry was performed on *Drosophila* Malpighian tubules to determine the cellular localisation of specific proteins. The primary polyclonal antibodies and secondary commercial antibodies used are described in Section 2.12.

2.15.1 Drosophila melanogaster

Hindguts were dissected with the Malpighian tubules still attached in Schneider's medium and transferred into 1.5 ml eppendorfs containing PBS in batches of 8-10. The PBS was carefully removed and the tubules fixed in 4% (w/v) paraformaldehyde in PBS at RT for 20 min. The tubules were washed 3 x 10 min in PBT (PBS with 0.05% (v/v) Triton X-100) and incubated at RT with PBT containing 10% (v/v) goat serum (Sigma-Alrdich, UK) for 4 hours. Primary

antibody, diluted to the desired concentration in PBT and 10% (v/v) goat serum, was applied and the samples incubated at 4°C overnight. The following day the tubules were washed in PBT 5 x 20 min and incubated in PBTA with 10% (v/v) goat serum for 3 to 4 h. Secondary antibody, diluted to the desired concentration in PBT with 10% (v/v) goat serum, was applied and the samples incubated overnight at 4°C. The tubules were washed in PBT 3 x 20 min and stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Alrdich, UK) for nuclei visualisation. DAPI was applied to the tissues at 500 ng ml^{-1} for 1 min in PBS after dilution from a 10 mg ml^{-1} stock solution (in H_20). The samples were washed 3 x 5 min in PBS before mounting. Slides were mounted in 70% glycerol after 30 min washes with 30% glycerol and 50% glycerol, covered with 22 mm square BDH coverslips and sealed with glycerol/gelatin (Sigma-Aldrich, UK).

Samples were viewed using either fluorescence microscopy or confocal microscopy. Fluorescence microscopy was performed using a Zeiss Axiophot microscope using either a fluorescein or rhodamine filter. DAPI was visualised using a UV filter. Most images were captured by a Zeiss Axiocam HRC system and processed using Axiovision 3.0.6 software. Confocal microscopy of samples is described in section 2.16.

2.16 Confocal Microscopy

Fixed samples were viewed and imaged using a Zeiss 510 Meta Confocal system. The lens magnification used is included in the figure legends. Images which were to be compared to each other were taken at the same gain and exposure. All images were processed using the LMS imaging software, in some cases using features for integrating the signals from different stains and producing single images or Z stacks.

2.17 Fluid Secretion Assays

Fluid secretion assays were used to determine the rate of fluid secretion from excised *Drosophila* Malpighian tubules for both GAL4-driver and UAS-RNAi fly-lines. The diuretic peptide Drosokinin was used to stimulate secretion above the basal rate.

2.17.1 Fluid Secretion Assays in Drosophila

The rate of fluid secretion from *Drosophila* tubules was measured using the fluid secretion assay (Dow *et al.*, 1994). Seven-day old flies of the relevant genotype were anaesthetised on ice, transferred to Schneider's medium, decapitated and the abdomen dissected to reveal the gut and tubules. Tubule pairs were removed from the midgut by contacting only the ureter. One tubule was wrapped around a metal pin under white, heavy mineral oil (Sigma-Aldrich, UK) whilst the other tubule was immersed in 9 µl of a 1:1 solution of *Drosophila* saline (Section 2.18) and Schneider's medium. Drops emerging from the ureter were removed every 10 min with a fine glass rod and measured under a microscope graticule (Figure 2.2). When required, Drosokinin was added to the reservoir bubble as a 10 times stock resulting in a final concentration of 10^{-7} mg ml^{-1}, usually after 30 minutes of consistent secretion.

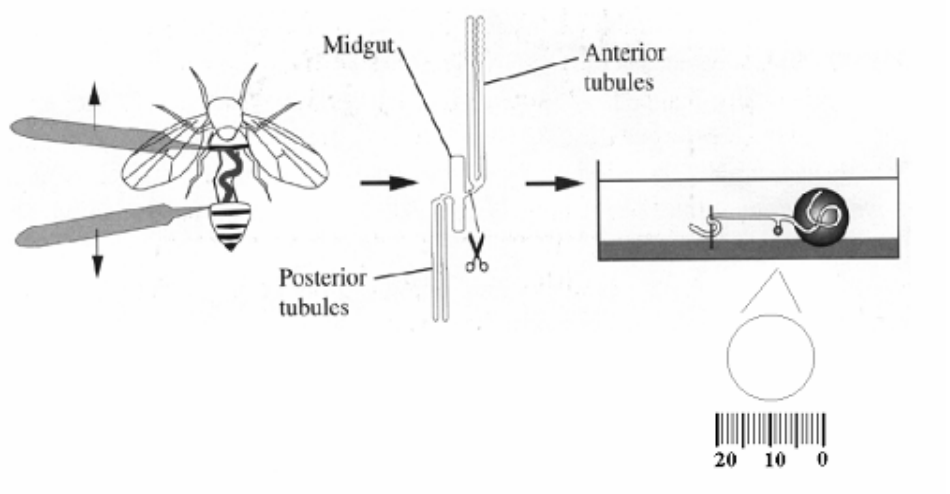


Figure 2.2 Diagram of the fluid secretion assay

2.18 Transport Assays

Transport assays were used to assess the up-take of glucose by the tubules, and to determine whether it is actively transported and secreted. Transport assays were also used to determine whether the glucose up-take of sugar transporter GAL4-driven UAS-RNAi lines was significantly different from their parental lines.

2.18.1 Transport Assays in Drosophila

Malpighian tubules were dissected and prepared in a similar manner as for standard secretion assays (Section 2.17.1). The secretion plate was set up similarly, and the reservoir bubble was minimal *Drosophila* saline (see Table 2.10). After 30 min of steady secretion from the tubules, ^{3}H-labelled glucose tracer (Sigma-Alrdich, UK) was added to the reservoir bubbles. After allowing a few minutes for mixing, a 1 µl sample of each reservoir droplet was removed to an Eppendorf tube containing 1 ml of scintillation fluid (Fisher Scientific, UK). The tubules were allowed to secrete for a further 2 h before the secreted droplet was measured and transferred to an Eppendorf tube containing scintillation fluid, and a 1 µl sample of each reservoir bubble removed to an Eppendorf tube containing scintillation fluid. The ^{3}H content of each sample was measured in a Beckman scintillation counter, with appropriate controls (Beckman, UK).

To determine whether glucose was being actively transported by the tubule a transport ratio was calculated. This was done by dividing the counts per min (cpm) from a measured volume of the secreted drop by the equivalent cpm from the same volume of reservoir bubble. As glucose is an uncharged substrate, active transport into the lumen would result in a transport ratio greater than 1. If glucose was not being actively transported across the tubule epithelium the transport ratio would be less than or equal to 1.

To determine whether glucose was being taken up by the tubule and metabolised rather than secreted, an uptake ratio was calculated. This was done by dividing the cpm from the 1 µl sample from the reservoir after 2 h of secretion by the cpm of the 1 µl sample from the reservoir taken 10 min after the ^{3}H glucose was added. A ratio of 1 would suggest that glucose was not being transported across the apical membrane. A ratio of less than one, combined with a transport ratio of less than 1, would suggest that a proportion of the available ^{3}H glucose was being taken up by the tubule but not secreted into the lumen.

Table 2.10 - Concentration of salts and amino acids used in the *Drosophila* salines. The minimal DS described (Linton and O'Donnell, 1999) was made without trehalose, and with the concentration of glucose varied during experimental optimisation. Schneider's medium as previously described (Ashburner, 1989; Dow *et al.*, 1994).

	Schneider's medium (mM)	DS (mM)	Minimal DS (mM)
NaCl	35.9	117.5	76.72
$Na_2HPO_4.2H_2O$	2.8	4.3	3.53
KH_2PO_4	5.0		2.50
$CaCl_2$	4.1	2	3.04
KCl	21.5	20	20.73
$MgSO_4$	37		18.50
$MgCl_2$		8.5	4.25
$NaHCO_3$		10.2	5.10
HEPES		15	7.50
α-ketoglutaric	1.4		
Succinic acid	0.8		
Fumaric acid	0.9		
Malic acid	0.7		
Glucose	11.1	20	0.05 – 15.55 mM
Trehalose	5.3		
Yeastole	2g		
B-alanine	5.6		
L-arginine	2.3		
L-asparagine	0.3		
L-aspartic acid	4.0		
L-systeine	0.5		
L-glutamic acid	5.4		
L-glutamine	12.3		6.16
Glutathione	0.1		
Glycine	3.3		1.67
L-histidine	2.6		1.29
L-isoleucine	1.5		
L-leucine	1.1		0.57
L-lysine	11.3		5.65
L-methionine	5.4		
L-phenylalanine	1.5		
L-proline	14.8		
L-serine	2.5		1.25
L-threonine	3.5		
L-tryptophan	1.0		
L-valine	2.6		1.28
FCS	18%		

2.19 *Drosophila* Crosses on Sugar-Modified Food

Oregon R wild-type flies were raised on fly-food with modified sugar content, and qPCR performed to determine whether it had any effect on the expression of the sugar transporter *CG15406*. GAL4-driven UAS-RNAi lines were also raised on *Drosophila* food with modified sugar content, to determine whether the sugars had any effect on the survival of flies in which the expression of a specific gene had been knocked-down.

2.19.1 Gene Expression on Sugar-Modified Food

Batches of food with glucose, fructose or sucrose as the main sugar component were prepared (Table 2.11). Oregon R wild-type flies were allowed to lay eggs on the normal, glucose, fructose and sucrose food. The parental flies were removed after 7 days, and the larvae left to develop into adult flies. Flies were collected as they emerged, and aged to 7-days old on the same sugar food. The Malpighian tubules were dissected out of the flies, the mRNA extracted and transcribed into cDNA, and qPCR performed (Sections 2.4.2, 2.4.3 and 2.6.3).

Table 2.11 Components of normal and sugar-modified *Drosophila* food. The quantities shown are for 1 l of food.

	Normal Food	Glucose Food	Fructose Food	Sucrose Food
Agar	10 g	10 g	10 g	10 g
Dried yeast	35 g	35 g	35 g	35 g
glucose	30 g	37.5 g	-	-
sucrose	15 g	-	-	75 g
Maize meal	15 g	15 g	15 g	15 g
Wheat germ	10 g	10 g	10 g	10 g
Treacle	30 g	30 g	30 g	30 g
Soya flour	10 g	10 g	10 g	10 g
fructose	-	-	37.5 g	-

2.19.2 Drosophila Survival on Sugar-Modified Food

Fly crosses of the relevant genotype were set-up on modified fly food and normal food (Table 2.11), and allowed to lay eggs. The parental flies were removed after 7 days, and the larvae left to develop into pupae. After eclosion, flies were counted as previously described (Section 2.10.2). The ratio of observed to expected flies for each genotype on each food type was then compared to determine whether altering the sugar component of the food had any effect on fly survival.

Chapter 3 - Validation and Analysis of the *Anopheles* Malpighian Tubule Microarray

3.1 Summary

Microarray analysis is a useful way of identifying transcripts which are highly expressed in a particular cell or tissue type (Fraser and Marcotte, 2004). In species such as *Drosophila* and *Anopheles*, which have had their genomes sequenced and annotated, the transcriptome can be mapped in specific tissues at time-points of interest to the experimenter. The tissue of interest in this work is the insect renal system, the Malpighian tubules. The *Drosophila* Malpighian tubules have been studied due to well understood genetics and the accessibility of mutant and transgenic stocks. Experimentation on the Malpighian tubules of *Anopheles* has been more difficult, due to the demanding nature of creating transgenic mosquitoes, and fewer catalogued mutant stocks. This chapter describes microarray experiments performed to identify genes which are highly expressed in the *Anopheles* Malpighian tubules. The microarrays compared the transcriptome of larval and adult Malpighian tubules to the transcriptome of the whole mosquito. Analysis of the microarray data identified genes which are highly enriched in the tubules while they are under specific pressures, such as when the larva is water-bound, or when the female is digesting a blood-meal. Genes which are highly enriched in the tubules are likely to be involved in processes important to the tubules, and their study increases understanding of renal function and physiology. The transcripts enriched in the *Anopheles* tubules were then compared to those enriched in the *Drosophila* tubules (Wang *et al.*, 2004), to identify homologous gene pairs which retain not only their coding sequence, but their expression pattern across ~150 million years of evolution (Yeates and Wiegmann, 1999). Four homologous genes pairs which were enriched in the *Anopheles* and *Drosophila* tubules were chosen for further study. The eight genes are all predicted to function as transporters, with putative roles in the transport of sugars, phosphate, zinc and monocarboxylates.

3.2 Introduction

Microarray technology has been developed primarily in the last decade. The basic function of a microarray is to quantify the expression of specific transcripts in an RNA sample from an organism or tissue of interest. From an organism with a sequenced and annotated genome it is possible to design a microarray containing probes for thousands of transcripts, including probes which differentiate between splice variants. As the expression of thousands of genes is measured simultaneously from the same sample, a massive amount of data is generated in a single set of experiments. Expression analysis arrays can be purchased from companies such as Affymetrix for many model organisms. In addition to the *Plasmodium*/*Anopheles* genome array used in this study, arrays are available for *Drosophila*, *Mus musculus, Rattus norvegicus, Arabidopsis thaliana, Caenorhabditis elegans* and *Escherichia coli*.

3.2.1 Microarray Technology

There are various ways of utilising microarray technology, depending on the aims and budget of the experimenter. Researchers can either use pre-designed chips such as those offered by Affymetrix, or design their own. Designing an array is useful when working on an organism which does not have a commercially produced chip. It can also be practical when working on a sub-set of genes which don't require tens of thousands of probes which cover the entire genome. These chips are usually produced by spotting or printing the oligonucleotide probe directly onto a substrate such as glass. The commercially produced Affymetrix GeneChips which are used in this thesis are expensive, but give reliable genome-wide coverage. They contain built-in controls which distinguish between perfect match (PM) oligonucleotide binding and binding with a one nucleotide mismatch (MM), to reduce the effect of non-specific binding on transcript quantification. Oligonucleotides are synthesized *in-situ* on a glass substrate and are 25 nucleotides in length, targeted towards the 3' of each gene. The whole genome arrays, such as those used in this study, are primarily for transcript expression analysis. DNA analysis and gene regulation arrays are also available, for locating polymorphisms and investigating gene promoter regions respectively. With time, high-throughput genome analyzers such as the Illumina Solexa technology will

succeed these methods, but currently the Affymetrix chips are the industry "gold standard".

3.2.2 Microarrays Utilized in this Study

The genes investigated in this study were chosen during the analysis of *Anopheles gambiae* microarrays which were performed in 2004 (Overend *et al.*, manuscript in preparation). Dissections for the microarray were performed by Pablo Cabrero (Dow-Davies Lab, University of Glasgow) and the Affymetrix chips processed by the Sir Henry Wellcome Functional Genomics Facility, University of Glasgow. Mosquitoes were obtained by kind donation from Lisa Ranford-Cartwright (Department of Infection and Immunity, University of Glasgow). The study used the Affymetrix GeneChip *Plasmodium*/*Anopheles* genome array, the first GeneChip to allow simultaneous coverage of two organisms on a single chip. This thesis also references data from a previously published microarray (Wang *et al.*, 2004) which utilized the GeneChip *Drosophila* genome array (Table 3.1).

Table 3.1 - Features of the *Anopheles* and *Drosophila* GeneChips used and referenced in this study (data as provided by Affymetrix).

GeneChip	Probe number	Probe pairs per probe set	Predicted number of transcripts
Plasmodium/ Anopheles	5,407 (*Plasmodium*) 16,941 (*Anopheles)*	11	~4,700 (*Plasmodium*) ~16,000 (*Anopheles*)
Drosophila	13,500	14	~12,100

3.2.3 The Anopheles Malpighian Tubule Microarray

The microarray experiment described was used to identify novel genes-of-interest which were enriched in the Malpighian tubule of the malarial mosquito *Anopheles gambiae* when compared to the average whole-mosquito expression.

The experiment was designed to compare the transcriptome of whole adult *Anopheles* with the transcriptome of the Malpighian tubules in larvae, adult males, adult females, and adult females 3 h after a human blood-meal (Table 3.2). The microarrays also allow comparison of the tubule transcriptome during

specific life-stages, such as the larval tubules versus the adult tubules, sugar-fed female tubules versus the tubules after a blood-meal, and male versus female tubules.

Table 3.2 - Transcriptomes compared in this study (The sample name for each group is used throughout this thesis).

Sample name	Gender of mosquitoes	Life-Stage of mosquitoes	Whole or tubules	Blood-fed (B) or sugar-fed (S)
Whole	males & females	adult	whole	sugar-fed
Larval	males & females	late-stage larvae	tubules	fed on fish food
MS	males only	adult	tubules	sugar-fed
FS	females only	adult	tubules	sugar-fed
FB	females only	adult	tubules	sampled 3 h after a blood meal

The adult mosquitoes were all 3-5 days old and from the same colony. The mosquitoes were not infected with malarial *Plasmodium*, although the GeneChip contains probe sets for over 5,000 *Plasmodium* transcripts. There was no significant binding to the *Plasmodium* probe sets. Four samples were dissected for each group of interest, requiring twenty GeneChip measurements in total. Clustering analysis showed no inconsistencies in gene expression between groups of samples, and all twenty arrays were used in subsequent analysis.

The *Anopheles* data-set allows several interesting comparisons across the life-cycle of the Malpighian tubules (Table 3.3). The microarrays are the first study of ontogeny, life-style, sex and feeding to be combined in one experiment.

Table 3.3 Interesting comparisons from the *Anopheles* microarray data-set.

Comparison	Data of interest
Whole versus larval tubule Whole versus male tubule Whole versus female tubule Whole versus female blood-fed tubule	Transcripts which are enriched in the tubules at specific stages in the life-cycle in comparison to the average whole mosquito expression
Larval tubule versus average adult tubule	Transcripts which are enriched in the larval tubules in comparison to the adult tubules
Male tubule versus female tubule	Transcripts which are tubule-enriched and sex-specifically expressed
Female tubule versus female blood-fed tubule	Transcripts which are enriched or down-regulated in the tubules in response to a blood-meal

3.3 Microarray Analysis

Analysis and validation of the microarray data took place in several stages; the data was initially analysed and validated using quantitative PCR (qPCR), which was followed by an in-depth analysis which identified genes for further study.

3.3.1 Initial microarray analysis

The initial GeneChip analysis was performed using Affymetrix software (MAS5). Using MAS5, individual probes are assigned a signal strength by calculating the 75th percentile of intensity within each probes binding area. Each probe set is annotated with a detection call of 'present', 'absent' or 'marginal'. This is determined by the difference between the PM and MM values, and reflects whether the signal strength is likely to be from specific or non-specific binding. After MAS5 analysis, computer packages such as the Affymetrix Data Mining Tool or GeneSpring (Agilent Technologies) can be used to filter and compare the data.

Microarray data is displayed in two different styles in this thesis. Firstly it is displayed as a signal strength with a standard of the mean (± S.E.M.). This is the average signal strength for a particular transcript or gene in the specified group. Microarray data is also displayed as an expression ratio (± S.E.M.). This number is the amount of transcript in one group (usually tubule) compared to the amount of transcript in another group (usually whole mosquito or fly), expressed as a ratio. Expressing microarray results by signal strength is useful as the experimenter can quickly determine whether a gene is highly transcribed or not. Expression ratios are useful as they allow the experimenter to see at a glance whether a transcript is differentially expressed between two tissues. They are also convenient when validating a microarray by qPCR, as the qPCR results can be shown as an expression ratio and the two compared. One-way ANOVA and Student's *t*-tests have been applied where necessary.

3.3.2 Validation of the Microarray

Although microarrays are a reliable way of measuring gene expression and have in-built experimental controls, it is prudent to validate that a subset of the

results is representative using other experimental techniques (Kothapalli *et al.*, 2002). This should be the first step when investigating a novel gene chosen from a microarray. Methods such as Northern blotting and serial analysis of gene expression (SAGE) can be used, but the simplest way to validate results is through Quantitative polymerase chain reaction (qPCR). qPCR is a modified version of PCR, which uses thermostable polymerases to amplify DNA through annealing and extension cycles. By incorporating a fluorescent dye such as SYBR green to calculate the amount of new DNA being generated, it is possible to quantify the amount of transcript which was originally present. This method was used to validate the integrity of the *Anopheles* microarray experiment.

3.4 Results

This section describes the findings from the analysis of the *Anopheles* microarray data-set. The genes which were most highly expressed in both the whole-mosquito and Malpighian tubule samples are described, as are the genes which were most highly enriched in the tubules in comparison to whole-mosquito. The *Anopheles* microarray data-set confirms that many of the genes which are highly enriched in the tubules are yet to be annotated and have their functions characterized. Four genes which were highly enriched in the *Anopheles* tubules with *Drosophila* homologues highly enriched in the fruit-fly tubules were chosen for further study, as possible insecticide targets.

3.4.1 Microarray Validation by Quantitative PCR

Eight genes were chosen to validate the *Anopheles* microarray data-set. Four genes were chosen as they were putative genes-of-interest, and four other genes chosen at random. The genes chosen at random were all up-regulated during at least one life-stage in the *Anopheles* Malpighian tubules, and had specific expression patterns which would test the level of agreement between the microarray and qPCR data. Gene expression was quantified in all five groups of interest and the data from the microarray and qPCR converted into expression ratios to make them analogous, by comparing tubule expression to whole mosquito expression in each sample. The expression profiles of four genes – *AGAP001858, AGAP010892, AGAP007495* and *AGAP008386* - can be found in Figure 3.1. The expression profiles of the other four genes –*AGAP007752, AGAP012251, AGAP009005,* and *AGAP002587* - can be found in Chapters 4, 5, 6 and 7 respectively.

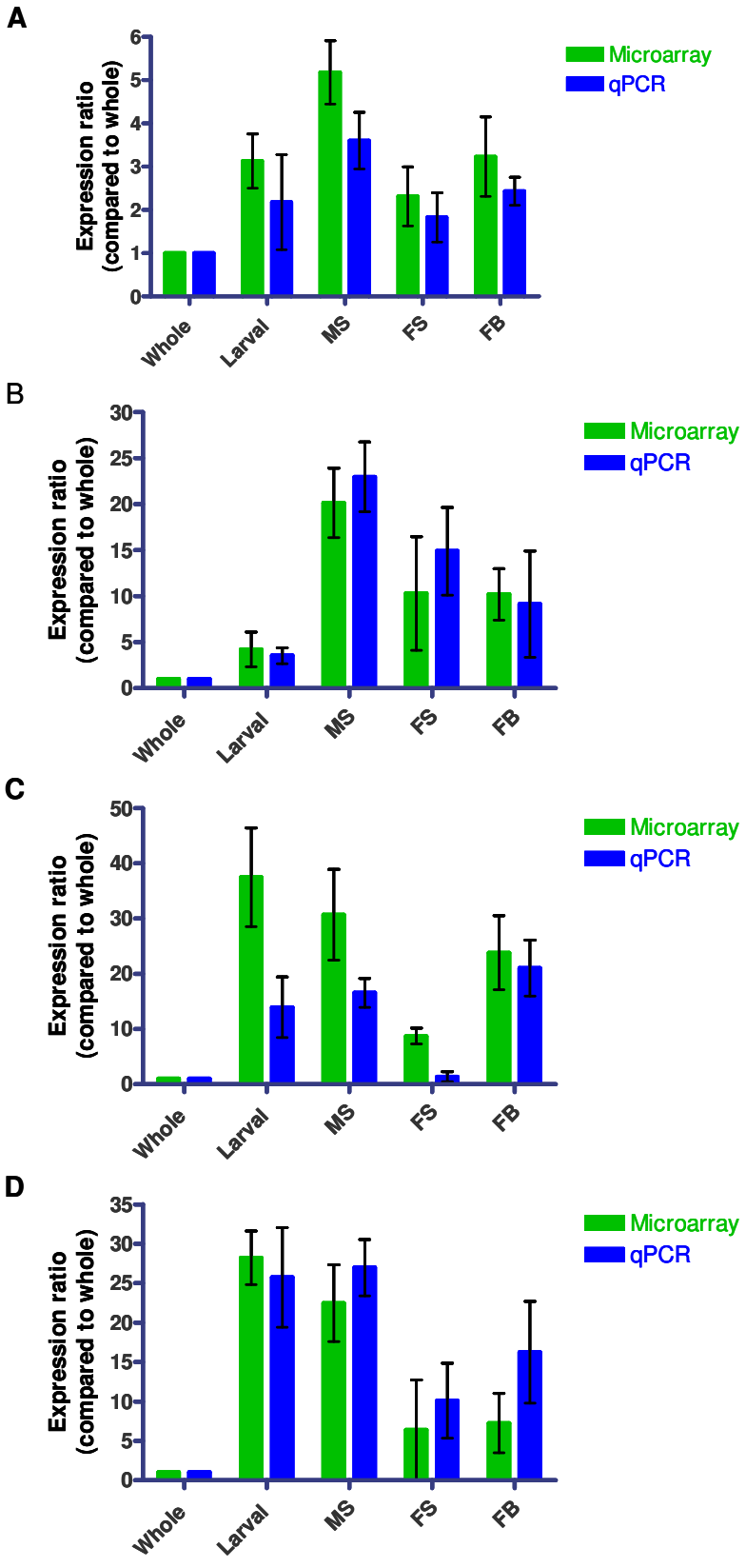


Figure 3.1 Comparison of *Anopheles* microarray and qPCR gene expression ratios for four genes. A: *AGAP002858*, B: *AGAP010892*, C: *AGAP007495* and D: *AGAP008386* (N=4, mean ± S.E.M.).

The eight *Anopheles* genes validated show good agreement between the microarray and qPCR expression ratios. The trends of up-regulation and down-regulation in comparison to the whole samples are consistent, although there are differences in the exact ratios. These are to be expected and could be for many reasons, such as different binding efficiencies of the microarray probes and qPCR primers, splice variants being detected in one set of experiments but not the other, or simple experimental error. As the trends in the eight genes checked are consistent we can assume that the microarray data-sets are a reasonably accurate representation of the expression pattern of transcripts in the *Anopheles* Malpighian tubules at the time-points assayed.

3.4.2 The Usefulness of the Data

Validation by qPCR shows that the *Anopheles* microarray data is a good representation of the Malpighian tubule transcriptome, but it is also important to determine that the microarray data is useful and novel. The hallmark of a well-designed microarray is that it contains information which could not be found elsewhere. In this case, we can determine this by comparing our data to that published by Dissanayake *et al*. They compared transcript expression in whole larvae, adult males, adult females, and adult females 3 h, 24 h, 48 h, 72 h, 96 h and 15 days after a blood-meal. By comparing gene data from both sets of microarrays, it is possible to determine whether our set of tubule-specific arrays tell us anything novel which could not be gotten from the whole-mosquito arrays. Figure 3.2 contains signal strength data for the *Anopheles* genes *AGAP002587* and *AGAP007495* for both whole mosquito, and the tubules, at different stages in the life-cycle. The whole-mosquito data is from the *Anopheles gambiae* Gene Expression Profile (Dissanayake *et al.*, 2006) which can be searched for gene-specific profiles. To date no research papers have been published containing microarray data primarily focused on the *Anopheles* Malpighian tubules.

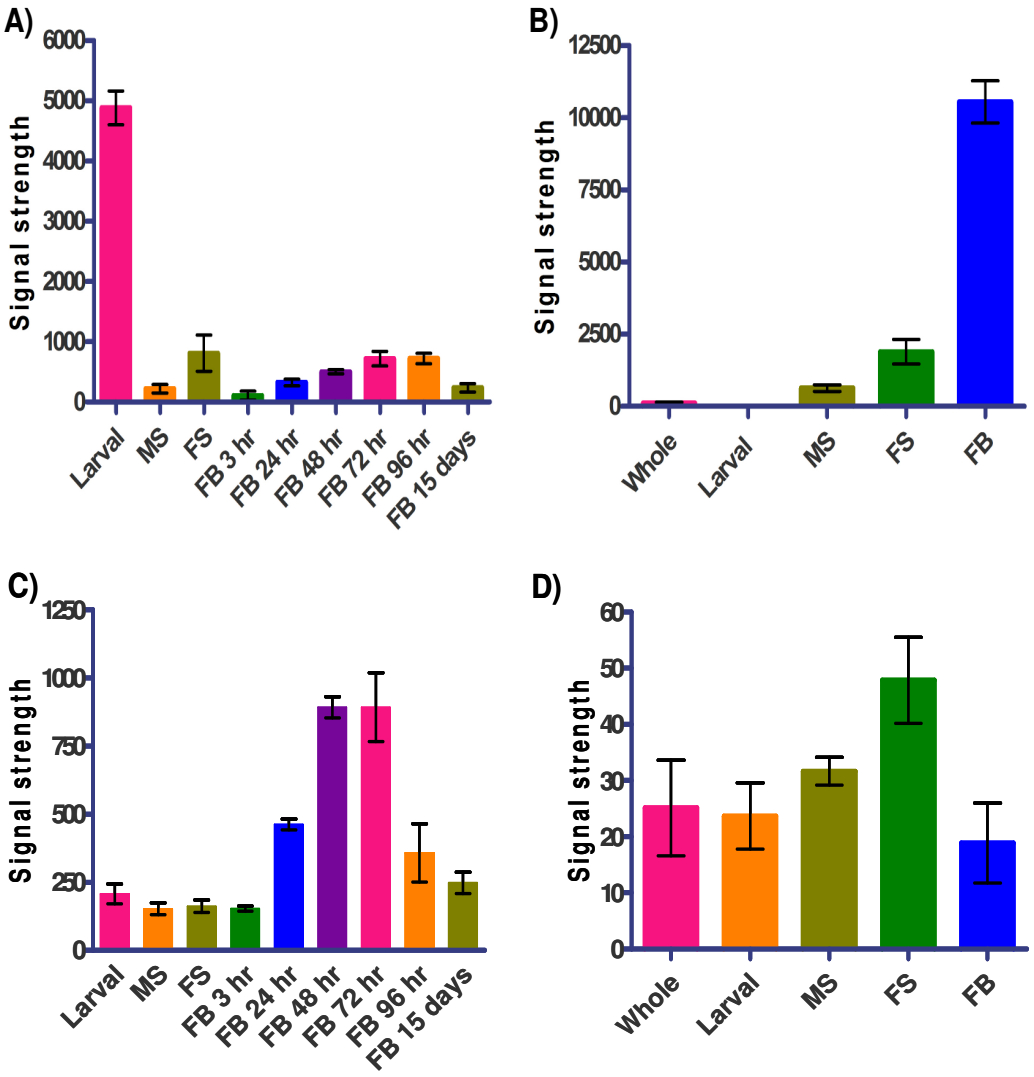


Figure 3.2 Signal strength comparison of *AGAP002587* and *AGAP007495* in whole-mosquito and Malpighian tubule microarrays. Expression of *AGAP002587* in A: whole mosquito and B: the *Anopheles* Malpighian tubules. Expression of *AGAP007495* in C: whole mosquito and D: the *Anopheles* Malpighian tubules (Dissanayake *et al.*, 2006). Signal strength was calculated as explained in Section 2.3. (mean ± S.E.M., N=4)

From the whole-mosquito data we would assume that *AGAP002587* was relatively larval specific, with a lesser role in adult. There is also no significant up-regulation of *AGAP002587* expression after a blood-meal. From our tubule-specific data it is apparent that although there may be high levels of transcription in larva, it is not in the larval Malpighian tubule. *AGAP002587* is highly up-regulated in the female tubules after a blood-meal, although from the data provided by Dissanayake *et al* we can determine that the up-regulation is not in all adult tissues. These microarrays are both informative, but they give completely different views of the gene expression profile of *AGAP002587*.

Conversely, the expression data show *AGAP007495* expression levels to be highly similar in the Malpighian tubules and whole-mosquito. Our tubule data is generally consistent with the whole-mosquito data, but offers some surprises and interesting expression patterns which could only be determined from a tissue-specific assay. Therefore, the microarrays in this study offer tubule-specific data which could not be obtained from whole-mosquito arrays. This is the argument of Chintapalli *et al*, that whole organism arrays under-represent tissue-specific changes in expression. Indeed in *Drosophila,* the tubules contribute only 5% of the whole-fly microarray signal (Chintapalli *et al.*, 2007).

3.4.3 Genes which are Highly Expressed in the Tubules

Although high levels of transcription do not always correlate with equivalent protein translation, genes which are highly expressed in a tissue are likely to be important to its function (Fagan *et al.*, 2007). In the *Drosophila* tubule microarray (Wang *et al.*, 2004) the most abundant transcripts are largely transporters and ion channels, as well as genes involved in transcription and translation. Transient genes such as transcription factors and cell-signalling genes do not have to be highly expressed to impact a tissue, and in terms of signal strength are often inconspicuous in a microarray dataset. The five genes with the largest signal strength for each mosquito life-stage can be found in Table 3.4. The genes which are annotated with an asterisk are transcripts which were originally mapped to the *Anopheles* genome, but have been removed from the latest gene build (Hubbard *et al.*, 2009).

The annotation of the *Anopheles* genome is at an early stage, and is therefore not yet as comprehensive as that of the *Drosophila* genome. Difficulty in predicting gene function arises from the low homology of many *Anopheles* transcripts to genes out-with the insects. It is also difficult to assign function using *in vivo* physiology in *Anopheles*, as the range of natural and transgenic mutant lines available in other species does not exist. Consequentially, approximately 35% of probe sets on the *Anopheles* GeneChip correspond to genes and transcripts which have not been electronically predicted to perform any known biological function, or belong to any protein family (Marinotti *et al.*, 2006).

Table 3.4 – Largest gene signal strengths in the *Anopheles* Malpighian tubule microarray at each life-stage. (N=4, mean ± S.E.M.). (Genes which appear in more than one category are colour-coded, function predicted by Ensembl (Hubbard *et al.*, 2009)).

Gene	Signal Strength	Function
Whole *Anopheles*		
AGAP010134	22239 ± 1371	Arrestin-like (signal transduction)
AGAP009863	21601 ± 1988	ATP dependent RNA helicase-like
AGAP009360	21382 ± 2922	Unknown
AGAP003768	21310 ± 1151.	Ribosomal subunit-like
AGAP001164	21015 ± 1117	Opsin-like (G-coupled protein receptor)
Larval tubules		
AGAP011061	25267 ± 1260	Unknown
AGAP012881	21986 ± 1285	Metallopeptidase-like
AGAP010469	20518 ± 934	Unknown
AGAP002599	20263 ± 1803	Ubiquitn-like
AGAP009441	20050 ± 125	Translation elongation factor-like
Male tubules		
AGAP010364	22392 ± 1652	Unknown
**ENSANGT00000011142*	21624 ± 1424	No longer mapped
AGAP003757	21305 ± 1758	Unknown
AGAP008219	20794 ± 1761	Cytochrome P450
AGAP000604	20206 ± 2450	Unknown
Female tubules		
AGAP010364	28314 ± 4680	Unknown
AGAP005802	24616 ± 3846	60s Ribosomal protein
AGAP004422	24386 ± 2632	Ribosomal protein-like
**ENSANGT00000011142*	23622 ± 3288	No longer mapped
AGAP004462	22691 ± 2634	Unknown
Female blood-fed tubules		
AGAP000604	27465 ± 973	Unknown
AGAP010364	24658 ± 654	Unknown
**ENSANGT00000011142*	20052 ± 691	No longer mapped
AGAP005802	19912 ± 790	60s Ribosomal protein
AGAP004422	19891 ± 917	Ribosomal protein-like

The function of around half of the genes in Table 3.4 is unknown, and cannot be speculated from sequence similarity to annotated proteins in other species. The genes from the microarray data-set which are highly expressed in the tubule and have an assigned function are largely involved in transcription (Hubbard *et al.*, 2009). Five genes are highly expressed in two or more of the adult tubule data-sets, which is slightly surprising as the pressures faced by the tubules change throughout the mosquito life-cycle. This suggests that these genes may essentially be tubule housekeeping genes. Although *ENSANGT00000011142* is present in all three adult tubule groups the transcript has been removed from the most recent GeneBuild, suggesting that annotation of this area of the genome is incomplete (Hubbard *et al.*, 2009). Whether *ENSANGT00000011142* is a genuine transcript which has been lost from the GeneBuild due to a highly specific expression pattern, or an artifact of non-canonical transcription or a similar process could be determined using Northern blotting. The microarray data-set supports the findings of other studies; that we know very little about the function of the genes which are most heavily transcribed in the Malpighian tubules of Dipterans (Wang *et al.*, 2004).

3.4.4 Genes which are Highly Enriched in the Tubules

By comparing the transcriptome of the tubules and whole mosquito it is possible to identify genes which have elevated expression in the tubules. This is one of the most effective ways of using microarrays, as in one set of experiments the researcher is able to unmask hundreds of genes which are putatively important for the functioning of a tissue. The microarray data-sets described in this thesis will not highlight every gene which plays a role in tubule development or physiology, but are hopefully a balanced view of the processes which are important for their everyday functioning at various life-stages. The five genes most highly up-regulated in the tubules in comparison to whole-mosquito at each life-stage can be found in Table 3.5. Transcripts which have been removed from the latest GeneBuild are marked with an asterisk (Hubbard *et al.*, 2009).

Table 3.5 – Genes highly enriched in the *Anopheles* Malpighian tubules in comparison to whole mosquito as determined by microarray (N=4, mean ± S.E.M.). (Genes which appear more than once are colour-coded, function predicted by Ensembl (Hubbard *et al.*, 2009)).

Gene	Enrichment (± S.E.M.)	Signal strength (± S.E.M.)	Putative Function
Larval tubules			
AGAP011682	4110 ± 768	14541 ± 1302	Unknown
AGAP010469	4034 ± 1628	20518 ± 934	Unknown
AGAP010361	3016 ± 1490	14338 ± 536	Unknown
AGAP005671	2720 ± 969	11096 ± 3123	Unknown
AGAP012881	2201 ± 915	1160 ± 291	Unknown
Male tubules			
AGAP000603	239 ± 75	4370 ± 635	Unknown
AGAP008731	116 ± 23	7244 ± 852	laccase-like
AGAP008593	89 ± 23	4552 ± 582	Unknown
AGAP004133	84 ± 8	8084 ± 757	Unknown
AGAP003758	62 ± 14	2209 ± 400	Unknown
Female tubules			
AGAP000603	393 ± 162	5119 ± 587	Unknown
**ENSANGT00000020967*	68 ± 16	679 ± 216	No longer mapped
AGAP010365	58 ± 11	6716 ± 1417	Unknown
AGAP006196	50 ± 5	965 ± 346	Unknown
AGAP008731	46 ± 9	2888 ± 496	laccase-like
Female blood-fed tubules			
AGAP004350	542 ± 142	16347 ± 1180	MFS-like
AGAP000603	1067 ± 480	12983 ± 502	Unknown
**ENSANGT00000020967*	624 ± 190	5098 ± 244	No longer mapped
AGAP008731	200 ± 38	13340 ± 262	laccase-like
AGAP003205	167 ± 21	503 ± 8	Unknown

One of the highly expressed genes which appear in Table 3.4 also appears in the Malpighian tubule up-regulated list. The gene *AGAP010469,* of unknown function, is both massively expressed and enriched in the larval tubule in comparison to the average whole-adult expression. The tubule-enriched list is dominated by genes of unknown function; only two genes have been annotated

and even then only by sequence similarity to genes in other species. The two genes have domains similar to those found in the Major Facilitator Superfamily (MFS) and the laccase family. The MFS is involved in the transport of small solutes in response to ion gradients, and is likely to be heavily represented in the tubules. The laccases are a family of copper-dependent enzymes involved in oxidation (see section 3.4.7). The S.E.M. calculated for the enrichment ratios are fairly substantial, due to very large tubule signal strengths being compared to very small whole mosquito signal strengths. This means that small inconsistencies in the whole mosquito signal strengths can result in highly variable enrichment ratios.

From the microarray data-set we can determine the number of transcripts which are up- or down-regulated in the tubules in comparison to the average expression in the whole-mosquito. Up-regulation in the tubules is defined as twice or greater the expression of whole mosquito, and down-regulation as half or less the expression of whole mosquito. Figure 3.3 illustrates the number of transcripts which are up- and down-regulated in comparison to whole mosquito at each life-stage.

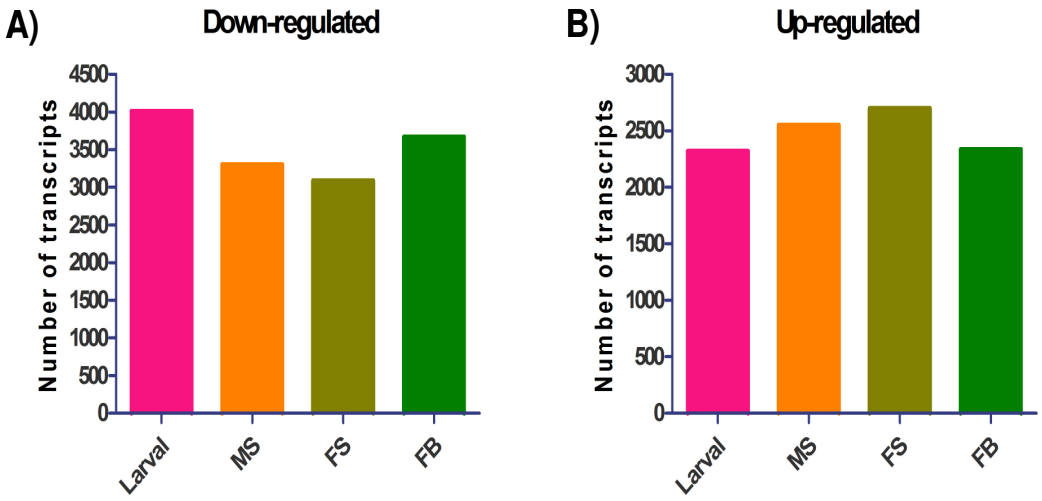


Figure 3.3 Expression of transcripts in the tubules in comparison to whole mosquito.
Number of transcripts which are A) down-regulated or B) up-regulated in each life-style.

The transcriptome of the *Anopheles* Malpighian tubules is very different from that of the whole organism. At each life-stage approximately 6,000 genes are either enriched or down-regulated in the tubules when compared to the average

whole-mosquito gene expression. The number of genes which are down-regulated is larger than the number up-regulated during all life-stages. It is unlikely that all of the genes which are up-regulated are tubule specific; some will be enriched in all epithelia, others will be involved in osmoregulation, detoxification or immune-challenge, functions which are shared with other tissues.

3.4.5 Comparison of the Malpighian Tubule Transcriptome During Different Life-stages

By comparing the transcriptome of the Malpighian tubules at different life-stages it is possible to predict the number of genes required to switch the focus of the tubules. Figure 3.4 shows the number of transcripts which are significantly up- or down-regulated in the tubules at the life-stages assayed (as determined by Student's *t*-test).

A surprising number of genes are involved in altering the function of the tubules. The largest change in the transcriptome appears to be between the larval and adult modes, when the tubules switch from water expulsion to conservation. The smallest change is in the female tubules after the mosquito takes on a blood-meal, and even this requires around 700 genes to be up-regulated and the same to be down-regulated. The transcriptome of the tubules in sugar-fed females is substantially different from that of males, probably in preparation for haematophagous feeding and sequestration of the nutrients required for oogenesis. Gene up-regulation ranges from small increases in lowly expressed genes to signal strength increases of tens of thousands. As annotation of the *Anopheles* genome and transcriptome improves, it will be possible to use this data to gain new insight into the functions of the tubule during each stage of the life-cycle.

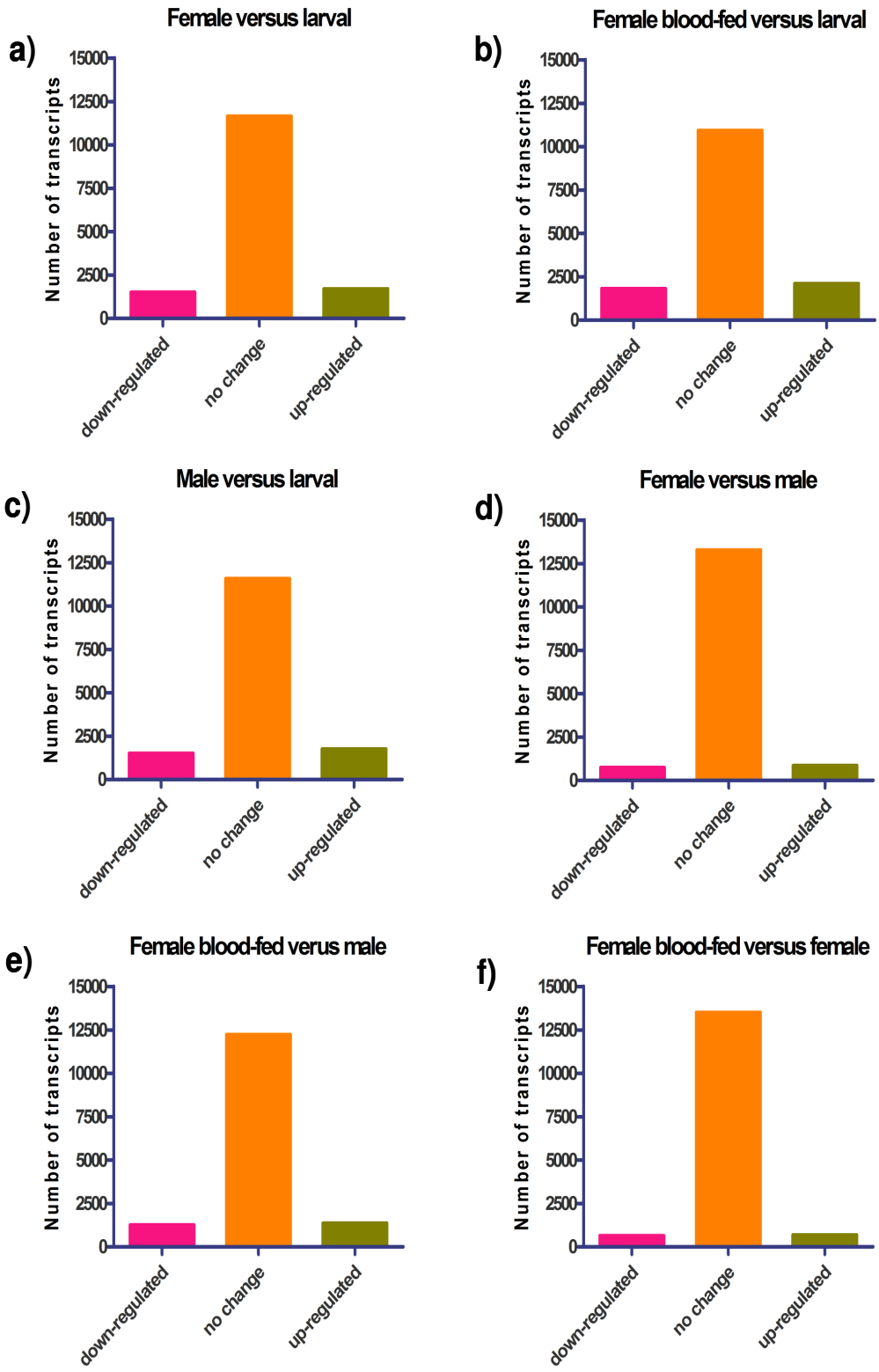


Figure 3.4 A comparison of the number of transcripts significantly up- or down-regulated in the *Anopheles* Malpighian tubules at the four life-stages assayed. (Statistical significance determined by Student's *t*-test, $p<0.05$).

3.4.6 Genes for Further Investigation

The information which can be gained from the *Anopheles* Malpighian tubule microarray data-set is useful in numerous ways. The data can be used to:

- Identify genes which are highly enriched in the *Anopheles* Malpighian tubules and are therefore likely to be functionally important

- Identify genes which are differentially expressed in the *Anopheles* tubules during the life-cycle. This can help determine processes which are important to the tubule while the mosquito is under different pressures during the life-cycle, such as post-haematophagy

- Identify genes with tubule-expressed homologues in other insects when analysed in combination with other Diptera Malpighian tubule microarrays. Conservation of these genes across species suggests that they may be important for renal function

- Identify genes vital for tubule function which could potentially be Diptera insecticide targets, when analysed in combination with other Diptera tubule microarrays. These could be genes with high homology to other Diptera genes for wide-action insecticides, or low homology for *Anopheles* or mosquito specific targeting

The aim of this investigation was to study genes which were highly enriched in the *Anopheles* tubule, differentially expressed across the life-cycle, and had a good *Drosophila* homologue which was also enriched in the tubules. The first stage of this process was to identify genes which met all of these criteria.

3.4.6.1 The Gene Selection Process

The initial microarray analysis was performed on the entire *Anopheles* data-set. The priority for the first round of selection was to choose genes which had strong signal strengths in the tubule in comparison to the average whole-mosquito expression. This highlighted genes which were highly expressed in the tubule but not in all tissues. It was also important to choose genes which had robust array data with consistent signal strengths and microarray ‘present’ calls

in the data from each life-stage. The next criterion for selection was that the gene should have an interesting expression pattern in the tubules during the life-cycle. This highlighted genes which were enriched or decreased in response to the changing life-style of the mosquito. This included genes which were heavily enriched in the larval tubules but not in the adult, genes which were adult specific but not larval, and genes which were heavily transcribed in one adult stage but not the others, such as after a blood-feed. After the initial analysis the pool was cut from ~15,000 genes to ~200 genes for further consideration.

As much of the experimental work would be carried out in *Drosophila*, the next stage of selection was to identify genes which had a good *Drosophila* homologue. A good homologue was defined as a highly conserved gene with enriched expression in the *Drosophila* tubules. Several databases available on the internet were used to find highly conserved homologues. The ENSEMBL Genome Browser is a joint project run between EMBL-EBI and the Sanger Institute, and provides automatic annotation on sequenced eukaryotic genomes (Hubbard *et al.*, 2002). ENSEMBL was used to determine which of the *Anopheles* genes had a recognisable *Drosophila* homologue. The basic local alignment search tool (BLAST) was also used to validate homologous genes by mutual BLAST 'best hit' (Benson *et al.*, 2009). The other website utilised at this stage of selection was the *Drosophila* Tubule Array Dataset (Wang *et al.*, 2004), which contains a searchable database of the *Drosophila* tubule microarray data-set. It is possible to search for genes of interest and determine whether they are up-regulated in the tubules in comparison to whole *Drosophila*. After the second stage of selection the number of genes under consideration was reduced from ~200 genes to 40 genes.

The final stage of selection involved a more detailed gene analysis on the genes under consideration. The gene analysis focused on:

- The predicted function of the gene in *Anopheles* and *Drosophila*
- Research which had been published on the *Drosophila* gene, and the mutant stocks and resources that were already available
- Human homologues of the gene, and research which had been published on their function

The *Drosophila* homologues were investigated to determine the extent of gene characterisation, and any resources which could be utilised in this study. The extent of homology to human genes was determined (Benson *et al.*, 2009), as it is preferable that any potential insecticide targets do not have a highly conserved human homologue.

3.4.6.2 Genes Selected for Study

Four homologous gene-pairs were chosen from the *Anopheles* and *Drosophila* Malpighian tubule microarrays for further investigation. Preference was given to gene-pairs which had not previously been studied, and had predicted functions which had not been investigated in the Malpighian tubules. The four gene-pairs chosen from the *Anopheles* and *Drosophila* microarray data-sets can be found in Table 3.6.

Table 3.6 The four homologous gene-pairs chosen from the *Anopheles* and *Drosophila* Malpighian tubule microarrays for further study (Benson *et al.*, 2009; Hubbard *et al.*, 2009).

***Anopheles* gene**	***Drosophila* gene**	**Putative function**
AGAP007752	*CG15406*	Sugar transporter
AGAP012251	*Picot (CG8098)*	Inorganic phosphate co-transporter
AGAP009005	*ZnT35C (CG3994)*	Zinc transporter
AGAP002587	*CG8028*	Monocarboxylate transporter

None of the genes had been previously investigated in either *Anopheles* or *Drosophila*. The sequence of *Picot* was submitted to FlyBase and the gene named (Da Lage, submission FBrf0114357) in 1997, but without further exploration. These eight genes and their potential as insecticide targets are discussed in Chapters 4, 5, 6 and 7. The evolutionary trees presented in Chapters 4, 5, 6 and 7 were compiled by ENSEMBL, and show genes which have diverged from a common ancestor. They were generated using the Gene Orthology/Paralogy prediction method, using protein homology to predict the most likely phylogenetic tree which represents the evolutionary history of a family (Vilella, 2009). The evolutionary trees are built from the annotation of all of the genomes

sequenced to date, and show both duplication events (gene is annotated with a red square) and speciation events (gene is annotated with a blue square).

3.4.7 Additional Genes of Interest from the Anopheles Microarray

Although not all of them reached our criteria for further study, numerous genes which warrant further investigation were identified during the microarray data-set analysis. Three particularly interesting genes are *AGAP008731, AGAP010725* and *AGAP004005*.

AGAP008731, which is from the laccase family, is highly up-regulated in the Malpighian tubules of male, female, and female blood-fed mosquitoes. Laccases catalyse the oxidation of phenol substrates and other aromatic compounds. They are part of the superfamily of multicopper oxidases, and usually contain several copper atoms as part of their catalytic centre. *AGAP003738*, a second gene from the laccase family, is also up-regulated in the adult *Anopheles* tubules, particularly in blood-fed females. These enrichments corroborate the work of Gorman *et al*, who found *AGAP008731* and *AGAP003738* to be up-regulated in the tubules in response to a blood-feed or immune challenge (Gorman *et al.*, 2008). The *Drosophila* gene *CG3759* is also a member of the laccase family, is up-regulated in the larval and adult tubules approximately seven-fold (Wang *et al.*, 2004), and is further up-regulated after an immune challenge (De Gregorio *et al.*, 2002). In conjunction with their role in sclerotization (Sugumaran *et al.*, 1992; Dittmer *et al.*, 2004), the laccase family may be involved in the detoxification of phenols in the tubules, and the function of the insect immune system. Detoxification and immune function are both important roles of the *Drosophila* Malpighian tubules (McGettigan *et al.*, 2005; Yepiskoposyan *et al.*, 2006).

Another gene of interest identified from the *Anopheles* microarray is *AGAP010725*, which is enriched in both the larval and adult tubules, particularly after haematophagy. The *Drosophila* homologue of *AGAP010725*, *Prestin,* is a member of the Solute Carrier 26 family, and is enriched in the larval and adult tubules. *Prestin* was named for its homology to a human gene which functions as a motor protein in cochlear outer hair cells (Zheng *et al.*, 2000). Although tenuous links have been made between *Prestin* and the *Drosophila* auditory

system (Weber *et al.*, 2003), the functional domains present in *AGAP010275* and *Prestin* suggest that they are involved in anion transport, and recent data suggests that *Prestin* is an oxalate transporter (M F Romero, personal communication). *Prestin* is an example of a gene which is published as having a highly-specific function in a particular tissue, when its expression is actually much more widespread.

AGAP004050 is also up-regulated in the adult *Anopheles* tubules, and is analogous to the *Drosophila* gene *Doublesex (dsx).Dsx* is a *Drosophila* transcription factor which regulates sexual somatic cell differentiation. Sex-specific isoforms of *dsx* are formed by RNA alternate splicing in both *Drosophila* and *Anopheles* (Coschigano and Wensink, 1993; Scali *et al.*, 2005). Microarray analysis shows *dsx* expression to be highly up-regulated in the *Drosophila* tubules (Wang *et al.*, 2004), which is surprising as hundreds of research papers have been published on *dsx*, but its function in the tubules has not been investigated. The up-regulation of the transcription factor in both the fly and mosquito renal system points towards a new area of investigation for *dsx* researchers.

3.5 Discussion

In this section the results of microarray experiments comparing the transcriptome of whole *Anopheles* to the Malpighian tubule transcriptome at different life-stages are discussed. The selection of four homologous gene-pairs for further investigation as possible insecticide targets is also examined. The genes-of-interest are all putative transporters highly expressed in the *Anopheles* and *Drosophila* Malpighian tubules.

3.5.1 Microarray Analysis and Validation

The *Anopheles* microarray data-sets give a novel insight into the mosquito Malpighian tubules, and how they cope with changing pressures during the life-cycle. The array also confirms that the *Anopheles* genome is not yet as comprehensively annotated as the *Drosophila* genome. That only two of the most highly enriched genes in the tubules have been assigned a putative function is not surprising, as *in vivo* physiological studies have been difficult in *Anopheles,* which suffers from a lack of reliable transgenics. Greater inroads have been made in characterizing the renal function of *Aedes aegypti* (Petzel *et al.*, 1987; Plawner, 1991; Pullikuth *et al.*, 2006). The majority of *Anopheles* studies are geared towards understanding the relationship between *Anopheles* and *Plasmodium*, and so renal physiology is relatively untouched. Microarray analysis of organs and tissues rather than whole-mosquito should help delineate genes which are specific to a particular tissue or function, such as those involved in insecticide resistance.

The qPCR experiments used to validate the *Anopheles* microarrays show a reliable and consistent agreement with the microarray data. Figure 3.5 compares the tubule enrichment ratios from the array and qPCR for the four *Anopheles* genes validated in this chapter.

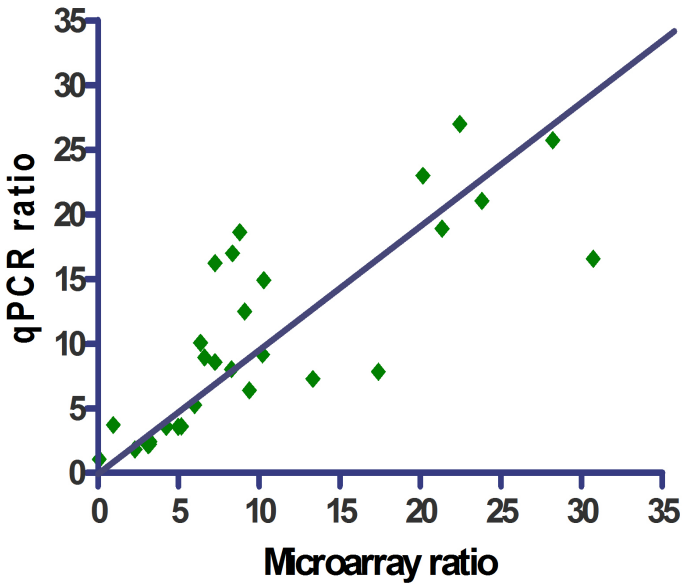


Figure 3.5 Comparison of the average microarray and qPCR tubule enrichment ratios for the four genes from the *Anopheles* microarray validated in this chapter. The line shown is y=x.

The ratio comparison showed that up- and down- regulation trends were consistent in all eight genes tested. The qPCR ratios varied slightly from those calculated from the microarray, but this may be for many reasons, such as probe saturation in the microarray, or the probe set not recognizing all of the isoforms of a transcript. The variation in the ratios has no consistent pattern, suggesting that neither the GeneChip nor qPCR is over-estimating the amount of transcript present.

The prevalence of ion transporters and channels among the genes enriched in the *Anopheles* tubule was expected. Several of these gene-types have already been studied in the *Drosophila* tubules, for example the V-ATPases, aquaporins and Na^+/K^+ -ATPases (Lebovitz *et al.*, 1989; Maddrell and O'Donnell, 1992; Dow, 1995). Other genes such as the monocarboxylate transporters, sugar transporters, zinc transporters, iron transporters, sodium-dependent phosphate transporters and ABC transporters are all represented by multiple, highly enriched genes, and are not as well characterized. Very little is known about the physiological role of these families in the Malpighian tubules, but their enrichment in both the *Anopheles* and *Drosophila* tubules suggests that they are functionally important.

A coordinated change in the expression of thousands of genes is required to alter the function of the tubules during the life-cycle. Phenotyping the function of these genes would provide insight into how the tubules are able to change from water excretion to conservation, and vice versa, with such speed. It seems likely that a combination of peptide hormones, post-translational protein modification, and gene transcription are responsible for altering tubule function.

Several Diptera microarrays have been published as free-to-access searchable databases, and this trend with hopefully continue (Dissanayake *et al.*, 2006; Chintapalli *et al.*, 2007). These data-sets provide a wealth of information on novel genes, as well as some surprising insight into genes which have been studied for many years. They also make it effortless for researchers from other fields to check the expression of homologous genes-of-interest in insect tissues. This may even lead them to consider using Dipteran insects as their model organism of choice.

3.5.2 Gene Selection

Choosing four genes-of-interest from the 12,000 which are annotated in *Anopheles* required a strict set of criteria. The first criterion was that the genes should be highly expressed in tubule in comparison to whole mosquito. We cannot assume that genes which are highly expressed are essential, but it seems unlikely that the mosquito would expend energy and transcriptional components enriching a protein-coding transcript which was of no use. Highly expressed genes were chosen in the hope that they were important, and therefore essential for survival, making them good insecticide targets. Genes with a good *Drosophila* homologue were chosen for two reasons. Firstly, sequence conservation over ~150 million years of divergence (Yeates and Wiegmann, 1999) would suggest these were important genes. Secondly, all of the transgenic work was to be carried out in *Drosophila*. A good homologue was defined as a gene with high sequence homology, which was also enriched in the *Drosophila* Malpighian tubules.

The genes chosen for further study were novel in both *Anopheles* and *Drosophila*. Although it may have been easier to choose genes which were

already partially studied, this would defeat the purpose of the microarray – to find novel renal genes which are important in Diptera.

Thus far the cellular processes which have been investigated in both the *Drosophila* and the *Anopheles* Malpighian tubules have shown highly conserved mechanisms of action. We cannot assume that this will happen for all processes or functions, but continued parallel studies of the two insects will help establish when *Drosophila* is a good model for *Anopheles*. Part of the aim of this study was to compare data for the two species wherever possible, and to determine whether it is feasible to study a gene which is, for example, highly-enriched after a blood-meal, in a species which is not haematophagous.

3.6 Conclusions

The *Anopheles* microarray data-sets provide a useful insight into the types of gene which are highly expressed in the Malpighian tubules. Although high levels of transcription do not always correlate with high levels of translation to protein, conservation of enrichment in homologues from two species suggests an important gene function. Homologous gene-pairs which are enriched in the renal system of *Anopheles* and *Drosophila* are likely to be functionally interesting, as well as possible insecticide targets. Performing comparable microarrays in both mosquito and fly results in a powerful data-set, for understanding genes with conserved expression patterns, as well as those gene families which have expanded or branched differently. Ion transporters and channels are particularly prevalent in both the *Anopheles* and *Drosophila* Malpighian tubules. Four homologous gene-pairs were chosen from the transport genes up-regulated in the renal system of *Anopheles* and *Drosophila*. The four pairs of genes are the sugar transporters *AGAP007752* and *CG15406,* the phosphate transporters *AGAP012251* and *Picot,* the zinc transporters *AGAP009005* and *ZnT35C,* and the monocarboxylate transporters *AGAP002587* and *CG8028.* These genes were selected for further study due to their massive up-regulation in the *Anopheles* and *Drosophila* tubules and association with functions poorly understood in the insect renal system.

Chapter 4 - *AGAP007752* and *CG15406* as Insecticide Targets

4.1 Summary

In Chapter 3 it was established that many inorganic and organic transporters are highly enriched in the *Anopheles* Malpighian tubules, and therefore may be effective insecticide targets. Particularly conspicuous among the tubule-enriched data-set are genes putatively involved in sugar transport. Little is understood about the functional role of sugar transporters in the tubules of *Anopheles* and *Drosophila*. The introduction to this chapter describes the principal sugar transporter families in Diptera and mammals, and our current understanding of their physiological role. The *Anopheles* gene *AGAP007752* and *Drosophila* gene *CG15406* are putative sugar transporters, and are the focus of this study. Analysis of microarray data suggests that they are highly expressed in the adult Malpighian tubules of *Anopheles* and *Drosophila*, and the results presented in this chapter confirm their expression as both transcripts and proteins in the tubules. Both genes are expressed throughout the length of the tubules in the principal cells, and *CG15406* is localised to the basolateral membrane. The importance of *CG15406* for fly survival through to adulthood is demonstrated using the UAS/GAL4 system to perform knockdown of gene expression. Knockdown of *CG15406* throughout the fly causes lethality at the pupal stage, and knockdown of *CG15406* solely in the Malpighian tubules reduces the viability of the fly. The importance of *CG15406* in tubule secretion is also investigated, although gene knockdown of *CG15406* has no significant effect on the rate at which fluid is secreted from the tubules. Further experiments to elucidate which sugars are being transported by *CG15406* in the tubules are also inconclusive. Finally, the *Anopheles* and *Drosophila* data collected for *AGAP007752* and *CG15406* is discussed, as is the expected function of *AGAP007752* and *CG15406*, and their likely success as insecticide targets.

4.2 Introduction

Sugars are not only a source of cellular energy, but form the building-blocks of the polysaccharides which provide cells with structural support. Although their uptake and metabolism is essential for survival, the processes by which insects sense, regulate and utilize sugars are not well understood. The genes involved, such as sugar transporters, are not only functionally interesting but may make attractive insecticide targets. Two such genes, *AGAP007752* and *CG15406*, are up-regulated in the Malpighian tubules of *Anopheles* and *Drosophila* respectively. *AGAP007752* and *CG15406* are unstudied, but contain motifs which are characteristic of sugar transporters in mammals.

4.2.1 The Importance of Sugars

Monosaccharides such as glucose and fructose are versatile molecules which can exist in many forms. They can isomerise to be galactose and mannose, or replace a hydroxyl group with a side chain and become sugar derivatives such as glucosamine and N-acetylglucosamine. They can form disaccharides such as sucrose, lactose and maltose by linking together two monosaccharides, or form oligosaccharides and polysaccharides such as glycogen. Sugars join together through condensation reactions between hydroxyl (–OH) groups. Each sugar contains multiple hydroxyl groups which can link to another monosaccharide, resulting in an extremely large number of potential polysaccharides. One of the most abundant polysaccharides in nature is chitin, which is found in insect exoskeletons and is a linear polymer of N-acetylglucosamine. Oligosaccharides can also be linked to lipids and proteins to form glycolipids and glycoproteins, which are a large component of cell membranes. In addition to their roles in cell structure, the monosaccharides are important as an energy source. They can be broken down to release small amounts of energy, or stored as glucose subunits in the branched polysaccharide glycogen. Glycogen can be synthesised or degraded rapidly depending on the energy need of the organism and the food sources available. Therefore, the transport and storage of sugars must be carefully regulated to ensure that all tissues have an adequate supply.

4.2.2 Sugar Uptake in Anopheles and Drosophila

Anopheles mosquitoes consume sugar from plant sources such as floral nectar and honeydew as part of their staple diet (Foster, 1995). Laboratory based studies suggest that males feed approximately twice nightly, with females consuming sugar meals before and between gonotrophic cycles (Gary and Foster, 2006). Sugar detection in *Anopheles* and *Drosophila* is through neurons clustered in gustatory sensilla (McIver, 1982; Nayak, 1983). In *Drosophila* gustatory sensilla are mainly found in the labellum, but are also situated on the male forelegs (Stocker, 1994). *Anopheles* and *Drosophila* contain 76 and 66 gustatory genes respectively, encoding taste receptors which enable them to differentiate between compounds (Clyne *et al.*, 2000; Hill *et al.*, 2002). The Anophelines show preference for particular plant varieties when feeding, evidently due to the type and abundance of sugar which is found in each plant (Manda *et al.*, 2007).

4.2.3 Sugar Transporters in Anopheles and Drosophila

Understanding of the *Anopheles* and *Drosophila* gustatory systems has increased greatly since the publication of their sequenced genomes. How other tissues sense, utilise and regulate sugars after consumption has not been explored. Several gene families have been electronically annotated as putative sugar transporters in *Drosophila*. The H^+/sugar symporter family which contains *sut1, sut2, sut3, Glut1* and *CG7882* has been annotated but not studied (Hubbard *et al.*, 2009). *Sut1*, *sut2* and *Glut1* are expressed throughout the fly with no obvious tissue specificity. *Sut3* is highly testis specific, and *CG7882* is highly tubule specific (Chintapalli *et al.*, 2007). The H^+/sugar symporter family is thought to be homologous to the human genes *GLUT1, GLUT2, GLUT3* and *GLUT4* (see section 4.2.4). Another family of genes classed as glucose transporters through sequence annotation are *CG1213, CG10960, CG6484, CG30035, CG8234, sut4* and *CG1208* (Hubbard *et al.*, 2009). These genes are expressed throughout the fly (Chintapalli *et al.*, 2007), and are annotated as being homologous to the human genes *GLUT6* and *GLUT8* (See section 4.2.4).

The *Drosophila* gene under investigation in this study, *CG15406*, is part of the sugar transporter family which also includes *CG15408, CG3285, CG14606, CG14605, CG33281* and *CG33282* (Hubbard *et al.*, 2009). These genes are all

highly specific in the adult fly to the tubules or testis, with minimal expression elsewhere (Chintapalli *et al.*, 2007), and as yet remain uncharacterised. Presently the only species which have had their genomes sequenced and are annotated as having a homologous gene family are *Anopheles* and *Aedes aegypti*, suggesting that the gene family is insect specific (Figure 4.1). The family are annotated as sugar transporters because they contain many of the classic motifs which define the mammalian GLUT genes (see Section 4.2.5).

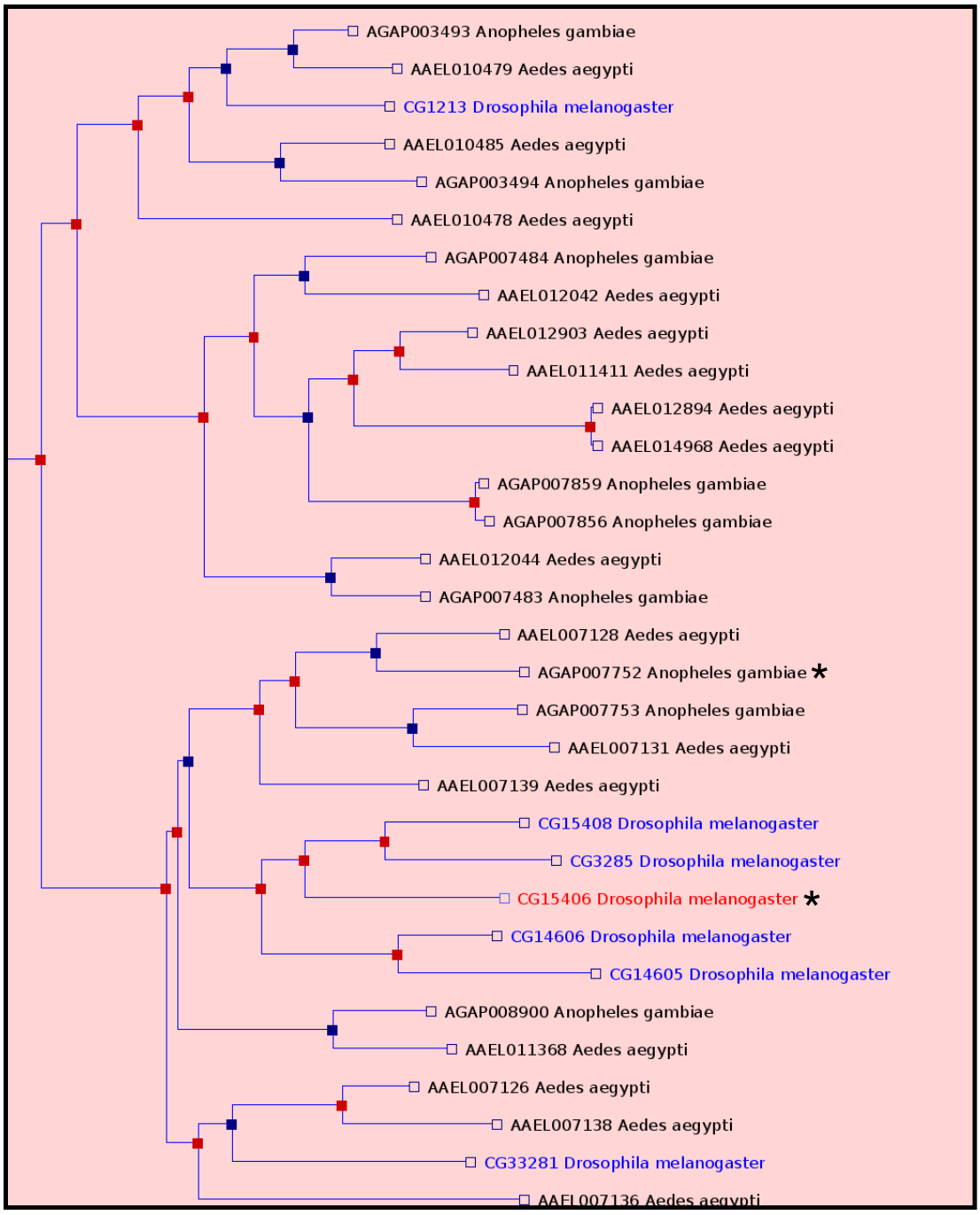


Figure 4.1 Gene tree image of *AGAP007752* and *CG15406* and their homologues. (Tree generated by Ensembl, see Section 3.4.6.2 (Hubbard *et al.*, 2009; Vilella, 2009)).

4.2.4 Sugar Transport in Mammals

The GLUTs are a family of integral membrane transporter proteins responsible for the facilitated diffusion of monosaccharides such as glucose, fructose, galactose, and mannose, across biological membranes. Members of the GLUT family have been identified in vertebrates and invertebrates, but have primarily been studied in human, mouse and rat. The human GLUT family has 13 members, at least one of which is expressed in every tissue in the body, where they transport sugars by electrochemical gradient. The GLUTs have been categorised into three classes by gene homology and function (Uldry *et al.*, 2001). Class I contains *GLUT1-4*, which have been characterized as glucose transporters (Keller and Mueckler, 1990). Class II contains *GLUT5, GLUT7, GLUT9* and *GLUT11*; *GLUT5* is a fructose transporter (Burant *et al.*, 1992), and *GLUT7* a fructose/glucose transporter (Cheeseman, 2008). *GLUT9* has recently been linked with regulation of serum urate levels in humans (Matsuo *et al.*, 2008).The class III genes are *GLUT6, GLUT8, GLUT10, GLUT12* and *HMIT*. *GLUT 8, GLUT10* and *GLUT12* are classed as glucose transporters (Doege *et al.*, 2000; Dawson *et al.*, 2001; Rogers *et al.*, 2003), and *HMIT* as an H^+/myo-inositol symporter (Uldry *et al.*, 2001).

The general structure of the GLUT family is twelve transmembrane domains with the carboxy- and amino-terminal ends on the cytosolic side of the membrane, and an N-linked oligosaccharide sidechain on the first or fifth extracellular loop (Uldry and Thorens, 2004). The three classes of GLUTs also exhibit a distinctive pattern of motifs on each of the intracellular loops (see Figure 4.2). Each gene has a unique expression pattern in humans; for example *GLUT1* is expressed ubiquitously, but *GLUT5* is intestine specific. One of the most striking features of the GLUTs is their ability to differentiate between sugars. Each transporter has a unique profile of sugars which it will transport, some with high affinity, and others with low affinity (Uldry and Thorens, 2004). Transport efficiency is likely to be determined by the border motifs of the water-filled passageway which joins the extracellular and intracellular domains in each transporter, as demonstrated in *GLUT1* (Salas-Burgos *et al.*, 2004). The GLUTs are currently being investigated as therapeutic targets for normalising glycaemia and other sugar-regulatory illnesses.

4.2.5 The Putative Sugar Transporters AGAP007752 and CG15406

The putative sugar transporters *AGAP007752* and *CG15406* are classed as gene homologues by ENSEMBL with an identity of 36% and an Expect value (e-value) of 2.9e-108 (Hubbard *et al.*, 2009). *AGAP007752* and *CG15406* contain many of the conserved motifs which define the human GLUT gene family, and have a similar predicted twelve transmembrane-domain structure (see Figure 4.2).

In mammalian GLUT genes the lack of a QLSG motif in the seventh transmembrane domain usually correlates with a higher affinity for fructose transport than glucose transport (Uldry and Thorens, 2004). Although *CG15406* and *AGAP007752* do not show conservation of the QLSG sequence most of the members of the *CG15406* gene family do, suggesting that *CG15406* is not necessarily a glucose transporter. The exposed amino acid sequences around the pore, such as the QLSG domain, are thought to determine which sugars are transported. Whether the *Anopheles* and *Drosophila* sugar transporters contain the N-linked oligosaccharide sidechain on the first or fifth extracellular loop which is found in human GLUTs is not known, although there are putative glycosylation sites in *CG15406* and *AGAP007752*.

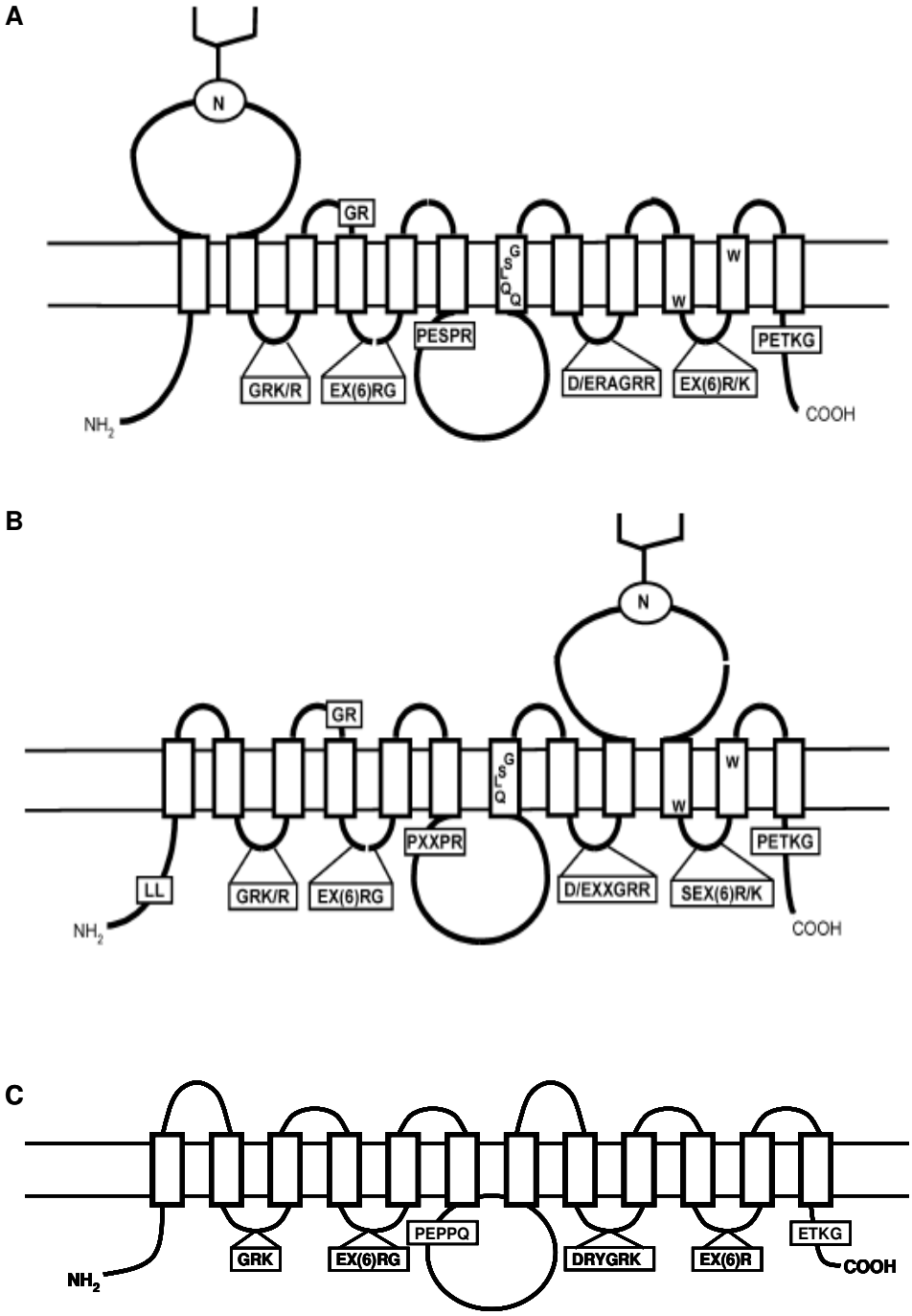


Figure 4.2 The predicted structures of the mammalian GLUT family and *CG15406*. The structures of A: Class I and Class II and B: Class III members of the human GLUT family (Uldry and Thorens, 2004); C: the predicted structure of *CG15406* showing conservation of human GLUT signature sequences. *CG15406* is predicted to have a similar 12 TM domain structure to the human GLUTs, with the carboxy- and amino-terminal ends on the cytosolic side of the membrane. All of the motifs found in the intracellular loops are at least partially conserved between the human GLUTs and *CG15406*. *CG15406* structure predicted using protein prediction tools provided by the Centre for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/TMHMM/) and ExPASy Proteomics Tools (http://www.expasy.ch/tools/).

The expression pattern of *CG15406* in adult and larval *Drosophila* can be found in Table 4.1. The *CG15406* transcript is up-regulated in two tissues when compared to the average expression in whole-fly; the adult and larval Malpighian tubules. The transcription pattern of *AGAP007752* in *Anopheles* is not yet known. The function of *AGAP007752* and *CG15406* in the tubules is not yet known. A likely role is the uptake of sugars from the haemocoel for use as a cellular energy source. The tubules are an extremely active tissue, which is known to survive much better *in vitro* if maintained in a bathing medium which contains an energy source such as glucose (Berridge, 1966). Therefore, it is likely that the tubules need a constant source of sugar to energise osmoregulation and ion transport *in vivo*.

Table 4.1 *CG15406* expression in larval and adult *Drosophila* (Chintapalli *et al.*, 2007).

Tissue	mRNA Signal	Present Call	Enrichment	Affy Call
Brain	3 ± 1	1 of 4	0	Down
Head	6 ± 0	0 of 4	0	Down
Thoracicoabdominal ganglion	5 ± 1	0 of 4	0	Down
Salivary gland	15 ± 2	0 of 4	0.09	Down
Crop	2 ± 0	0 of 4	0	Down
Midgut	19 ± 1	4 of 4	0.1	Down
Tubule	**4941 ± 253**	**4 of 4**	**29.8**	**Up**
Hindgut	8 ± 1	0 of 4	0	Down
Heart	11 ± 6	3 of 4	0.07	Down
Fat body	29 ± 29	3 of 4	0.18	Down
Ovary	3 ± 0	0 of 4	0	Down
Testis	10 ± 0	4 of 4	0.1	Down
Male accessory glands	8 ± 2	0 of 4	0.1	Down
Virgin spermatheca	26 ± 17	3 of 4	0.16	Down
Mated spermatheca	12 ± 1	3 of 4	0.07	Down
Adult carcass	18 ± 4	2 of 4	0.1	Down
Larval CNS	3 ± 2	0 of 4	0.02	Down
Larval Salivary gland	3 ± 0	0 of 4	0.02	Down
Larval midgut	9 ± 2	1 of 4	0.06	Down
Larval tubule	**4193 ± 108**	**4 of 4**	**25.3**	**Up**
Larval hindgut	63 ± 3	4 of 4	0.39	Down
Larval fat body	14 ± 3	0 of 4	0.1	Down
S2 cells (growing)	3 ± 0	0 of 4	0.02	Down
Whole fly	**165 ± 22**	**4 of 4**		

4.3 Results

In this section the results from the investigation into the genes *AGAP007752* and *CG15406* as possible insecticide targets are described. The expression and localisation of *AGAP007752* and *CG15406* was explored at both the transcript and protein level using microarrays, qPCR, *in situ* hybridisation, Western blotting and immunocytochemistry. *CG15406* was then further investigated in *Drosophila*, where RNAi-mediated gene knockdown was used to demonstrate that its expression is required for survival through to adulthood. The effect of *CG15406* knockdown on the rate of fluid secretion from the tubules in *Drosophila* was also determined. Further investigations into the sugar or sugars likely to be transported by *CG15406* and *AGAP007752* are also presented, although they proved inconclusive.

4.3.1 AGAP007752 Expression in the Anopheles Tubules

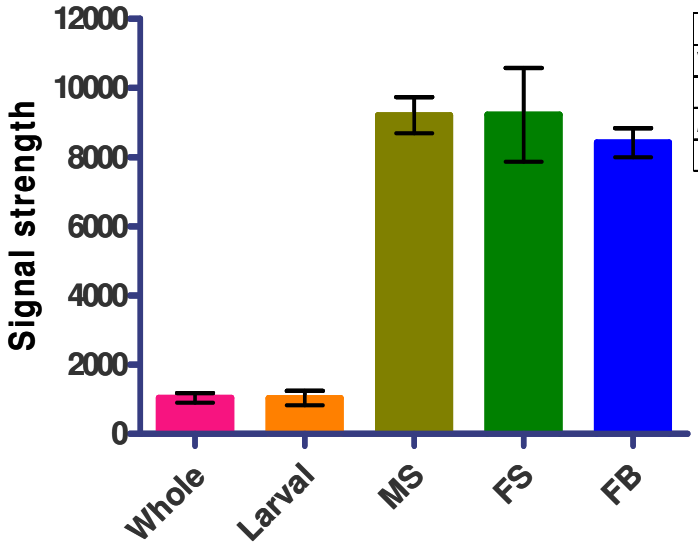
The expression of *AGAP007752* as both a transcript and a protein in the *Anopheles* Malpighian tubules is described in this section. This relevant microarray data is presented, as is its subsequent verification by qPCR. The expression pattern of the transcript in the adult tubules was determined using RNA probe *in situ* hybridisation. The translation of the transcript to a protein was verified through Western blotting, using an AGAP007752-specific antibody.

4.3.1.1 Expression of the Transcript

The putative sugar transporter *AGAP007752* was chosen for investigation from the *Anopheles* microarray due to its strong signal strength in the adult tubules in comparison to the average whole mosquito expression (Figure 4.3). The statistical significance of the microarray data was determined using one-way ANOVA analysis and is presented in the attached table.

The signal strength of *AGAP007752* in the whole mosquito microarray is ~1000. This is a relatively large signal, suggesting that although the gene is highly enriched in the tubules, there is significant expression of *AGAP007752* elsewhere in the mosquito. There is also a strong signal in the larval tubules, suggesting that *AGAP007752* is functional in the tubules before adulthood. There is no

significant difference in transcription of *AGAP007752* between the sexes and sugar- and blood-fed mosquitoes, suggesting that the gene is always present in the adult tubules, most likely to transport sugars ingested from plant sources.



	FB	FS	MS	Larval
Whole	***	***	***	-
Larval	***	***	***	
MS	-	-		
FS	-			

Figure 4.3 Microarray data for *AGAP007752* expression in the *Anopheles* tubules at different stages in the life-cycle. The attached table shows the statistical significance between life-stages. (N=4, mean ± S.E.M.) One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

Quantitative PCR (qPCR) was used to validate the microarray data and ensure it was representative of *AGAP007752* transcript expression in the tubules (Figure 4.4A). qPCR was also used to extend the microarray data by quantifying expression in the female tubules 24 h after a blood-meal (Figure 4.4B).

The qPCR data agrees reasonably well with the microarray data, although it shows almost double the expression ratio in the female tubules than is predicted by the microarray. It also suggests a higher level of transcription in the larval tubules than is forecast by the microarray. As demonstrated by Marinotti *et al*, haematophagous behaviour alters the whole-animal transcriptome of *Anopheles* for up to 96 h (Marinotti *et al.*, 2006). As we would expect the tubules to be especially active in the first 24 h after a blood-feed, during natriuresis and diuresis, it is interesting to consider transcription at both 3 h and 24 h after a blood-feed (Williams, 1983; Petzel *et al.*, 1987; Adams, 1999; Marinotti *et al.*, 2006). Our data suggests that *AGAP007752* is not up-regulated in response to haematophagous behaviour. Expression remains stable 3 h after a blood-feed and

drops between 3 h and 24 h. This may be because *AGAP007752* is expressed to transport sugars normally absorbed during sugar-feeding, which the female is unlikely to do until the end of the gonotrophic cycle. It may also be because the tubule is transcribing genes vital for dealing with a blood-meal, and so *AGAP007752* is transcribed at a lesser level.

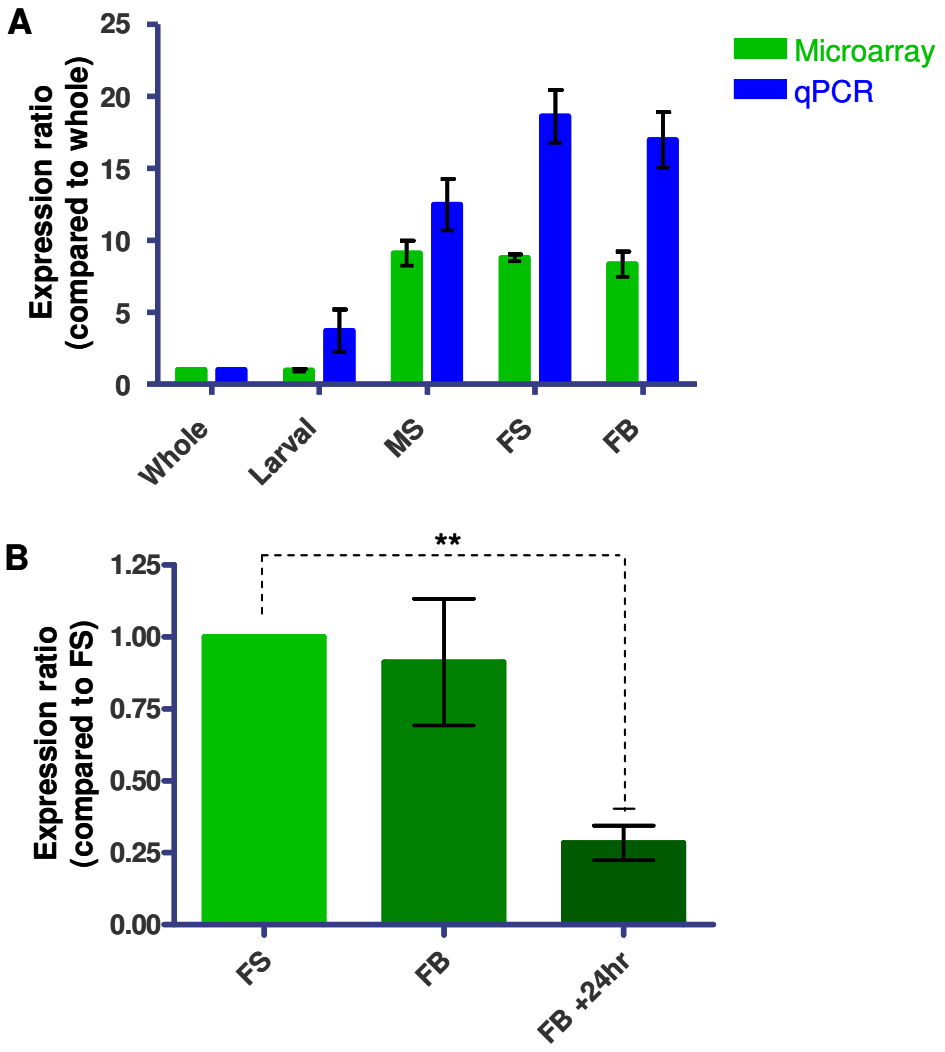


Figure 4.4 qPCR validation of *AGAP007752* expression in *Anopheles*. A: ratio of expression as calculated from microarray and qPCR data. B: ratio of expression in female tubules 3 h and 24 h after a blood-feed (N≥4, mean ± S.E.M.). One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

It is of interest to determine whether *AGAP007752* is expressed throughout the tubules, or in specific functional domains. Although the functional domains of mosquito Malpighian tubules are not well understood, we know that in other species such as *Drosophila* there are dedicated regions for absorption from the haemocoel and re-absorption into the haemocoel (Sozen *et al.*, 1997), as well as

multiple cell types which perform specific functions. *In situ* hybridisation was used to determine the expression pattern of the *AGAP007752* transcript in the *Anopheles* Malpighian tubules (Figure 4.5).

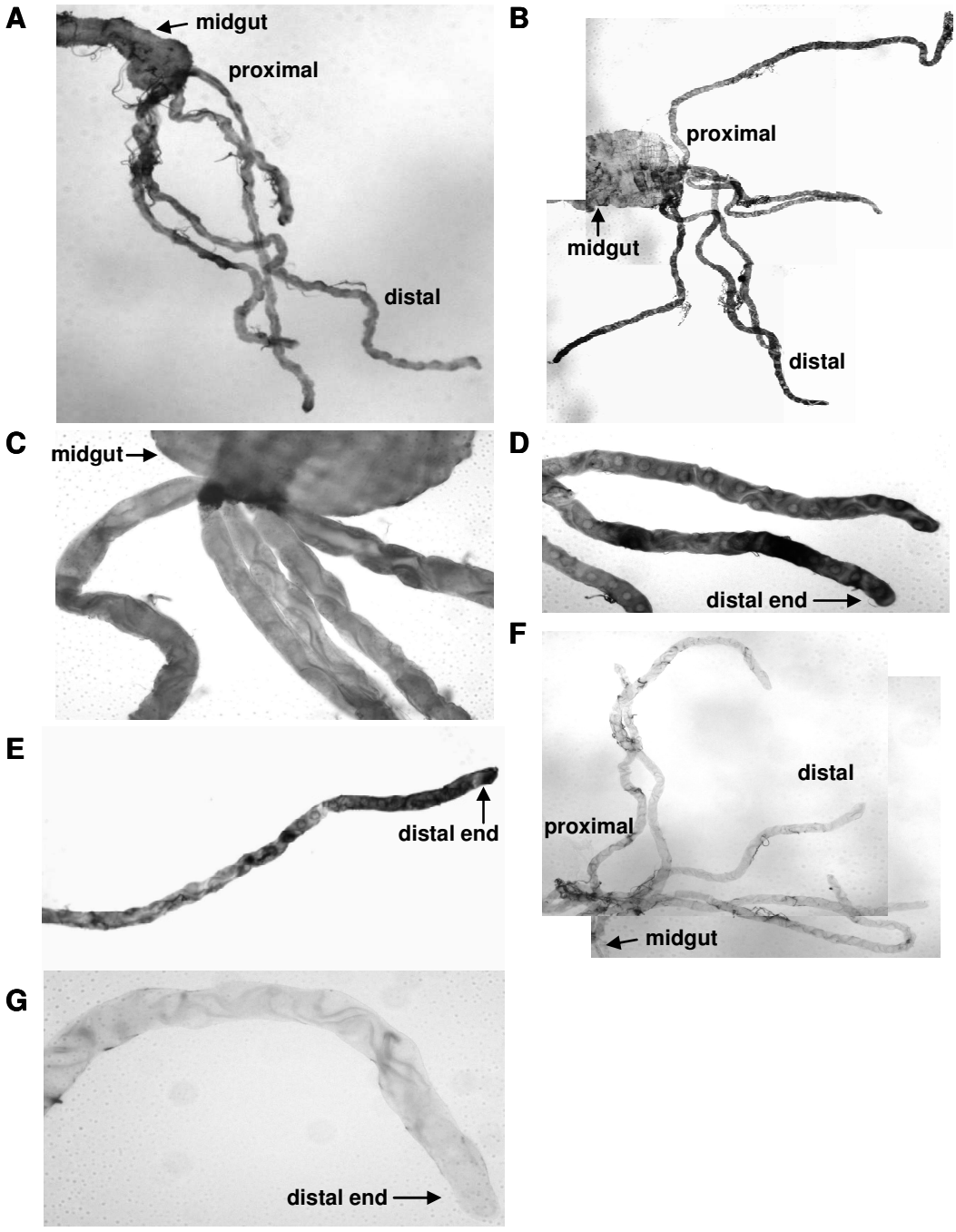


Figure 4.5 Expression of *AGAP007752* in the adult *Anopheles* tubules as determined by *in situ* hybridisation. A: low power view of the male tubules showing expression throughout. B: low power view of the female tubules showing expression throughout. C: high power view showing expression in the female lower tubule where it attaches to the gut. D: high power view of the distal end of the female tubules showing strong expression in the principal cells. E: main section of the male tubule showing expression in the principal cells. F: low power view of female tubule stained with sense probe as a control, showing no expression. G: high power view of the female distal end stained with sense probe as a control, showing no expression.

These experiments confirm that *AGAP007752* is expressed in all five tubules in both males and females. The transcript was abundant in all regions of both the 3 mm and 5 mm tubules, from where the tubule attaches to the gut to the blind distal end. Staining appears to be darker in the principal cells than in the stellate cells, suggesting that *AGAP007752* has higher expression in the principal cells. The *AGAP007752* probe also stained the gut, suggesting that there is expression in other areas of the alimentary canal. The sense control probe produced no staining in the tubules or gut, although there does appear to be non-specific staining in the trachea which wrap around the tubules.

4.3.1.2 Expression of the Protein

The expression of the *AGAP007752* transcript throughout the length of the adult Malpighian tubules in the principal cells was established in the previous section through *in situ* hybridisation. As *in situ* hybridisation assesses the existence of the transcript but not the protein, an antibody specific for the AGAP007752 protein was designed. A fourteen amino-acid antigenic sequence was chosen from the AGAP007752 sequence (Hubbard *et al.*, 2002) which would be available for antibody binding and had limited homology to other proteins in *Anopheles*. Western blotting was used to determine whether the antibody bound to a protein of correct size for AGAP007752 (~50 kDa) in *Anopheles* Malpighian tubule, head and whole-body samples (Figure 4.6).

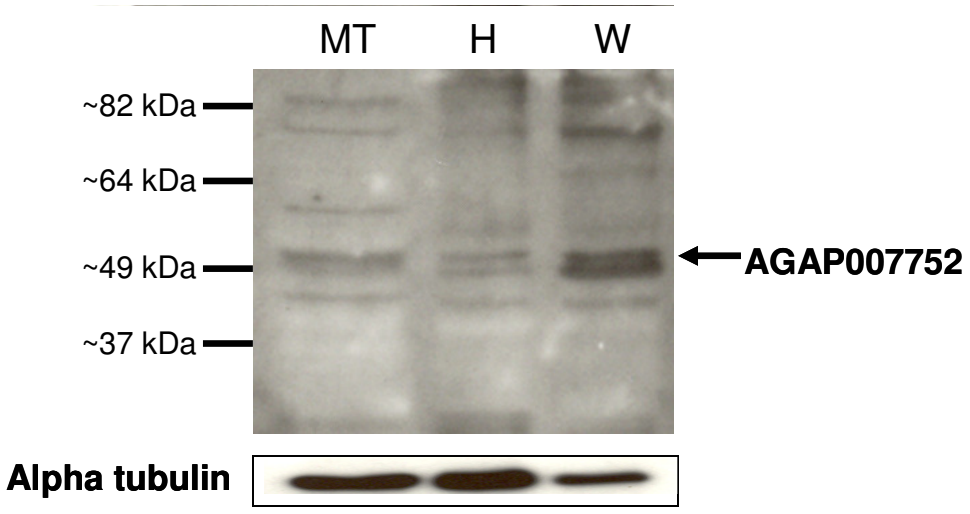


Figure 4.6 Expression of the AGAP007752 protein in *Anopheles* as determined by Western blotting. A band the correct size for the AGAP007752 protein (~50 kDa) can be seen in protein extracted from the Malpighian tubules (MT), head (H) and whole-body (W). Alpha tubulin was also blotted for as a control for protein integrity.

A band of the appropriate size was found in all three protein samples, suggesting that AGAP007752 is expressed in, but not confined to, the tubules. The AGAP007752 antibody also bound to several additional bands of protein, suggesting that it is not specific to AGAP007752. Equivalent samples probed with pre-immune rabbit serum instead of AGAP007752-antibody produced no protein binding. Immunolocalisation studies performed to identify the cellular location of AGAP007752 proved inconclusive, due to the high level of background auto-fluorescence in the *Anopheles* tubules.

4.3.2 CG15406 Expression in the Drosophila Tubules

The expression of *CG15406* as both a transcript and a protein in the *Drosophila* Malpighian tubules is described in this section. Expression of the transcript in the tubules was verified by qPCR. RNA probe *in situ* hybridisation was then used to determine which tubule domains the transcript is expressed in. Western blotting was used to verify that the transcript is translated to a protein. The cellular site of the protein was localised to the basolateral membrane of the tubules using immunocytochemistry.

4.3.2.1 Expression of the Transcript

CG15406 was identified as a homologue of *AGAP007752* in *Drosophila* due to its high sequence homology and enriched expression in the tubules (Wang *et al.*, 2004). The enrichment of the *CG15406* transcript in the larval and adult tubules in comparison to whole-fly was verified using qPCR (Figure 4.7A). Expression of the transcript was also compared in the tubules of adult males and females, to determine whether *CG15406* expression is sex-specific (Figure 4.7B).

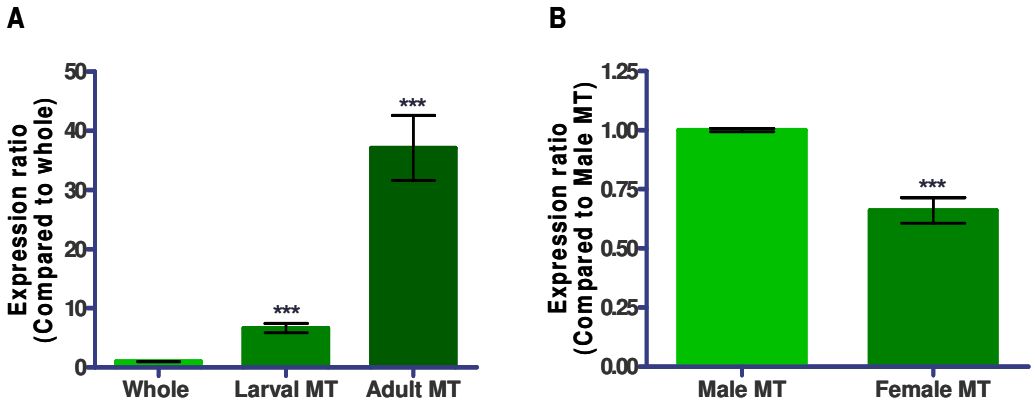


Figure 4.7 Expression of *CG15406* in the *Drosophila* Malpighian tubules as determined by qPCR. A: expression in the larval and adult Malpighian tubules in comparison to whole-fly. B: expression in the female tubules in comparison to the male tubules (N=4, mean ± S.E.M.). Student's t-test, *p<0.05, **p<0.01, ***p<0.001.

Expression of *CG15406* is substantially up-regulated in both the larval and adult Malpighian tubules in comparison to the average expression in whole-fly. It is also significantly greater in the tubules of adult males than adult females, which is surprising as mated female *Drosophila* show increased foraging in comparison to males, and are therefore likely to ingest a greater sugar-load (Barnes *et al.*, 2008).

In situ hybridisation was performed on the Malpighian tubules of adult *Drosophila* to determine whether transcript expression is localised to a specific domain or cell type (Figure 4.8).

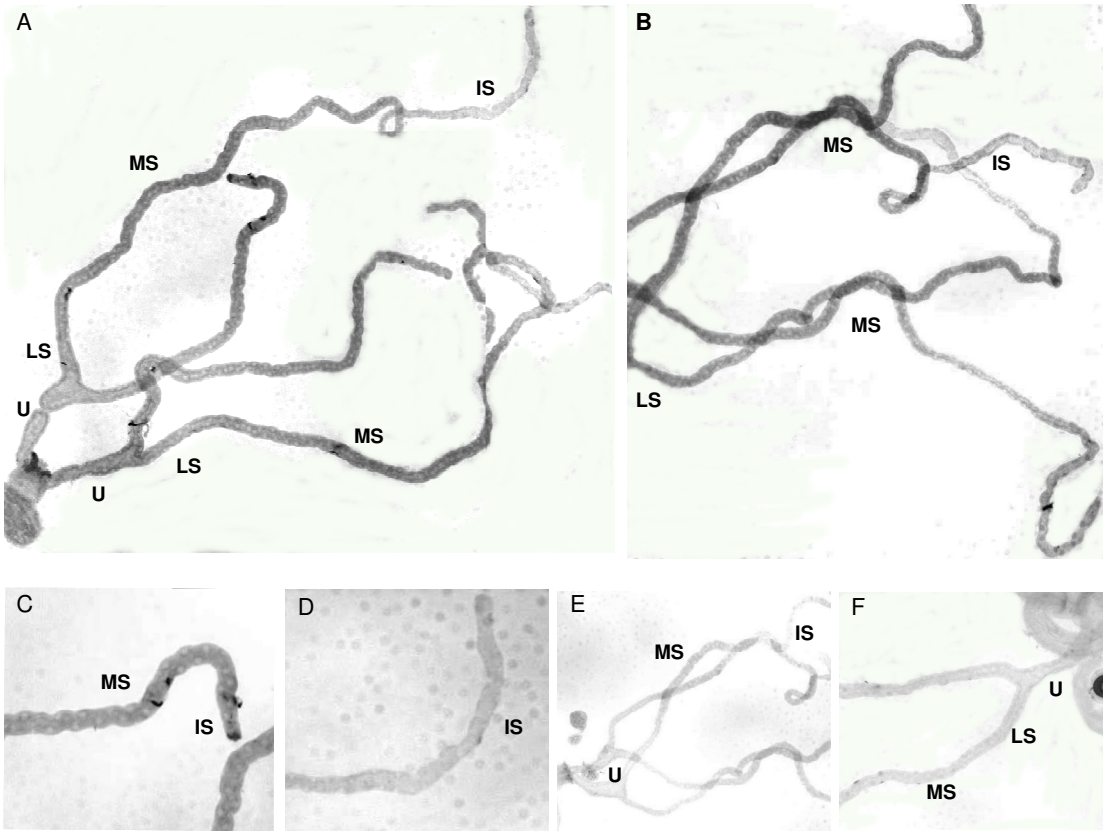


Figure 4.8 Expression of *CG15406* in the adult tubules as determined by *In situ* hybridisation. Low power views showing expression in the ureter (U), lower segment (LS), main segment (MS) and initial segment (IS) in the male (A) and female (B) tubules. C: high power shot of the posterior distal tubule showing staining in the principal cells. D: high power shot of the anterior distal tubule showing faint staining in the initial segment. E: and F: sense controls showing no staining in the tubules.

CG15406 is abundantly expressed in all regions of the Malpighian tubules in both male and female *Drosophila*. The probe staining is lighter in the initial and transitional segments than in the main segment, but darker than in the sense probe controls. There is strong *AGAP007752* staining in the principal cells, although it is difficult to determine whether it is also present in the stellate cells.

4.3.2.2 Expression of the Protein

An antibody was raised against an antigenic fourteen amino acid sequence from the CG15406 protein, for use in protein localisation. Western blotting was performed on protein samples extracted from whole-fly, Malpighian tubule and head, from 2-day and 7-day old flies (Figure 4.9).

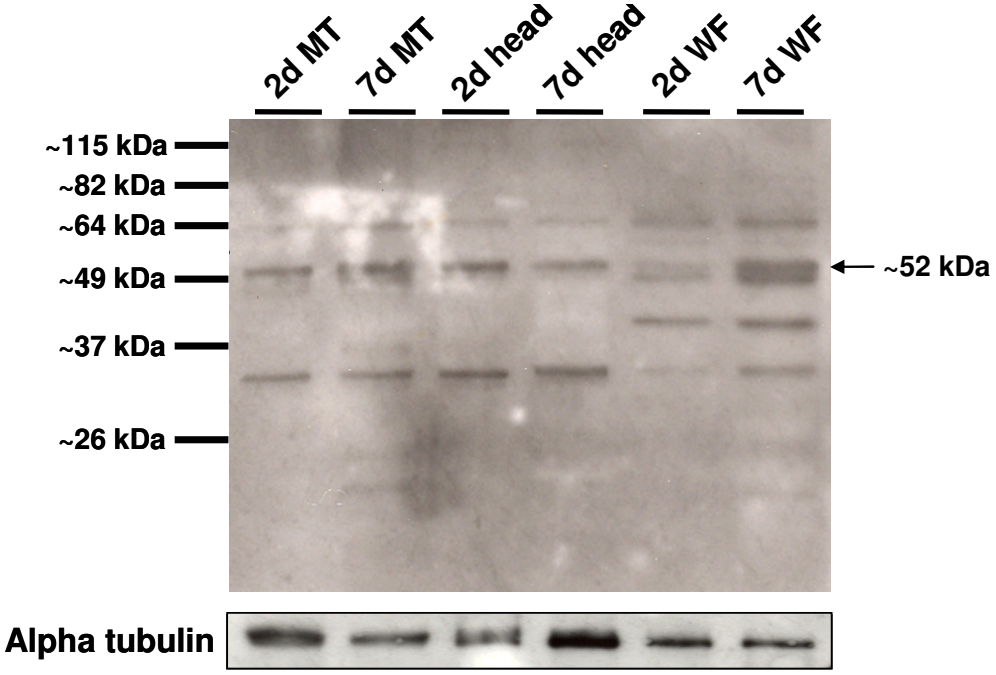


Figure 4.9 Expression of CG15406 in *Drosophila* as determined by Western blotting. A protein band the correct size for CG15406 (~52 kDa) can be seen in the Malpighian tubules of 2-day old (2d MT) and 7-day old (7d-MT) flies, the head of 2-day old (2d head) and 7-day old (7d head) flies, and whole-flies aged to 2-days (2d WF) and 7-days old (7d WF). Alpha tubulin was probed as a control for protein integrity.

A band of the correct size, ~52 kDa, was obtained in all six samples, suggesting once more that *CG15406* expression is not confined to the tubule. Bands of CG15406 protein can be seen in both the 2-day and 7-day old samples, suggesting that CG15406 is functional from the early stages of adulthood. The CG15406 antibody also binds to multiple bands of proteins, suggesting that it is not specific to CG15406. The same experiment repeated with pre-immune serum instead of CG15406-specific antibody showed no protein bands.

Immunocytochemistry (ICC) was performed to identify the cellular localisation of CG15406. Tubules were dissected from adult male and female flies, stained with CG15406-specifc antibody, and then with Texas Red (TR) anti-rabbit (Invitrogen, UK) as a secondary antibody to add a fluorescent tag (Figure 4.10).

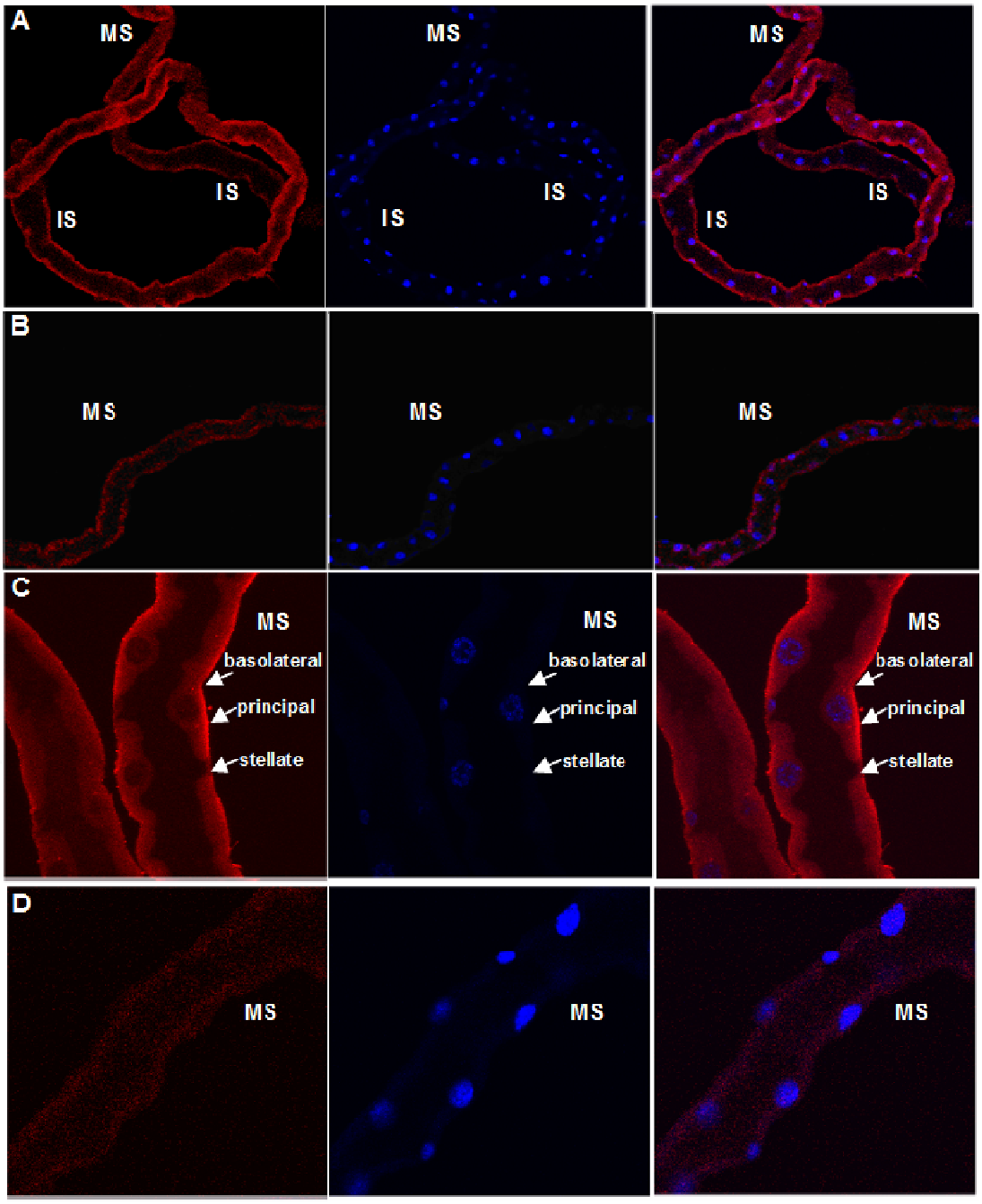


Figure 4.10 Localisation of CG15406 in adult *Drosophila* tubules by immunocytochemistry. A: and B: Initial segment (IS) and main segment (MS) showing binding at the basolateral membrane (20x magnification). C: Antibody binding at the basolateral membrane of the main segment showing binding in the principal cells, but not the stellate cells (40x magnification). D: Control tubules treated with pre-immune serum and Texas Red (40x magnification). Left hand panel – Texas Red staining of CG15406 (red); middle panel – DAPI nuclear staining (blue); right hand panel – Texas Red and DAPI merged.

ICCs performed with the CG15406 antibody produced staining at the basal membrane along the entire length of the tubules, including the initial and transitional segments of the anterior tubule. Small gaps in the fluorescent

staining at the basal membrane correlate with the placement of stellate cells, once more implying that *CG15406* is expressed in the principal cells, but not the stellate cells. The precise localisation of CG15406 to the apical, lateral or basal membrane of the tubules could be determined using co-labelling experiments to known membrane proteins. The immunolocalisation data broadly agrees with the transcription pattern suggested by *in situ* hybridisation. The immunolocalisations also suggest that *CG15406* is involved in the transport of sugars into the tubules for energy metabolism, as the basolateral membrane is where we would expect to find such transporters. There is no staining to suggest that CG15406 is expressed at the lumen or in cytoplasmic structures.

4.3.3 RNAi Knockdown of CG15406

As our understanding of small RNA-mediated cellular processes has advanced, it has been possible to incorporate and adapt them for research purposes. RNAi is the process of using a specific double-stranded RNA to target the degradation of homologous mRNAs. In *Drosophila* this process is combined with the UAS/GAL4 system to direct gene knockdown in a specific spatial and temporal pattern. As no RNAi lines were publicly available for targeting *CG15406*, the pWIZ transgene (Lee and Carthew, 2003) was used as a vector. Three constructs were cloned, each targeted to a different part of the *CG15406* transcript. The constructs were microinjected into w^{1118} flies and balanced over the homozygous lethal *CyO* on the second chromosome and TM3,*Sb* on the third chromosome. Three balanced lines were selected for each construct, resulting in nine UAS-RNAi fly-lines in total. Further studies were performed to determine the effect of *CG15406* knockdown on fly survival and secretion rate from the Malpighian tubules.

4.3.3.1 Effect on Fly Survival

For a gene to be an appropriate insecticide target it should be functionally essential to the species. The nine UAS-RNAi fly-lines under study were driven by four different GAL4 drivers (Table 4.2) to determine the effect of gene knockdown in specific tissues on fly development through to adulthood.

Table 4.2 Pattern of expression of GAL4 drivers used to drive UAS-RNAi fly-lines.

Driver line	Pattern of expression
Actin-GAL4	Ubiquitous expression throughout the life-cycle
C42-GAL4	Principal cells in the main and lower segments and bar-shaped cells in the initial and transitional segments of the tubules
Uro-GAL4	Principal cells in the main segment of the tubules
C724-GAL4	Stellate cells in the main segment and bar-shaped cells in the initial and transitional segments of the tubules

Fly survival in GAL4-driven UAS-RNAi lines was classified as normal, semi-lethal, or lethal. Normal was defined as the expected ratio of driven-RNAi to non-driven-RNAi progeny being observed in a cross between a parental RNAi line and a driver line. Semi-lethal was defined as significantly fewer RNAi-driven progeny being observed than expected (as determined by Chi-squared test). Lethal was classified as no driven-RNAi progeny emerging from a cross. The results can be found in Table 4.3.

Table 4.3 Effect of *CG15406* RNAi knockdown on fly survival (cross N=3). (Raw data is presented in Appendix 2).

Fly-line	*Actin-GAL4*	*C42-GAL4*	*Uro-GAL4*	*C724-GAL4*
CG15406.c1.l1	lethal	semi-lethal	normal	normal
CG15406.c1.l4	lethal	semi-lethal	normal	normal
CG15406.c1.l9	lethal	normal	normal	normal
CG15406.c2.l4	semi-lethal	normal	normal	normal
CG15406.c2.l7	normal	normal	normal	normal
CG15406.c2.l8	normal	normal	normal	normal
CG15406.c3.l3	lethal	normal	normal	normal
CG15406.c3.l4	lethal	normal	normal	normal
CG15406.c3.l5	semi-lethal	normal	normal	normal

When crossed to the *Actin-GAL4* driver, 7 out of 9 fly-lines showed greatly reduced fly numbers or no surviving adult flies. This coincided with large numbers of late-stage pupae which failed to eclose, suggesting that *CG15406* is critical for survival beyond eclosion. The number of lines in which RNAi

expression results in partial or full lethality indicates that it is unlikely to be off-target silencing or insertional effects of the transgene which are responsible. The tubule specific drivers did not have such a powerful effect on survival to adulthood, with only two of the fly-lines driven by *c42-GAL4* showing slight lethality. As *Actin-GAL4* is the only driver which expresses ubiquitously in the tubule we cannot discount tubule knockdown as the cause of lethality.

CG15406 knockdown was quantified using qPCR for each GAL4-driven UAS-RNAi line (Figure 4.11). The average amount of *CG15406* transcript in the parental line (as determined using the gene standard curve, see section 2.6.3) was compared to the average amount of *CG15406* transcript in the GAL4-driven UAS-RNAi lines, and an expression ratio calculated. The RNAi lines which were not classified as lethal or semi-lethal were driven with *Actin-GAL4* to quantify *CG15406* knockdown in the tubules. Where expression driven by *Actin-GAL4* was lethal or semi-lethal for the progeny, knockdown was quantified in the tubules of *c42-GAL4* driven lines. *CG3285,* the closest homologue of *CG15406*, was also quantified in RNAi-driven flies, to check for off-target gene silencing (Figure 4.11D).

All nine of the driven RNAi lines had significantly lower *CG15406* expression than their parental lines, as determined by Student's *t*-test. The average knockdown was to <25% of the parental transcript level, with variation between the nine lines resulting from insertional effects and different efficiencies of the constructs at targeting the transcript for degradation. The RNAi lines driven with *c42-GAL4* show a good knockdown, and as *c42-GAL4* only promotes expression in the principal and bar-shaped cells of the tubule, the knockdown supports the *in situ* hybridisation and immunocytochemistry data which show expression in the principal cells.

All of the lines tested for off-target gene-silencing effects showed no significant decrease in *CG3285* expression. This is encouraging as *CG3285* is the most homologous *Drosophila* gene to *CG15406*. In theory, the reduced fly survival which results from interfering with *CG15406* function suggests that it is a valid insecticide target.

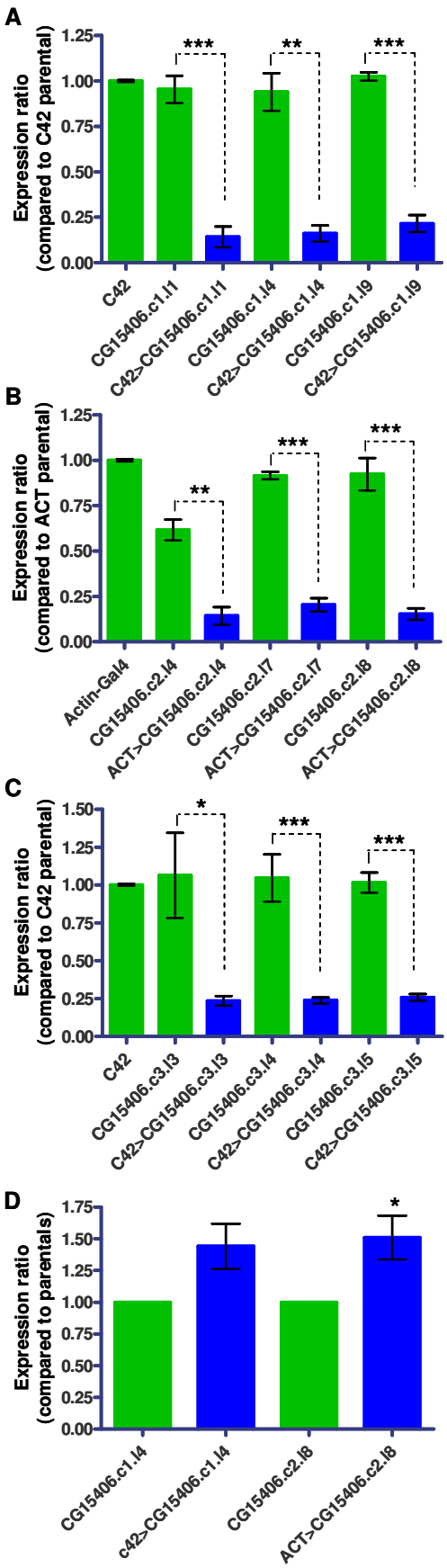
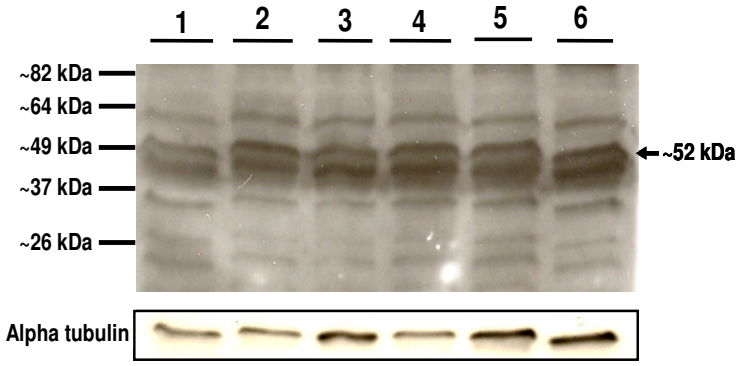


Figure 4.11 qPCR verification of *CG15406* knockdown in the tubules. *CG15406* expression in A: RNAi lines from construct 1, B: RNAi lines from construct 2, and C: RNAi lines from construct 3. D: Quantification of *CG3285* expression in driven RNAi lines (N=3, mean ± S.E.M.) (Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001).

Western blotting was used to assess CG15406 protein expression in the tubules of three GAL4-driven UAS-RNAi fly-lines in comparison to their parental fly-lines (Figure 4.12).



Lane	Fly-line
1	c42>CG15406.c1.I10
2	CG15406.c1.I10
3	ACT>CG15406.c2.I4
4	CG15406.c2.I4
5	c42>CG15406.c3.I4
6	CG15406.c3.I4

Figure 4.12 Protein expression of CG15406 in three RNAi fly-lines. Three RNAi lines driven by *Actin-GAL4* or *c42-GAL4*, and their respective parental lines, were blotted for CG15406 expression in the tubules. Alpha tubulin was blotted as a control for protein integrity.

The results of the Western blot are inconclusive, although CG15406 expression appears to be slightly reduced in the three driven RNAi lines. That there is no clear reduction may be due to differences in the amount of protein loaded, or transcript knockdown not correlating directly with reduced protein translation.

4.3.3.2 Effect on Fluid Secretion from the Tubules

The effect of *CG15406* knockdown on the rate at which the tubules are able to secrete was investigated using a modified Ramsay secretion assay (Dow *et al.*, 1994). Tubules dissected from the GAL4 driven UAS-RNAi line, the UAS-RNAi parent and the GAL4 driver parent were allowed to secrete steadily for 30 min before the diuretic peptide Drosokinin was added to the reservoir bubble. Three driven RNAi lines were tested; one line for each of the three RNAi constructs (Figure 4.13).

Knockdown of *CG15406* has no significant effect on basal or stimulated secretion. Although the driven RNAi line had elevated secretion compared to the RNAi parental line in each experiment, it was still lower than the parental driver secretion rate. An increase in secretion rate requires increased energy expenditure, and therefore a greater supply of the energy source. The

knockdown of *CG15406* does not seem to limit the energy available to the tubules. This may be due to a redundancy of sugar transporters which are able to maintain an adequate supply of sugars to the tubules to power secretion.

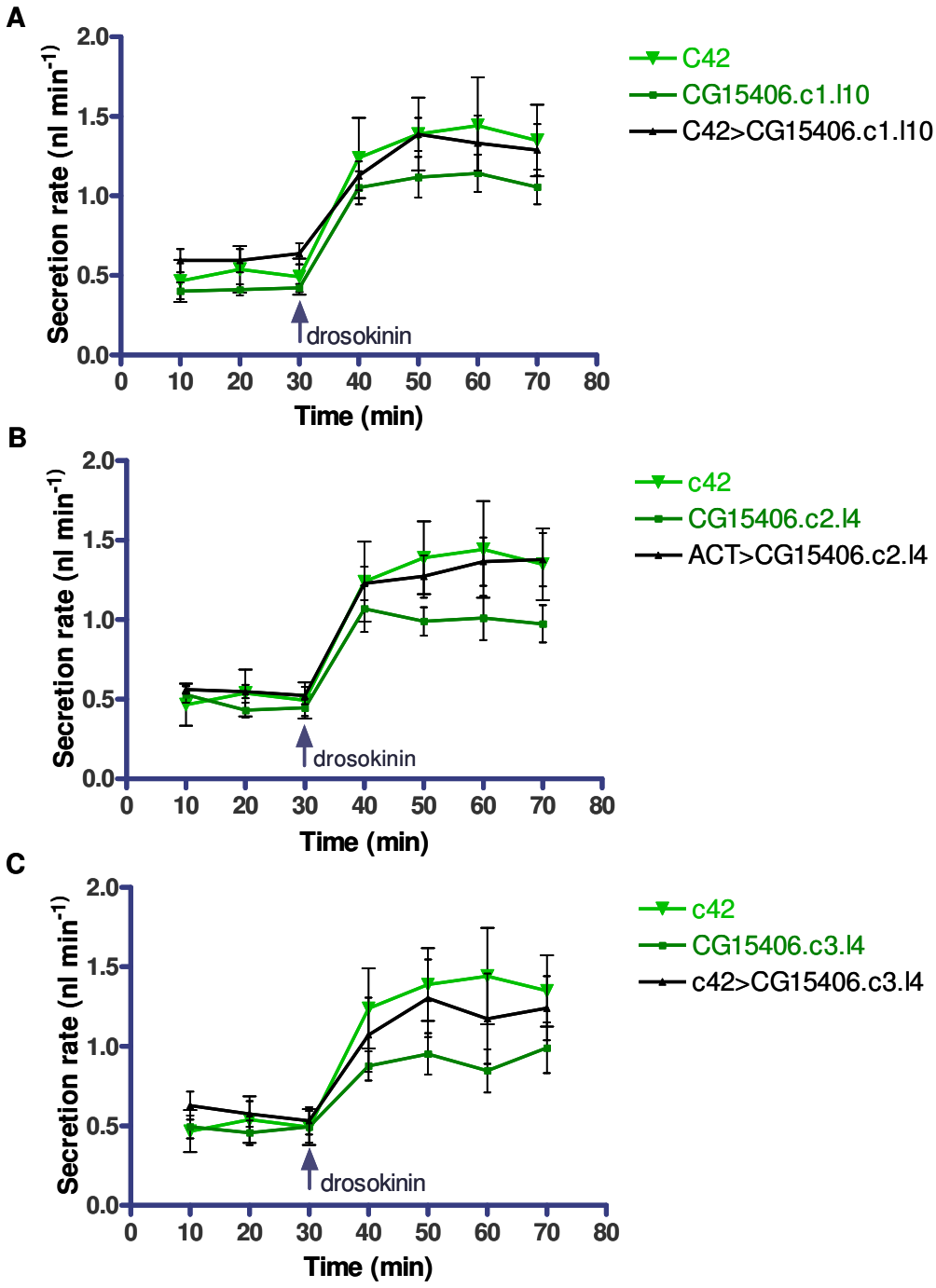


Figure 4.13 Effect of *CG15406* knockdown on the rate of fluid secretion from the tubules. Typical experimental results, showing the response of three driven RNAi lines and the appropriate parental lines to Drosokinin after 30 min of steady secretion (mean ± S.E.M., N≥10).

4.3.4 Sugars Transported by CG15406

CG15406 is localised to the basolateral membrane along the length of the tubules, and is most likely to be involved in sugar uptake. This section describes experiments performed to elucidate which sugars are transported from the haemocoel by *CG15406,* with glucose, fructose and sucrose being the most likely substrates. Glucose and fructose are the main sugar components of laboratory fly-food, and are also found in the decaying fruit which *Drosophila* feeds on in nature. Studies show that *Drosophila* is able to survive for more than two weeks on pure solutions of all three sugars (Hassett, 1948).

4.3.4.1 Fly Survival on Glucose, Fructose and Sucrose

To determine whether *CG15406* is transcribed in response to the presence of a particular type of sugar, Oregon R flies were raised on food in which the sugar content was either glucose, fructose or sucrose (Section 2.19.1), and expression of *CG15406* in the tubules measured by qPCR (Figure 4.14).

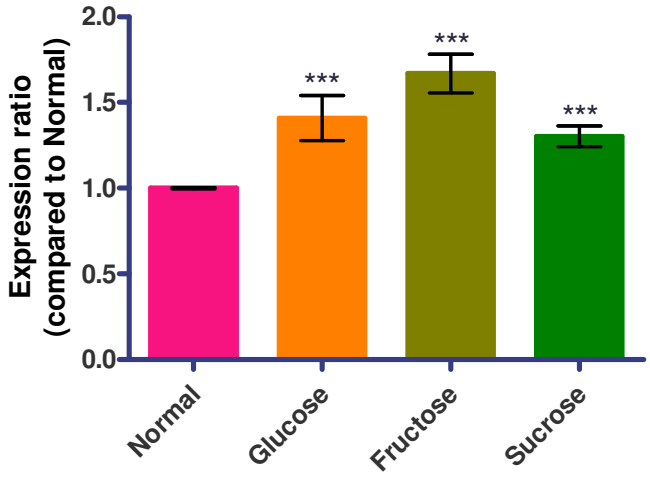


Figure 4.14 *CG15406* expression in the tubules of flies raised on fly-food with modified sugar content. Expression is compared in flies raised on normal, glucose-rich, fructose-rich, and sucrose-rich food (N=4, mean ± S.E.M.). Student's *t*-test comparing normal food to modified food, *p<0.05, **p<0.01, ***p<0.001.

Expression of *CG15406* increased significantly when flies were raised on the glucose, fructose and sucrose foods, compared with normal food containing the same molarity of sugar. There was no significant difference in expression between the three sugar-specific foods, suggesting *CG15406* expression is not

triggered by one specific sugar. Fly eclosion and survival was similar for all four food types on which the flies were reared.

It is also of interest to determine whether the lethality of knocking down *CG15406* expression can be altered by changing the sugar content of the food. Two RNAi lines were crossed to the GAL4-drivers *Actin-GAL4* and *c42-GAL4*, and allowed to lay eggs on normal, glucose, fructose and sucrose food. The proportion of driven-RNAi flies which emerged on each food-type was counted, to determine whether glucose, fructose and sucrose enhanced or reduced fly survival (Figure 4.15).

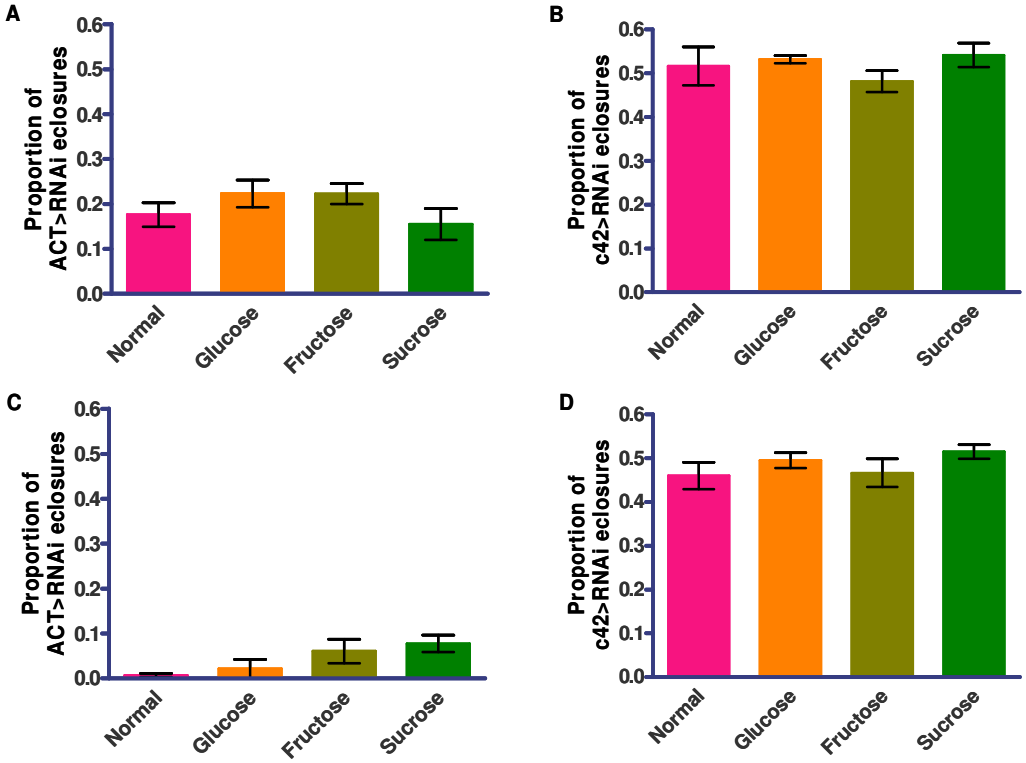


Figure 4.15 Survival of RNAi fly-lines on sugar-modified food. A: and B: *CG15406.c2.l4* driven by *Actin-GAL4* and *c42-GAL4*. C and D: *CG15406.c3.l4* driven by *Actin-GAL4* and *c42-GAL4*. (N=4, mean ± S.E.M.). Student's t-test, *p<0.05, **p<0.01, ***p<0.001.

There is no significant difference between the proportion of driven-RNAi progeny eclosures on normal, glucose, fructose and sucrose enriched food. The survival of flies containing *CG15406.c2.l4*, which is semi-lethal when driven throughout the fly, and *CG15406.c3.l4*, which is almost lethal when driven throughout the

fly, is not affected by changing their principal source of sugar. This suggests that *CG15406* is not a simple, single sugar transporter.

4.3.4.2 Glucose Transport by the Tubules

A transport assay was designed to determine whether glucose is transported into the Malpighian tubules of wild-type flies, and if so, whether it is also secreted by them. The experiment was then repeated using GAL4-driven UAS-RNAi flies, to determine whether *CG15406* knockdown has any effect on the amount of glucose transported into the tubules. D-Glucose-1-^{3}H (Sigma-Aldrich, UK) was used as a tracer to measure glucose intake and excretion from the tubules.

Radiolabelled glucose was included in the bathing medium of the transport assays. The amount of radiation in the secreted drop of wild-type Oregon R tubules, as well as three driven-RNAi and parental lines, was measured after 1 h of secretion. The measurements revealed only trace amounts of radioactivity in the drop secreted by the tubules in all of the fly-lines tested (Figure 4.16).

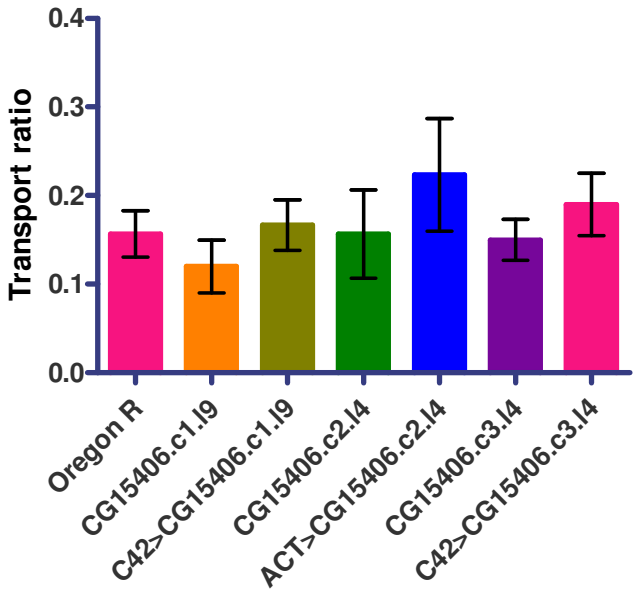


Figure 4.16 Transport ratio of ^{3}H-labelled glucose in wild-type and RNAi fly-lines. (mean ± S.E.M., N>12).

As each of the fly-lines had only trace amounts of sugar in their secreted droplets, which correlated with a transport ratio of <1, it would appear that

glucose is not being actively secreted by the tubules. The trace levels of ^{3}H could be secreted glucose, or the ^{3}H-labelled by-product of glucose metabolism.

To determine whether glucose is transported into the tubules of Oregon R flies, the amount of radiolabelled glucose present in the bathing solution was measured at the beginning of the experiment, and then after 3 h of secretion (data not shown). This allowed the amount of ^{3}H-labelled glucose up-take by the tubules over a 3 h period to be quantified. These experiments showed that glucose is taken-up by the tubules from the bathing medium, as the amount of ^{3}H-labelled glucose in the bathing medium decreased with time. The mode of sugar transport into the tubules is not yet known, and glucose could move into the tubules via concentration-dependent passive transport or through active transport by a sugar transporter such as *CG15406*. The amount of glucose taken up by the tubules of *Oregon R* flies was variable, apparently due to fluxes in room temperature.

If glucose is actively transported by a sugar transporter such as *CG15406*, we might expect the up-take of glucose by the tubules to be decreased when *CG15406* expression is decreased. The transport assay was repeated, comparing the glucose up-take rate of GAL4-driven UAS-RNAi flies to their parental fly-line. These experiments were performed to determine whether the driven RNAi lines had reduced glucose up-take from the bathing medium when compared to the parental fly-lines. Typical results for three of the GAL4-driven UAS-RNAi lines tested can be found in Figure 4.17, showing the proportional decrease in glucose in the bathing medium after 3 h of secretion. Secretion rate was also measured during each experiment, and was similar for the GAL4-driven UAS-RNAi flies and their parental lines.

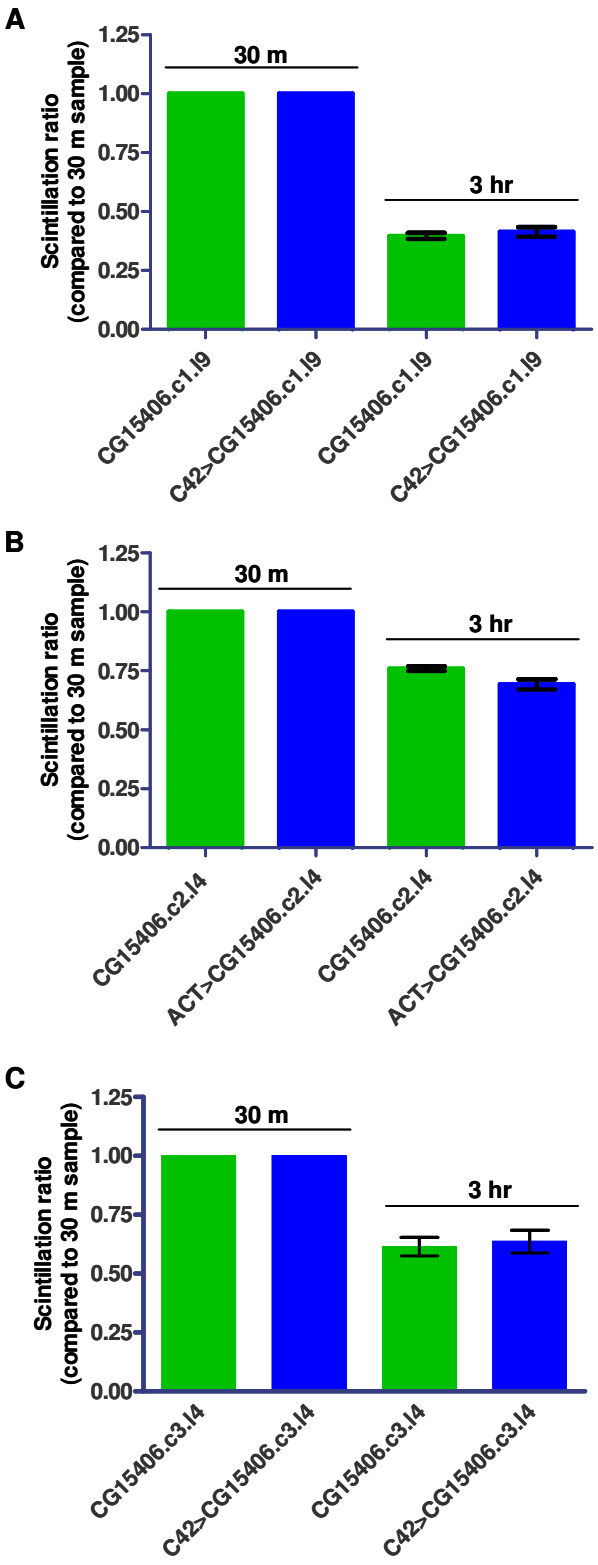


Figure 4.17 Uptake of ^{3}H-labelled glucose by *CG15406* RNAi lines. Uptake of glucose after 3 h by three different GAL4-driven UAS-RNAi lines and their parental fly-lines. (mean ± S.E.M., N>12)

The transport assay data shows that knockdown of *CG15406* expression has no significant effect on glucose movement into the tubules. There was no difference in glucose uptake between the driven-RNAi, RNAi parental lines and wild-type Oregon R flies in any of the experiments. This may be because of the redundancy of sugar transporters in the tubules, or may be because *CG15406* does not principally transport glucose. It is also possible that glucose moves into the tubules passively on a concentration gradient, and is not actively transported by *CG15406*. It is also unclear what happens to the ^{3}H label of the glucose when it is metabolised in the tubules. The ^{3}H could be secreted in a water molecule, or retained in the tubules as one of the products of glycolysis. Using a glucose tracer which is radioactively labelled on a carbon atom may be a more reliable way of tracing glucose transport and metabolism.

4.4 Discussion

The uptake, transport, regulation and storage of sugars is vital to all living organisms, from unicellular bacteria to complex mammals. Understanding of how the insect gustatory system detects sugars is increasing, but little is known about their regulation thereafter. This section discusses the enriched expression of the putative sugar transporters *AGAP007752* and *CG15406* in the Malpighian tubules of *Anopheles* and *Drosophila*, and their likely function. The usefulness of *CG15406* as a model for *AGAP007752* is also discussed, as is their likely success as putative insecticide targets.

4.4.1 AGAP007752 and CG15406 in the Tubules

The spatial and temporal expression of *AGAP007752* and *CG15406* in the tubules was investigated for two reasons; to determine how similar gene expression of the homologues is, and to gain further understanding of the function of the two genes. Both genes are greatly enriched in the adult tubules, with a slight enrichment in the larval tubules in comparison to the average expression in the adult. The strong microarray signal in the whole-body samples suggests that expression of *AGAP007752* and *CG15406* is not confined to the tubules. *In situ* hybridisation confirms that the transcripts are expressed in the principal cells of the tubule in both male and female *Anopheles* and *Drosophila*. The immunocytochemistry experiments show *CG15406* to be expressed at the basolateral membrane in the principal cells but not the stellate cells along the entire length of the tubule. This would suggest that *CG15406* is involved in the transport of sugars into the tubule cells, rather than the secretion of sugars into the lumen. This seems likely, as it is improbable that the insect would want to excrete a valuable source of energy.

Expression of *AGAP007752* does not increase after the mosquito consumes a blood-meal, when the tubules are known to be highly active, performing both natriuresis and diuresis. This is slightly surprising, as we would expect the energy requirement – and therefore the sugar requirement - of the tubules to peak after haematophagous behaviour. The Western blot results show that CG15406 is expressed when the fly is 2-days old and 7-days old in the tubules. This suggests that *AGAP007752* and *CG15406* are present in the tubules throughout adulthood.

The Western blots also show expression of the two genes in head and whole-body protein samples, agreeing with the microarray data that they are enriched in, but not confined to the tubules. Attempts to localize the AGAP007752 protein in the *Anopheles* tubules using immunocytochemistry were unsuccessful due to the high level of auto-fluorescence in the tubules (data not shown).

The microarray, qPCR, *in situ* hybridisation and Western blotting data are in good agreement with one another, for both *AGAP007752* and *CG15406*. It is reassuring to find that transcript expression in the tubules correlates with protein expression in the tubules for both of these genes. Although whole genome protein arrays would in many ways be preferable to transcript arrays, they are presently not a feasible alternative, and so it is encouraging to note that they would be likely to show similar results. There is also good agreement between *AGAP007752* and *CG15406* expression in *Anopheles* and *Drosophila* for all five types of experiment performed. The spatial expression pattern of the two genes in the tubules is very similar, an additional level of homology on top of sequence conservation and transcript enrichment in the tubules.

Knockdown of *CG15406* expression using the UAS/GAL4 system was used to mimic the effect that insecticide targeting would have if the compound prevented transporter function. RNAi was the preferred technique as it can be tailored to mimic what would happen during insecticide targeting. Expression can be decreased by variable amounts by designing multiple RNAi constructs which insert into the genome at random points. Gene knockdown is also defined temporally and spatially by GAL4-driver choice, and this can be utilised to gain better understanding of where and when the gene is important. Knockdown of *CG15406* throughout the fly using the *Actin-GAL4* driver line had a lethal or semi-lethal effect on the progeny of 7 out of 9 lines tested. Lethality mainly occurred at the late pupal stage, when the adult fly was on the verge of eclosion. This suggests that although *CG15406* is expressed during larval stages it is most important during adulthood, which the fly prepares for during pupation. Knockdown of *CG15406* in specific tubule cell-types was not lethal to the fly, although driving RNAi flies with *c42-GAL4* was semi-lethal in 2 lines. The *c42-GAL4* driver knocks down expression in the principal cells in the lower and main segments, and the bar-shaped cells of the initial segment. Survival of *c42-GAL4* driven flies suggests that *CG15406* expression has to be highly depleted in the

principal cells throughout the tubule for it to have a lethal effect. It could also mean that it is depletion in another tissue entirely which is lethal to the fly. Either way, expression of *CG15406* is essential for survival through to adulthood.

4.4.2 AGAP007752 and CG15406 as Sugar Transporters

AGAP007752 and *CG15406* contain motifs highly similar to those which define the sugar transporter GLUT family in vertebrates. There is some variation in the peptide motifs, but their conservation over hundreds of millions of years of separate evolution points towards a shared function. The predicted protein structures of AGAP007752 and CG15406 are similar to the 12 transmembrane-domain configuration which defines the GLUT family. The most obvious disparity is the lack of a 'QLSG' motif, usually found in the seventh transmembrane domain. The human GLUTs which do not have this domain show greater affinity for fructose transport over glucose transport (Uldry and Thorens, 2004). Of the other six *Drosophila* genes which are classed as paralogues, only *CG3285* has no recognisable 'QLSG' motif (Hubbard *et al.*, 2009). Similarly to *CG15406*, *CG3285* is an uncharacterised gene which is specifically expressed in the larval and adult tubules (Chintapalli *et al.*, 2007). This suggests that the affinity of the *Drosophila* sugar transport family for different sugars is determined by small changes in important motifs. Also of note is that as of yet, there are no homologues of *AGAP007752* and *CG15406* outside of the insects. This highly expressed insect tubule and testis-specific family may have become specialised to deal with the demands of these high-energy tissues.

If *CG15406* is a ubiquitous transporter supplying sugar for energy metabolism in the tubules, we might expect that decreasing its expression would affect the rate at which fluid is secreted from the tubules. Surprisingly, the secretion assays show no difference in the basal or stimulated fluid secretion rate between GAL4-driven UAS-RNAi fly-lines and their parental lines. Analogous experiments using a fructose-based secretion bathing medium gave similar results (data not shown). This suggests that even with a decrease in *CG15406* expression the tubules are able to energise secretion to a normal level. This could be due to a redundancy of sugar transporters, as five other genes from the same family as *CG15406* are heavily expressed in the tubules. It is also worth noting that the

secretion assays are being performed on flies which have developed through to adulthood, and are therefore transcribing enough *CG15406* for survival.

The modified sugar-food and transport assay experiments performed were inconclusive as to what sugars *CG15406* is transporting. Altering the main dietary sugar did not aid survival of *CG15406* knockdown flies. The ^{3}H-glucose transport assays showed that glucose moves into the tubules, but is not secreted. They also showed no difference in the amount of glucose transported by parental and GAL4-driven *CG15406* RNAi lines. This suggests that either *CG15406* does not transport glucose, or that the tubules are able to import enough glucose via other transporters to energise secretion. It is also possible that sugars such as glucose move passively into the tubules on a concentration gradient, and are not transported actively. It would be useful to repeat the transport assays using other tracer sugars such as fructose, to determine whether *CG15406* knockdown has any effect on their uptake.

4.4.3 AGAP007752 and CG15406 as Insecticide Targets

AGAP007752 and *CG15406* appear to make good putative insecticide targets. The lethal effect of reducing *CG15406* expression suggests that it is an essential gene for fly development through to adulthood. The availability of a whole family of highly expressed sugar transporters in the tubules and testis also allows the possibility of designing a compound which targets the family rather than a single gene. Not only could this result in a more potent insecticide, but it may reduce the risk of the development of populations of insects which are resistant through gene mutation. The high degree of similarity in tubule enrichment and expression between *Anopheles* and *Drosophila* also increases the likelihood of any developed insecticides being useful for multiple Diptera species. That there is no homologous gene family annotated in humans or any other organism out-with insects would be beneficial, as it decreases the likelihood of an insecticide compound being dangerous to other species. The most effective life-stage to target *AGAP007752* and *CG15406* would likely be during the larval or pupal stages, before eclosure to adulthood.

4.5 Conclusion

The results presented in Chapter 4 indicate that the putative sugar transporters *AGAP007752* and *CG15406* would be interesting insecticide target candidate genes. The agreement between the five experiments performed in both *Anopheles* and *Drosophila* is encouraging, as the expression of the two genes is very similar. The massive enrichment of these genes in the *Anopheles* and *Drosophila* Malpighian tubules and their expression in the principal cells throughout the length of the tubules suggests that they are functionally active. The conservation of this homologous gene pair over ~150 million years of Dipteran evolution also suggests that they are important (Yeates and Wiegmann, 1999). That they maintain the main motifs which define mammalian GLUTs but do not have obvious mammalian homologues suggests a family of Dipteran testis and tubule-specific transporters which may have evolved to deal with the specific energy need of these tissues. The lack of a secretion or transport phenotype is most-likely due to the redundancy of highly-expressed members of this family in the tubules. Transport assays using fructose and sucrose tracers could help elucidate the sugars which are transported by *CG15406*. Further functional studies, such as in *Xenopus* oocyte, would also confirm whether the physiological properties of *AGAP007752* and *CG15406* are homologous. The sugars transported by *CG15406* and *AGAP007752* could be determined, and the system could also be used to find insecticides and pharmacological agents which disrupt their function. Alternative transcripts of *CG15406* and *AGAP007752* with mutations in the channel border motif could also be expressed, and the effect of the channel border motif on the uptake of specific sugars investigated.

Chapter 5 - *AGAP012251* and *Picot* as Insecticide Targets

5.1 Summary

In Chapter 3 it was established that inorganic and organic transporters are highly enriched in the *Anopheles* Malpighian tubules, and therefore may be effective targets for new insecticides. Phosphate co-transporters are conspicuous among the genes enriched in the *Anopheles* and *Drosophila* Malpighian tubule microarray data-sets (Wang *et al.*, 2004), although little is understood about their functional role. The introduction to this chapter describes the known phosphate co-transporters in Diptera and mammals, and our current knowledge of their physiological role. The *Anopheles* gene *AGAP012251* and the *Drosophila* gene *Picot* are putative inorganic phosphate co-transporters, and are the focus of this study. The results presented in this chapter confirm the expression of *AGAP012251* and *Picot* as both transcripts and proteins in the tubules of *Anopheles* and *Drosophila*. In *Drosophila* expression of *Picot* is largely in the initial and transitional segments of the anterior tubule, whereas *AGAP012251* expression appears to be throughout the length of the *Anopheles* tubule. Preliminary localisation of the Picot protein suggests that it is found at the basolateral membrane of the initial and transitional segments of the anterior tubule. Furthermore, *Picot* is essential for fly development through to adulthood; UAS/GAL4-based knockdown of gene expression is lethal. The role of *Picot* in osmoregulation is also investigated, although gene knockdown of *Picot* has no significant effect on tubule fluid secretion rates. Finally, the *Anopheles* and *Drosophila* data collected for *AGAP012251* and *Picot* are discussed, as is the expected function of *AGAP012251* and *Picot*, and their likely success as insecticide targets.

5.2 Introduction

Phosphate is an essential compound which must be actively transported into cells against its electrochemical gradient. As well as being a component of bones, it plays a role in almost all aspects of molecular life. From storage and release of energy through hydrolysis of phosphoanhydride bonds, to a structural role in the phospholipids found in biological membranes, phosphates are important and ubiquitous.

The role of inorganic phosphate co-transporters in the insect Malpighian tubule is not yet understood. The tubules are known to store ions such as calcium in phosphate-containing concretions, and inorganic phosphate co-transporters are therefore likely to play a role in the import of inorganic phosphate into the tubules. The massive up-regulation of multiple inorganic phosphate co-transporters in the tubules suggests that they are functionally important, and could be effective insecticide targets.

5.2.1 Inorganic Phosphate

Phosphorus is an essential element utilised by all living organisms, for both metabolic and structural purposes. As the roles of phosphorus are widespread and numerous, its transport and storage must be carefully regulated. Phosphorus is transported into cells from the extracellular environment in the form of negatively charged inorganic phosphate (P_i).The inorganic phosphate co-transporter families studied in vertebrates use an inwardly directed electrochemical gradient of Na^+ ions to transport P_i. The gradient is maintained by Na^+- K^+- ATPases in the tissues which have been studied (Gmaj and Murer, 1986). In *Aedes aegypti* and *Drosophila* it has previously been shown that Na^+- K^+- ATPase subunits are highly expressed in the Malpighian tubules (Weng *et al.*, 2003; Wang *et al.*, 2004), but are not sensitive to pharmacological block by ouabain. In the *Drosophila* Malpighian tubules ouabain is actively excreted by an active transport system which co-localises with the Na^+- K^+- ATPase subunits (Torrie *et al.*, 2004). The presence of these ATPases suggests that a Na^+ gradient is utilised by co-transporters in the fly tubules, although it has not yet been proven to be a mechanism for phosphate co-transport.

In mammals three solute carrier families have been studied for their role in phosphate transport, the SLC17, SLC20 and SLC34 families.

5.2.2 Inorganic Phosphate Co-transporters

Phosphate transporters are categorized as members of the Major Facilitator Superfamily (MFS), which is observed ubiquitously in bacteria, archaea and eukarya. The MFS is involved in solute:cation (H^+ or Na^+) symport, as well as solute:H^+ and solute:solute antiport. Members of the MFS exhibit specificity for many compounds, such as sugars, osmolites, organic anions and inorganic ions.

A homology search of the *Drosophila* genome found 131 proteins which contain the MFS domain, 21 of which also contain an anion/cation symport (ACS) consensus sequences (Laridon *et al.*, 2008). ACS consensus sequences are present in genes which transport organic or inorganic substrates in response to chemiosmotic cation gradients, and can be found in all kingdoms. The 21 ACS genes in *Drosophila* mainly belong to two families, the inorganic phosphate co-transporter family and the vesicular glutamate transporter family (Hubbard *et al.*, 2009). They have been classified as such due to their similarity to the mammalian transport families SLC17, SLC20 and SLC34.

5.2.2.1 Mammalian Phosphate Transporters

The kidneys are the central effector organ for phosphate and calcium homeostasis in humans. Regulation is through controlled reabsorption and excretion of phosphate and calcium, as signalled by hormonal peptides such as calcitonin (Austin and Heath, 1981) and alpha-Klotho (Nabeshima and Imura, 2008). Three unrelated protein families have been linked with phosphate transport in mammals (Virkki *et al.*, 2007). The SLC20 family (also known as the Type III Na^+/P_i co-transporters) is comprised of *PiT-1* and *PiT-2* (Collins *et al.*, 2004). Expression of *PiT-1* and *PiT-2* is ubiquitous in humans, and has been linked to a housekeeping role in phosphate regulation, as well as involvement in bone P_i metabolism and vascular calcification (Caverzasio and Bonjour, 1996; Jono *et al.*, 2000; Yoshiko *et al.*, 2007). Transport of P_i by the SLC20s is coupled to a H^+ gradient in prokaryotics and plants, but in animals and fungi the influx is

driven by Na^+ (van Veen, 1997; Martinez *et al.*, 1998; Harris *et al.*, 2001; Collins *et al.*, 2004).

The second class of P_i transporters, the SLC34 family (also known as the Type II Na^+/P_i co-transporters), contains three genes in humans; *NaPi-IIa*, *NaPi-IIb* and *NaPi-IIc*. Studies have largely concentrated on their expression in epithelial and epithelial-like cells in the kidney and small intestine, where they maintain P_i homeostasis (Murer *et al.*, 2000). Discovery of additional roles, such as P_i regulation in the central nervous system (Hisano *et al.*, 1997) and salivary glands (Homann *et al.*, 2005), suggests that the physiological roles of the SLC34 family are more widespread than first assumed. The SLC34 family is known to have a high affinity for HPO_4^{2-}, in contrast to the SLC20 family, which preferentially transports $H_2PO_4^-$.

The third transporter family, the SLC17s, were originally thought to be Na^+-dependent P_i transporters, but have subsequently been re-classified. No SLC17 family member is known to be a strict Na^+/P_i co-transporter, and instead they transport compounds such as inorganic and organic ions (Busch *et al.*, 1996), sialic acid (Verheijen *et al.*, 1999), glutamate, and monocarboxylic acids (Bellocchio *et al.*, 2000). Mutations in the *sialin* gene from the SLC17 family can result in sialic acid lysosomal storage diseases in humans (Verheijen *et al.*, 1999; Ruivo *et al.*, 2008).

There is low homology between the mammalian inorganic phosphate co-transporters and annotated genes in Diptera. The SLC34 family has no homologues in *Anopheles* and *Drosophila*, while the SLC20 family has one homologue in each species, the novel genes *AGAP004257* and *CG7628* (Hubbard *et al.*, 2009). *CG7628* is expressed throughout the fruit-fly, but is highly enriched in the nervous tissues such as brain and thoracicoabdominal ganglion (Chintapalli *et al.*, 2007). There are no known Dipteran homologues to the SLC17 genes involved in inorganic ion and sialic acid transport, but the *Drosophila* gene *VGlut* and the *Anopheles* gene *AGAP007992* are similar to *SLC17A6, SLC17A7* and *SLC17A8*. *VGlut* is expressed in the nervous system of the fruit-fly, and similarly to its human homologues, appears to be involved in the transport of glutamate into synaptic vesicles at glutamatergic synapses (Daniels *et al.*, 2004).

The genes studied in this chapter, *AGAP012251* and *Picot*, are most similar in sequence to the mammalian SLC17 family (Benson *et al.*, 2009). Genes which are highly homologous to *AGAP012251* and *Picot* can be found in the gene alignment tree in Figure 5.1.

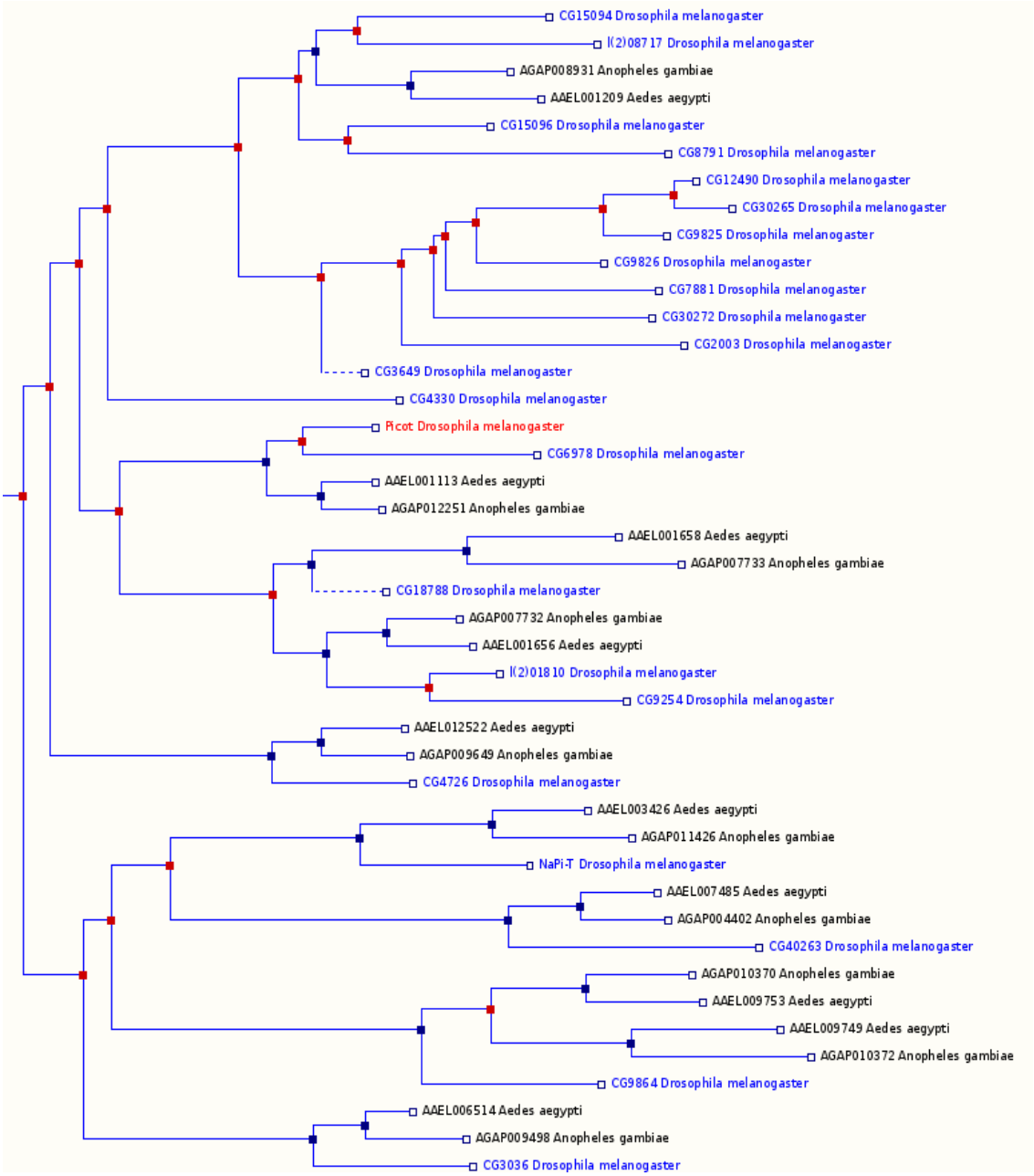


Figure 5.1 Alignment tree of genes closely related to *AGAP012251* and *Picot*. All of the highly homologous genes are from *Anopheles*, *Aedes* and *Drosophila*, suggesting a protein family which is highly insect-specific. Evolutionary tree generated by Ensembl, see Section 3.4.6.2 (Hubbard *et al.*, 2009; Vilella, 2009).

All of the highly related genes are insect-specific, belonging to *Anopheles*, *Drosophila* or *Aedes aegypti*, implying an adaptive radiation in the insects, and possibly suggesting that they could be good targets for selective insecticides. No human genes cluster with the insect-specific inorganic phosphate and vesicular glutamate transporters.

5.2.3 Inorganic Phosphate in the Malpighian Tubules

The insect Malpighian tubules, which are akin to the human renal system, are similarly involved in phosphate and calcium regulation and storage. Concretions of metallo-organic aggregates, often containing calcium and P_i, have been identified in the Malpighian tubules of *Drosophila* (Hevert, 1973), *Rhodnius prolixus* (Maddrell *et al.*, 1991), *Aedes aegypti* (Wessing, 1992) and *Anopheles albimanus* (Martinez-Barnetche *et al.*, 2007).

In *Drosophila* two types of concretions have been identified. Type I concretions are composed of calcium, magnesium, P_i and bicarbonate (Hevert, 1973; 1974; Wessing, 1992), and are located in the initial segment of the anterior tubule. The number of Type I concretions in the initial segment increases in parallel with increased calcium in the *Drosophila* diet (Wessing and Zierold, 1999). Type II concretions in *Drosophila* are thought to contain potassium, calcium and magnesium (Wessing, 1992) and are found in the transitional segment of the anterior tubule. In *Rhodnius prolixus* calcium is stored in concretions in the upper tubule, similarly to *Drosophila* (Maddrell *et al.*, 1991). There are differences between the species however; *Rhodnius* appears to sequester almost all calcium into concretions, whereas *Drosophila* secretes ~15% of dietary calcium in its urine and sequesters ~85% in the tubules (Dube *et al.*, 2000). In the mosquito *Anopheles albimanus,* metallo-organic concretions are found in the principal cells of the Malpighian tubules (Martinez-Barnetche *et al.*, 2007). In female *Anopheles albimanus*, there is also an increase in transcription of some of the genes involved in aggregate formation after a blood-meal (Martinez-Barnetche *et al.*, 2007). It has been suggested that excess ions such as iron may be stored in the concretions as a storage/deposit excretion method (Maddrell, 1971). The concretions would therefore be a way of isolating materials which are toxic in excess, by confining them to the tubules. Alternatively the concretions may be a useful store of ions, which can then be re-directed as needed to

sustain biological processes, such as oogenesis in the ovaries. Although P_i is prevalent in the *Drosophila* concretions, it is not known whether it is also abundant in the *Rhodnius* and *Anopheles* aggregates.

In *Drosophila* a small proportion of calcium is secreted by the main and lower segments of the tubule (Dow *et al.*, 1994; O'Donnell and Maddrell, 1995), with the vast majority being stored with P_i in the initial segment concretions (Wessing, 1992). In *Drosophila hydei,* the initial segment of the anterior tubule imports calcium at a much higher rate than any other segment in the tubules (Dube et al., 2000). Basolateral flux of calcium in the tubules is reduced when availability of P_i is reduced, suggesting that P_i is essential for the successful formation of concretions. Inorganic phosphate co-transporters are therefore likely to be required for the import of P_i into the Malpighian tubules, to allow calcium aggregate formation.

5.2.4 The Putative Inorganic Phosphate Co-transporters AGAP012251 and Picot

The genes studied in this chapter, *AGAP012251* and *Picot* (*CG8098*), are classed as homologues by ENSEMBL with an identity of 65% and an Expect value (e-value) of 2.5e-268 (Hubbard et al., 2009). Almost all differences in the coding sequences of *AGAP012251* and *Picot* are found in the N- and C-terminals of the proteins. *Picot* has twenty paralogues in *Drosophila*, and *AGAP012251* has eight paralogues in *Anopheles*. *AGAP012251* and *Picot* are classified as part of a protein cluster, which as of yet, has no members out-with the insect world.

The *Drosophila* genes which contain an anion/cation symport sequence, and have sequence similarity to the mammalian inorganic phosphate co-transporters, are split into two families; the Vesicular Glutamate Transporters and the Inorganic Phosphate Co-transporters (Hubbard *et al.*, 2009). *Picot* and *AGAP012251* are members of the Inorganic Phosphate Co-transporter family.

Picot and *AGAP012251* are novel genes with no published function. *Picot* was sequenced and named in a study of the neighbouring *AMYREL* gene in 1997 (Da Lage, submission FBrf0114357), but has not been functionally characterised. *Picot* is expressed in the embryonic salivary glands, midline glial cells, hindgut

and Malpighian tubules (Laridon *et al.*, 2008), as well as throughout the adult fly (Table 5.1).

Picot is transcribed in all of the adult tissues assayed, suggesting an involvement in P_i transport throughout *Drosophila* (Chintapalli *et al.*, 2007). It is highly enriched in the midgut, hindgut, and larval and adult Malpighian tubules, which are likely to be the main organs of P_i regulation. The transcription pattern of *AGAP012251* in *Anopheles* is not yet known.

Table 5.1 *Picot* expression in adult and larval *Drosophila* (Chintapalli *et al.*, 2007).

Tissue	mRNA Signal	Present Call	Enrichment	Affy Call
Brain	835 ± 37	4 of 4	1.80	Up
Head	682 ± 17	4 of 4	1.50	Up
Thoracicoabdominal ganglion	1058 ± 13	4 of 4	2.30	Up
Salivary gland	703 ± 26	4 of 4	1.50	Up
Crop	101 ± 5	4 of 4	0.20	Down
Midgut	1652 ± 92	4 of 4	3.50	Up
Tubule	**3621 ± 397**	4 of 4	**7.70**	**Up**
Hindgut	3336 ± 95	4 of 4	7.10	Up
Heart	58 ± 18	4 of 4	0.68	Down
Fat body	150 ± 6	4 of 4	0.12	Down
Ovary	175 ± 5	4 of 4	0.40	Down
Testis	150 ± 6	4 of 4	0.30	Down
Male accessory glands	85 ± 7	4 of 4	0.20	Down
Virgin spermetheca	573 ± 37	4 of 4	1.22	None
Mated spermetheca	769 ± 92	4 of 4	1.64	Up
Adult carcass	262 ± 16	4 of 4	0.60	Down
Larval CNS	1012 ± 118	4 of 4	2.15	Up
Larval salivary gland	285 ± 23	4 of 4	0.61	Down
Larval midgut	1433 ± 52	4 of 4	3.05	Up
Larval tubule	**2982 ± 136**	**4 of 4**	**6.30**	**Up**
Larval hindgut	3329 ± 125	4 of 4	7.08	Up
Larval fat body	7 ± 0	0 of 4	0.00	Down
S2 cells (growing)	13 ± 1	4 of 4	0.03	Down
Whole fly	**470 ± 26**	**4 of 4**		

5.3 Results

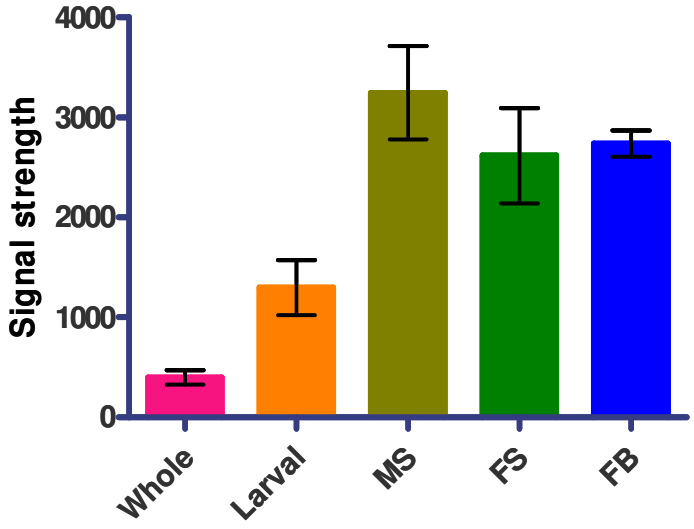
In this section the results from the investigation into the genes *AGAP0012251* and *Picot* as possible insecticide targets are described. The expression of *AGAP012251* and *Picot* was explored at both the transcript and protein level using microarrays, qPCR, *in situ* hybridisation, Western blotting and immunocytochemistry. *Picot* was then further investigated in *Drosophila*, where RNAi-mediated gene knockdown was used to demonstrate that its expression is required for development through to adulthood. The effect of decreasing expression of *Picot* on the rate of fluid secretion from the tubules was also determined in *Drosophila*.

5.3.1 AGAP012251 in the Tubules

The expression of *AGAP012251* as both a transcript and a protein in the *Anopheles* Malpighian tubules is described in this section. The initial microarray data which highlighted *AGAP012251* as highly enriched in the tubules is described, as is its subsequent verification by qPCR. The spatial expression pattern of the transcript in the tubules was determined by *in situ* hybridisation with RNA probes, in adults of both genders. Western blotting was used to verify that the *AGAP012251* transcript is translated to a protein, and to explore protein expression out-with the tubules.

5.3.1.1 Expression of the Transcript

The putative inorganic phosphate co-transporter *AGAP012251* was chosen for investigation from the *Anopheles* microarray due to its strong signal strength in the adult Malpighian tubules in comparison to the average expression in the whole mosquito (Figure 5.2). The statistical significance of the microarray data was determined using one-way ANOVA analysis and is presented in the attached table.



	FB	FS	MS	Larval
Whole	***	***	***	-
Larval	*	*	**	
MS	-	-		
FS	-			

Figure 5.2 *Anopheles* Malpighian tubule microarray data for *AGAP012251.* The attached table shows the statistical significance between life-stages. (N=4, mean ± S.E.M.) One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

The average signal strength for *AGAP012251* expression in whole mosquito is ~400. This is a relatively strong signal, suggesting that although *AGAP012251* is highly enriched in the tubules, there is expression elsewhere in the mosquito. Expression in the larval tubules is not significantly higher than the average expression in the adult mosquito, but the signal strength suggests that *AGAP012251* is functional in the tubules before adulthood.

Quantitative PCR was used to validate the microarray data and ensure it is representative of *AGAP012251* transcript expression (Figure 5.3A). The microarray data was also extended by measuring *AGAP012251* expression in the female tubules 24 h after a blood-meal (Figure 5.3B).

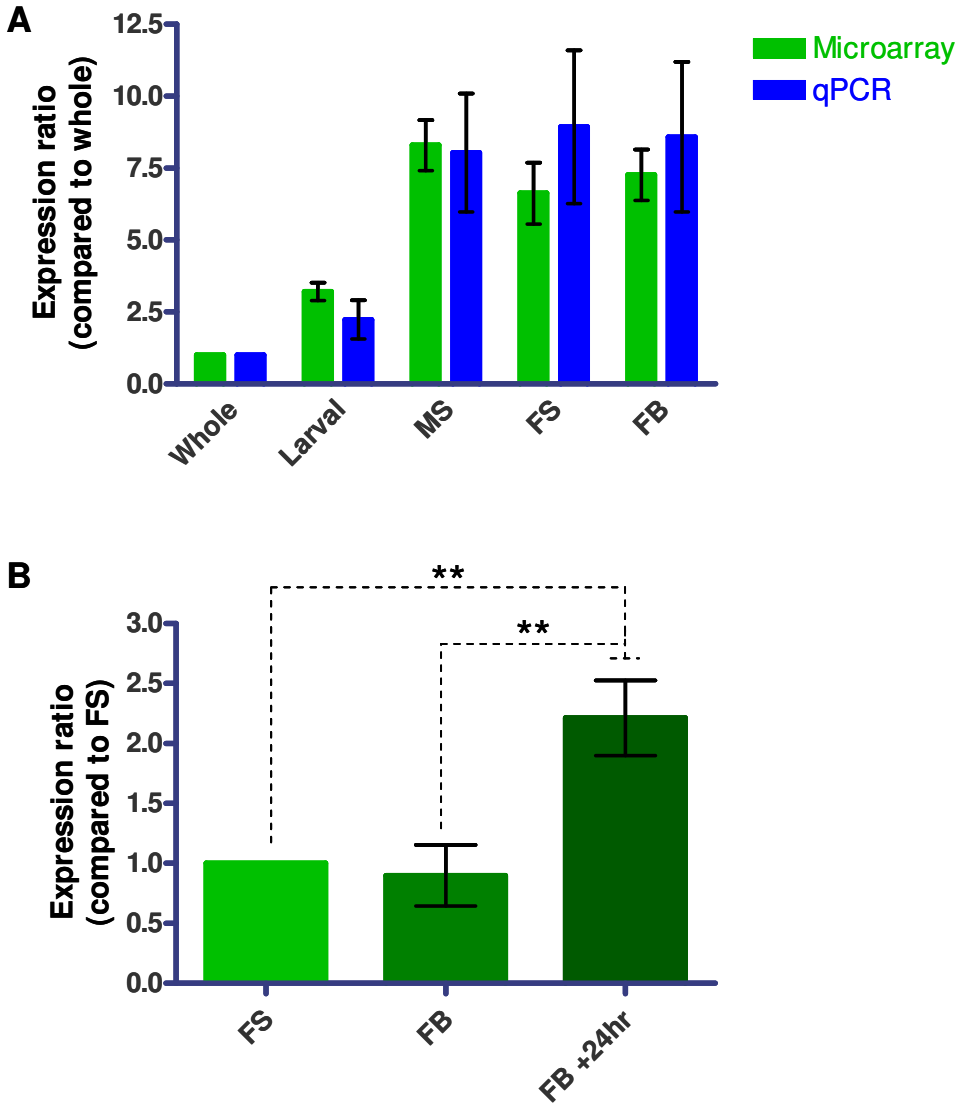


Figure 5.3 qPCR validation of *AGAP012251* expression in the *Anopheles* tubules. A: ratio of expression as calculated from microarray and qPCR data. B: ratio of expression in female tubules 3 h and 24 h after a blood-meal (N≥4, mean ± S.E.M.). One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

The validation data produced by qPCR agrees well with the microarray data, showing very similar expression ratios. In each adult life-stage the tubules have approximately 7- to 10-fold enriched expression over the average whole-mosquito sample. The qPCR data also shows *AGAP012251* to be enriched ~2-fold 24 h after a blood-meal, when the mosquito is digesting and regulating the protein and ion-load associated with haematophagous behaviour.

It is also of interest to determine whether *AGAP012251* is expressed ubiquitously in the Malpighian tubules, or in specific areas such as the distal tubule. Although the functional domains of mosquito Malpighian tubules are not well understood,

it is known that in other species such as *Drosophila* there are dedicated regions for absorption from the haemocoel and re-absorption into the haemocoel (Sozen *et al.*, 1997), as well as multiple cell types. *In situ* hybridisation was used to determine the expression pattern of *AGAP012251* in adult *Anopheles* tubules (Figure 5.4).

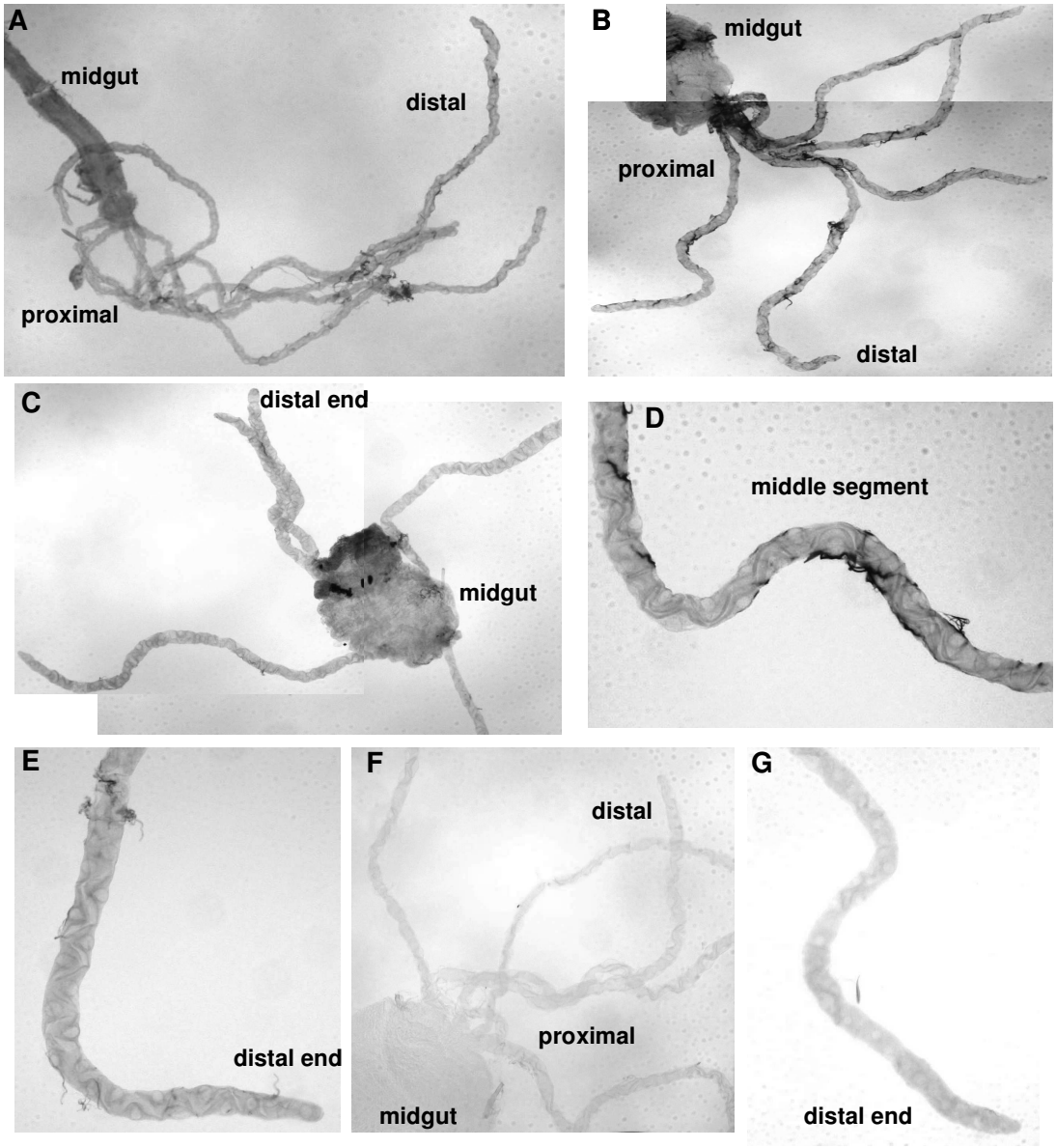


Figure 5.4 Expression of *AGAP012251* in the adult *Anopheles* tubules. A: low power view of the male tubules showing expression throughout. B: low power view of the female tubules showing expression throughout. C: low power view showing expression in the female lower tubule where it joins the gut. D: high power view of the middle of the female tubule showing expression. E: high power view of the distal end of the female tubule showing expression. F: low power view of the female tubules stained with sense probe as a control, showing no expression. G: high power view of the male distal end stained with sense probe as a control, showing no expression.

The *in situ* hybridisations confirmed that *AGAP012251* is expressed in all five tubules in adult males and females. The *AGAP012251* probe stained the *Anopheles* tubules from where they attach to the gut to the blind distal end, but showed much weaker staining than the other *in situ* hybridisations performed during this investigation. The anti-sense probe staining is darker than that produced by the control sense probe, but it is difficult to determine whether expression is in the stellate cells, or solely in the principal cells. The *AGAP012251* anti-sense probe also stained the midgut, suggesting that there may be expression in other areas of the alimentary canal.

The sense control probe showed no staining in the tubules or gut, although there was strong non-specific staining of the trachea which wrap around the tubules.

5.3.1.2 Expression of the Protein

In situ hybridisation of the *AGAP012251* transcript established that it is expressed throughout the length of the adult tubule in the principal cells, although it is unclear whether it is also in the stellate cells. As qPCR and *in situ* hybridisation assess the existence of the transcript but not the protein, an antibody specific for the AGAP012251 protein was designed. A fourteen amino-acid antigenic sequence was chosen which would be available for antibody binding and had limited homology to other proteins in *Anopheles*. Expression of *AGAP012251*, which is ~58 kDa in size, was assessed in protein extracted from *Anopheles* Malpighian tubules, head and whole-body, by Western blotting (Figure 5.5).

A band of the appropriate size was found in all three *Anopheles* samples, suggesting that *AGAP012251* expression is not confined to a specific tissue. The strongest band of AGAP012251 protein was in the tubules, although there is also protein present in the head and whole-mosquito samples. Although the Western blotting was optimised, numerous protein bands were found in all three samples, suggesting that the antibody is not specific to a single protein. Equivalent samples probed with pre-immune rabbit serum instead of AGAP012251-antibody produced no protein binding (data not shown).

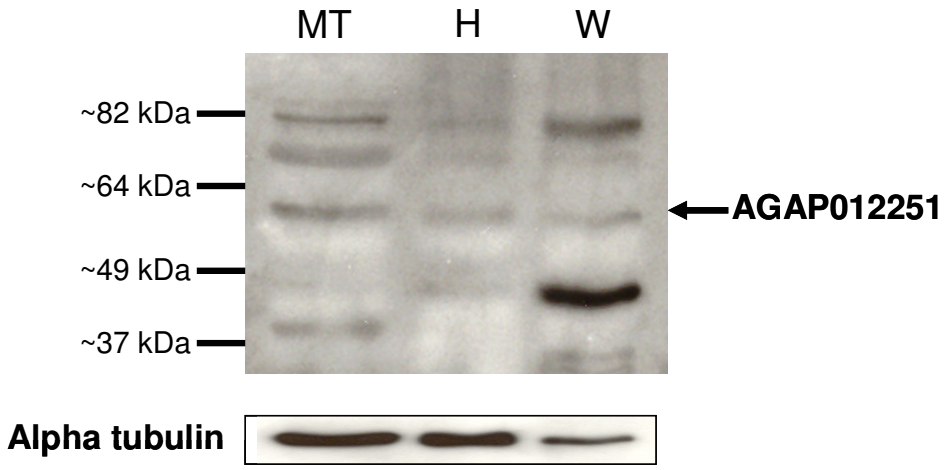


Figure 5.5 Protein expression of AGAP012251 in *Anopheles* as determined by Western blotting. A band the size of AGAP012251 (~58 kDa) can be seen in protein extracted from the Malpighian tubules (MT), head (H) and whole mosquito (W). Alpha tubulin was blotted as a control for protein integrity.

5.3.2 Picot in the Tubules

The expression of *Picot* as both a transcript and a protein in the *Drosophila* Malpighian tubules is described in this section. Transcription of *Picot* in the adult and larval tubules was verified by qPCR. The spatial expression of *Picot* in the tubule was determined using RNA probe *in situ* hybridisation. Western blotting and immunocytochemistry were used to determine that the transcript is translated to a protein, and to localise the cellular site of Picot in the tubules.

5.3.2.1 Expression of the Transcript

Picot was identified as a homologue of *AGAP012251* in *Drosophila* from its high sequence homology and enriched tubule expression (Wang *et al.*, 2004; Hubbard *et al.*, 2009). Enrichment of *Picot* in the larval and adult Malpighian tubules was verified by qPCR (Figure 5.6A). Expression of the transcript was also compared in the tubules of adult males and females, to determine whether its expression is sex-specific (Figure 5.6B).

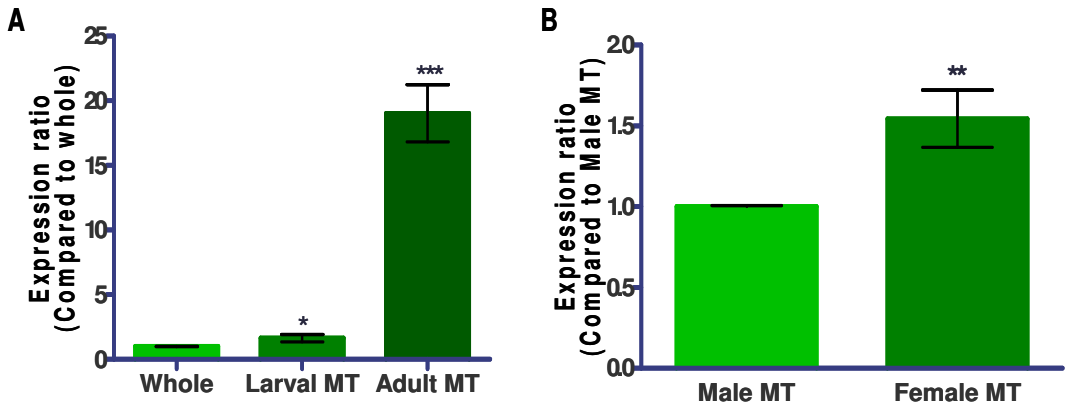


Figure 5.6 Expression of *Picot* in the *Drosophila* Malpighian tubules as determined by qPCR. A: expression in the larval and adult Malpighian tubules in comparison to the average whole-fly expression, as determined by qPCR. B: expression in the female tubules in comparison to the male tubules as determined by qPCR (N=4, mean ± S.E.M.). Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001.

Expression of *Picot* is up-regulated ~2-fold in the larval tubules and ~19-fold in the adult tubules in comparison to the average expression in the whole-fly. *Picot* transcription is also significantly greater in the tubules of adult females than males. In *Anopheles albimanus* some of the genes involved in metallo-organic concretion formation are enriched in the tubules of females compared to males (Martinez-Barnetche *et al.*, 2007). The genes are thought to be enriched to aid the regulation and storage of ions ingested during a blood-meal.

In situ hybridisation was performed on adult *Drosophila* tubules to determine whether *Picot* expression is localised to a particular domain or cell type (Figure 5.7).

The results of the *Picot in situ* hybridisations are similar to those of *AGAP012251*, although transcript staining is much lighter than in the other genes assayed. There is light staining along the length of the tubule, with no obvious differentiation between stellate and principal cells in both males and females. Additionally, there is strong staining in the anterior tubule initial and transitional segments, suggesting that this is the main site of *Picot* transcription. The sense control probe did not produce staining in the tubules.

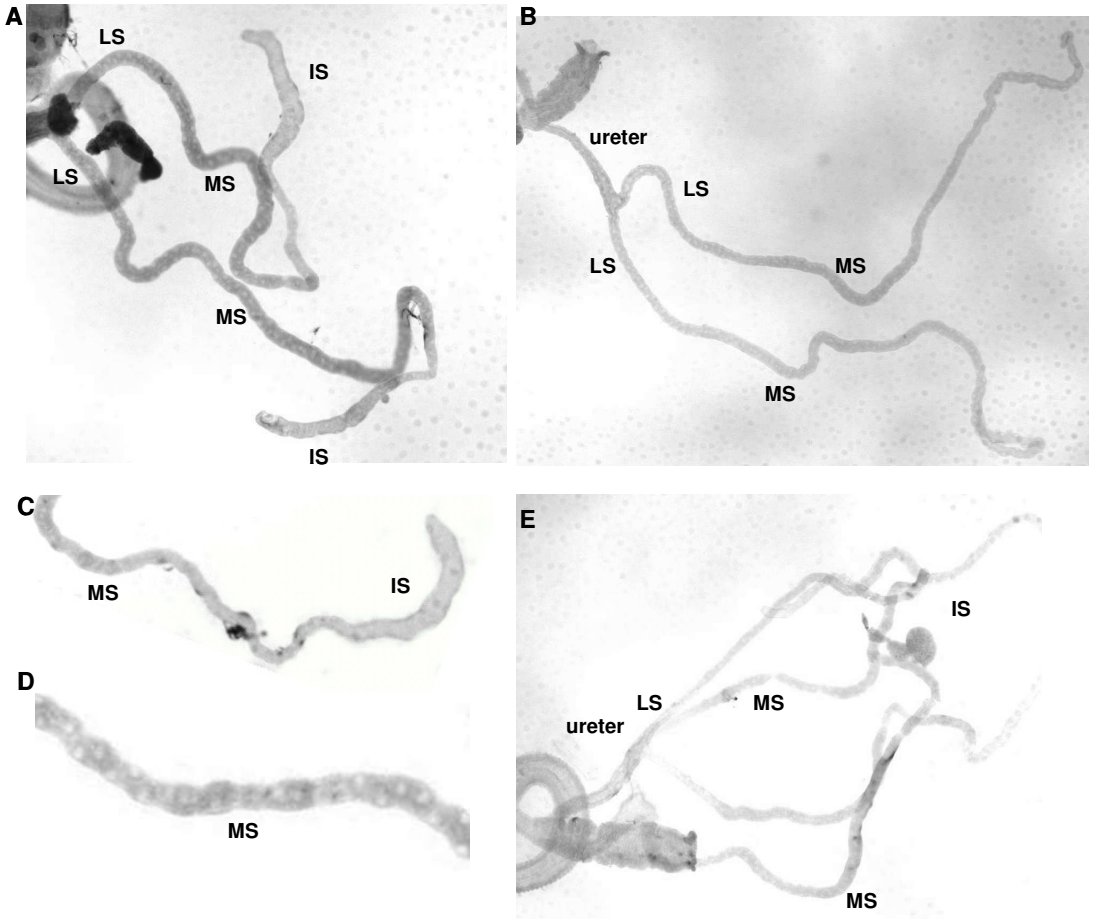


Figure 5.7 Expression of *Picot* in the *Drosophila* adult tubules as determined by *in situ* hybridisation. Low power views showing expression in the ureter, lower segment (LS), main segment (MS) and initial segment (IS) in the anterior (A) and posterior (B) tubules. C: high power shot of the anterior initial segment showing staining. D: high power shot of the posterior main segment showing faint staining. E: sense probe control showing no staining in the tubules.

5.3.2.2 Expression of the Protein

An antibody was raised again an antigenic fourteen amino acid sequence from the Picot protein. Western blotting was performed on protein samples extracted from whole-fly, Malpighian tubule and head, from 2-day and 7-day old flies (Figure 5.8).

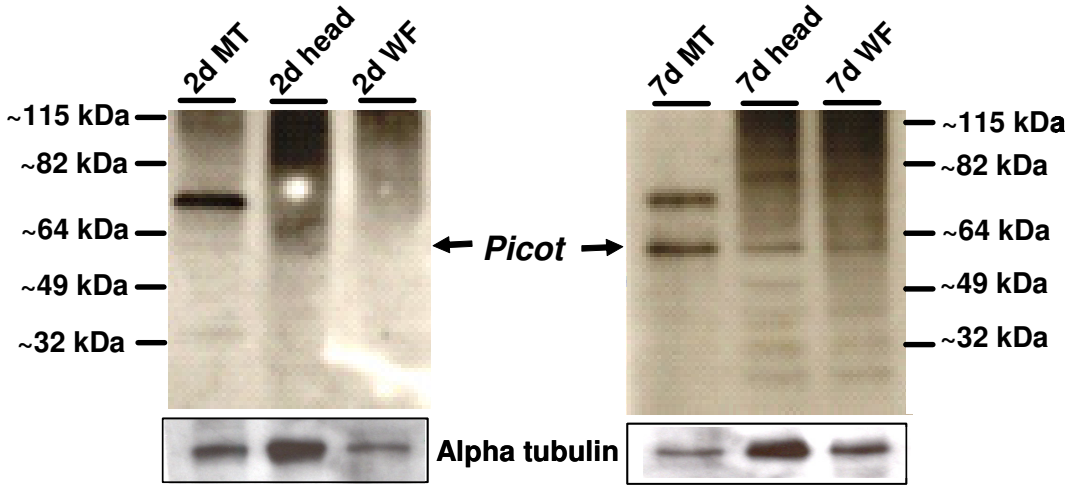


Figure 5.8 Expression of Picot in *Drosophila* as determined by Western blotting. A strong protein band the correct size for Picot (~58 kDa) can be seen in the Malpighian tubule, head and whole-fly samples of 7-day old flies, but only a faint band of Picot can be seen in the Malpighian tubules of 2 day-old flies. Alpha tubulin was probed as a control for protein integrity.

A faint band of the correct size, ~58 kDa, was found in the tubule sample of 2-day old flies, with no band in the head or whole-fly samples. A strong band of Picot protein was found in the tubule sample of 7-day old flies, with fainter bands in the head and whole-fly samples. This suggests that Picot expression is not prolific in the adult fly until at least 2 days after eclosion. As with the AGAP012251 antibody, the Picot antibody is not specific to a single protein, and identifies a second strong band of protein in the tubules. No Picot splice variants of the appropriate size (~74 kDa) have been sequenced, and the second strong band remains unidentified. The same experiment repeated with pre-immune serum instead of *Picot* antibody showed no protein bands (data not shown).

Immunocytochemistry was performed to identify the cellular structure to which the Picot protein localises. Tubules from adult males and females were dissected and stained with *Picot* antibody as a primary, and Texas Red (Invitrogen, UK) as a secondary antibody (Figure 5.9).

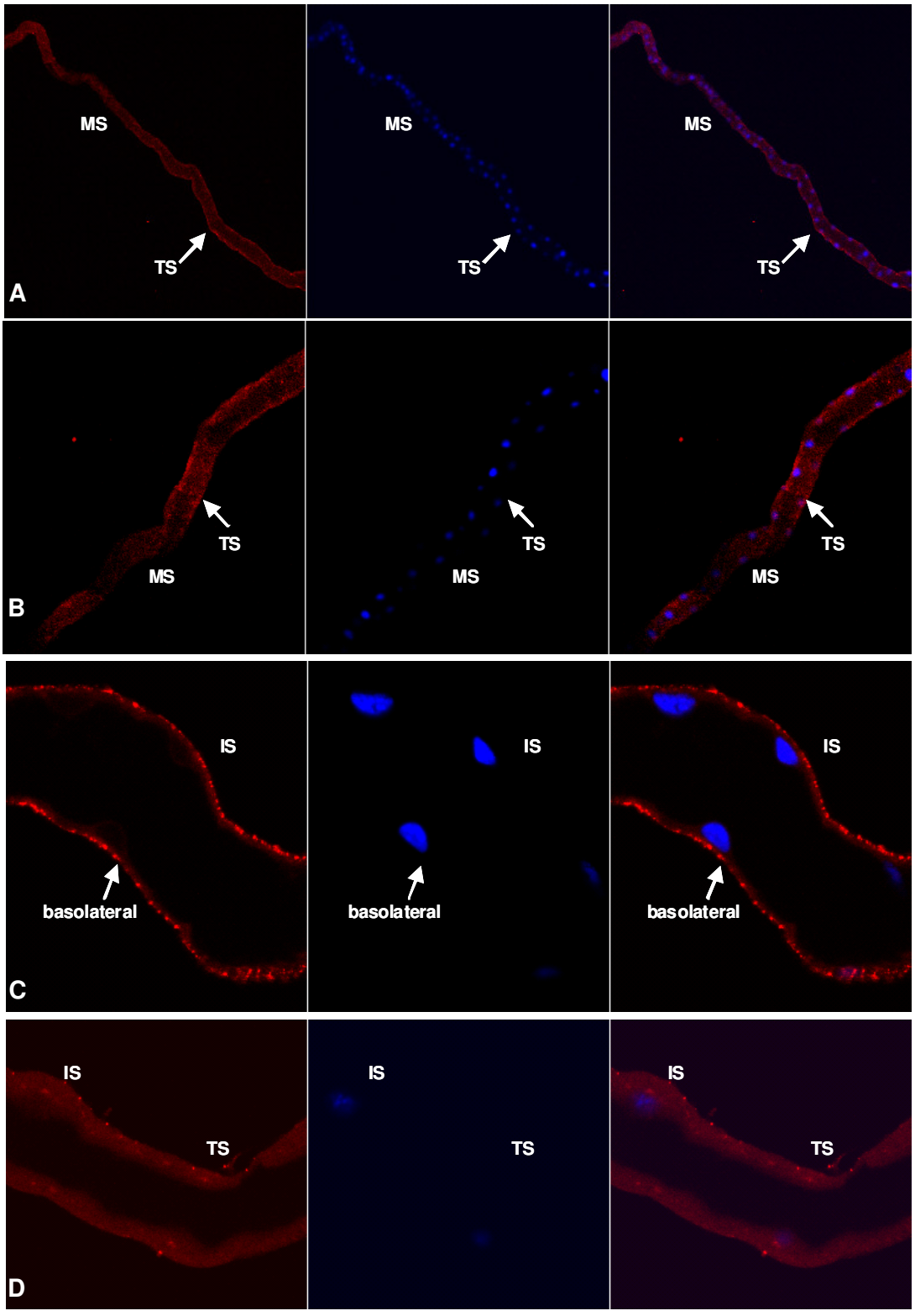


Figure 5.9 Localisation of Picot in the *Drosophila* adult tubules by immunocytochemistry. A: and B: antibody binding at the basolateral membrane of the transitional segment (TS) but not the main segment (MS) (20x magnification). C: antibody binding at the basolateral membrane of the initial segment (IS) (40x magnification). D: initial and transitional segment of control tubules treated with pre-immune serum and Texas Red (40x magnification).

Staining is present at the basolateral membrane of the initial and transitional segments of the anterior tubule. Whether Picot protein is specific to one cell-type is difficult to determine, although there is a continuous border of expression at the distal end of the tubules. The antibody staining in the main and lower segments appears to be background from the secondary Texas Red antibody. This data agrees with the *in-situ* hybridisation findings that *Picot* is strongly expressed in the initial and transitional segments of the anterior tubule. *Picot* may therefore be involved in the movement of P_i into vesicles at the basolateral membrane in the initial segment, where P_i is aggregated into concretions with calcium.

5.3.3 RNAi Knockdown of Picot

RNAi knockdown was used to determine whether *Picot* is an essential gene for *Drosophila* development to adulthood. As no RNAi lines were publicly available for targeting *Picot*, the pWIZ transgene (Lee and Carthew, 2003) was used as a vector. Three constructs were cloned, each targeted to a different part of the *Picot* transcript. The constructs were microinjected into w^{1118} flies and balanced over the homozygous lethal *CyO* on the second chromosome and TM3,*Sb* on the third chromosome. Three balanced lines were selected for each construct, resulting in nine RNAi fly-lines in total. Further studies were performed to determine the effect of *Picot* knockdown on fly survival and the rate of fluid secretion from the Malpighian tubules.

5.3.3.1 Effect on Fly Survival

For a gene to be an effective insecticide target it should be functionally essential to the species. The nine UAS-RNAi fly-lines under study were driven by four different GAL4 drivers (Table 5.2) to determine the effect of decreasing *Picot* expression on fly development through to adulthood. As the FlyAtlas (Chintapalli *et al.*, 2007) data suggests that *Picot* is highly expressed in *Drosophila* nervous tissues, the ELAV GAL4-driver was used to drive expression of the RNAi constructs in the fly nervous system.

Table 5.2 Pattern of expression of UAS/GAL4 drivers used to knock-down *Picot*.

Driver line	Pattern of expression
Actin-GAL4	Ubiquitous expression throughout the life-cycle
C42-GAL4	Principal cells in the main and lower segments and bar-shaped cells in the initial and transitional segments of the tubules
C724-GAL4	Stellate cells in the main segment and bar-shaped cells in the initial and transitional segments of the tubules
ELAV-GAL4	Nervous system

Progeny survival in GAL4-driven UAS-RNAi lines was classified as normal, semi-lethal, or lethal. Normal was defined as the expected ratio of driven-RNAi to non-driven-RNAi progeny being observed in a cross between a parental UAS-RNAi line and a GAL4-driver line. Semi-lethal was defined as significantly fewer RNAi-driven progeny being observed than expected (as determined by Chi-squared test). Lethal was classified as no driven-RNAi progeny emerging from a cross. The results can be found in Table 5.3.

Table 5.3 Effect of *Picot* RNAi knockdown on fly survival (cross N=3) (Raw data is presented in Appendix 2).

Fly-line	*Actin-GAL4*	*C42-GAL4*	*C724-GAL4*	*ELAV-GAL4*
Picot.c1.l3	lethal	semi-lethal	normal	normal
Picot.c1.l5	lethal	semi-lethal	normal	normal
Picot.c1.l6	lethal	normal	normal	normal
Picot.c2.l3	semi-lethal	normal	normal	normal
Picot.c2.l5	semi-lethal	normal	normal	normal
Picot.c2.l6	lethal	normal	normal	normal
Picot.c3.l2	semi-lethal	normal	normal	normal
Picot.c3.l3	semi-lethal	normal	normal	normal
Picot.c3.l5	lethal	normal	normal	normal

When crossed to the *Actin-GAL4* driver, all nine fly-lines had greatly reduced progeny numbers, or no progeny which survived beyond 5-days. Where there was complete lethality, it occurred in the embryonic or early larval stages, when *Picot* is known to be expressed (Chintapalli *et al.*, 2007; Laridon *et al.*, 2008). In the lines which were classed as semi-lethal, the progeny which eclosed to

adulthood died after ~5 days. This lethality correlates with the increase in *Picot* expression which occurs when flies are between 2-days and 7-days old. The number of lines in which GAL4-driven UAS-RNAi expression results in partial or full lethality indicates that it is unlikely to be off-target silencing or insertional effects of the transgene which are responsible.

The tubule specific GAL4-drivers did not have such an obvious effect on survival to adulthood, with only two fly-lines driven by *c42-GAL4* showing reduced progeny numbers. *C42-GAL4* drives expression in the bar-shaped cells of the initial and transitional segments of the tubules, in which *Picot* appears to be expressed. Reducing *Picot* expression in the nervous system by driving the RNAi constructs with ELAV had no effect on fly survival.

Picot knockdown was quantified in each GAL4-driven UAS-RNAi line using qPCR (Figure 5.10 A-C). Knockdown was quantified in the tubules of UAS-RNAi fly-lines driven with *c42-GAL4*, as fly-lines driven with *Actin-GAL4* were not viable. *CG6978,* the closest *Drosophila* homologue of *Picot*, was quantified in two GAL4-driven UAS-RNAi lines to determine whether there was any off-target gene silencing (Figure 5.10 D).

All nine of the GAL4-driven UAS-RNAi lines had significantly lower *Picot* expression than their parental lines, as determined by Student's *t*-test. The knockdowns ranged from ~50% to ~25% of parental transcript levels, with variation between the lines resulting from insertional effects and different efficiencies of the constructs at targeting the transcript for degradation. The lines tested for off-target gene-silencing showed no significant decrease in *CG6978* expression. In principal, the reduced fly survival which results from interfering with *Picot* function suggests that it would be a valid insecticide target.

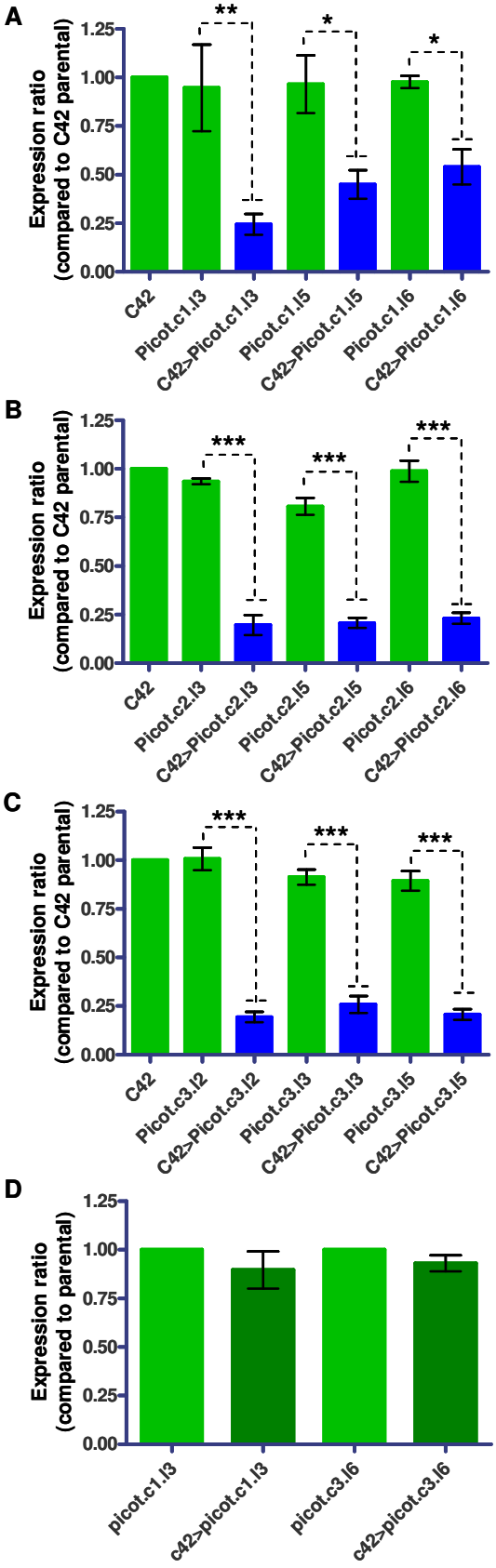
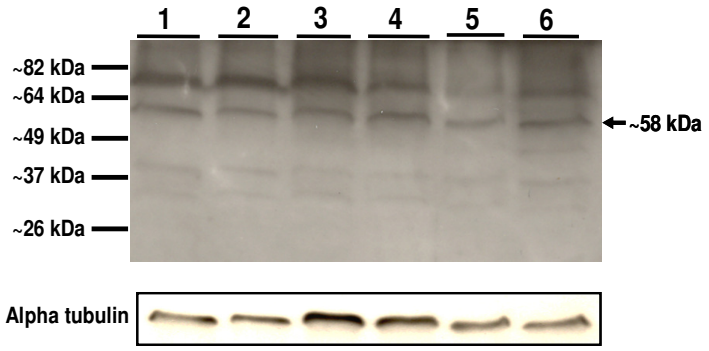


Figure 5.10 qPCR verification of *Picot* knockdown in RNAi fly-line tubules. *Picot* expression in A: RNAi lines from construct 1, B: RNAi lines from construct 2, and C: RNAi lines from construct 3. D: Quantification of *CG6978* expression in driven RNAi lines (N=3, mean ± S.E.M.) (Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001).

Western blotting was used to assay Picot protein expression in three GAL4-driven UAS-RNAi fly-lines (Figure 5.11).



Lane	Fly-line
1	c42>picot.c1.l6
2	picot.c1.l6
3	c42>picot.c2.l3
4	picot.c2.l3
5	c42>picot.c3.l3
6	picot.c3.l3

Figure 5.11 Picot expression in three GAL4-driven UAS-RNAi fly-lines and their parental fly-lines as determined by Western blotting. Alpha tubulin was blotted as a control for protein integrity.

The Western blot does not show a convincing down-regulation of Picot between the GAL4-driven and parental fly-lines. This may be because the *c42-GAL4* driver does not drive knockdown in all the cells of the initial and transitional segments where Picot is expressed or because the knockdown is partial and not all of the *Picot* transcripts are degraded.

5.3.3.2 Effect on Fluid Secretion from the Tubules

The effect of *Picot* RNAi knockdown on the rate of fluid secretion from the tubules was investigated using Malpighian tubule secretion assays (Dow *et al.*, 1994). Tubules dissected from the GAL4 driven UAS-RNAi line, the UAS-RNAi parent and the GAL4 driver parent were allowed to secrete steadily for 30 min before the diuretic peptide Drosokinin was added to the reservoir bubble. Three GAL4-driven UAS-RNAi lines were tested; one line for each of the three RNAi constructs (Figure 5.12).

Knockdown of *Picot* has no statistically significant effect on the rate of basal or stimulated fluid secretion from the *Drosophila* Malpighian tubules. If the role of *Picot* is the transport of P_i into the initial segment of the anterior tubule for storage it is not surprising that knockdown has no effect on secretion rates, as the two are unlikely to be directly related.

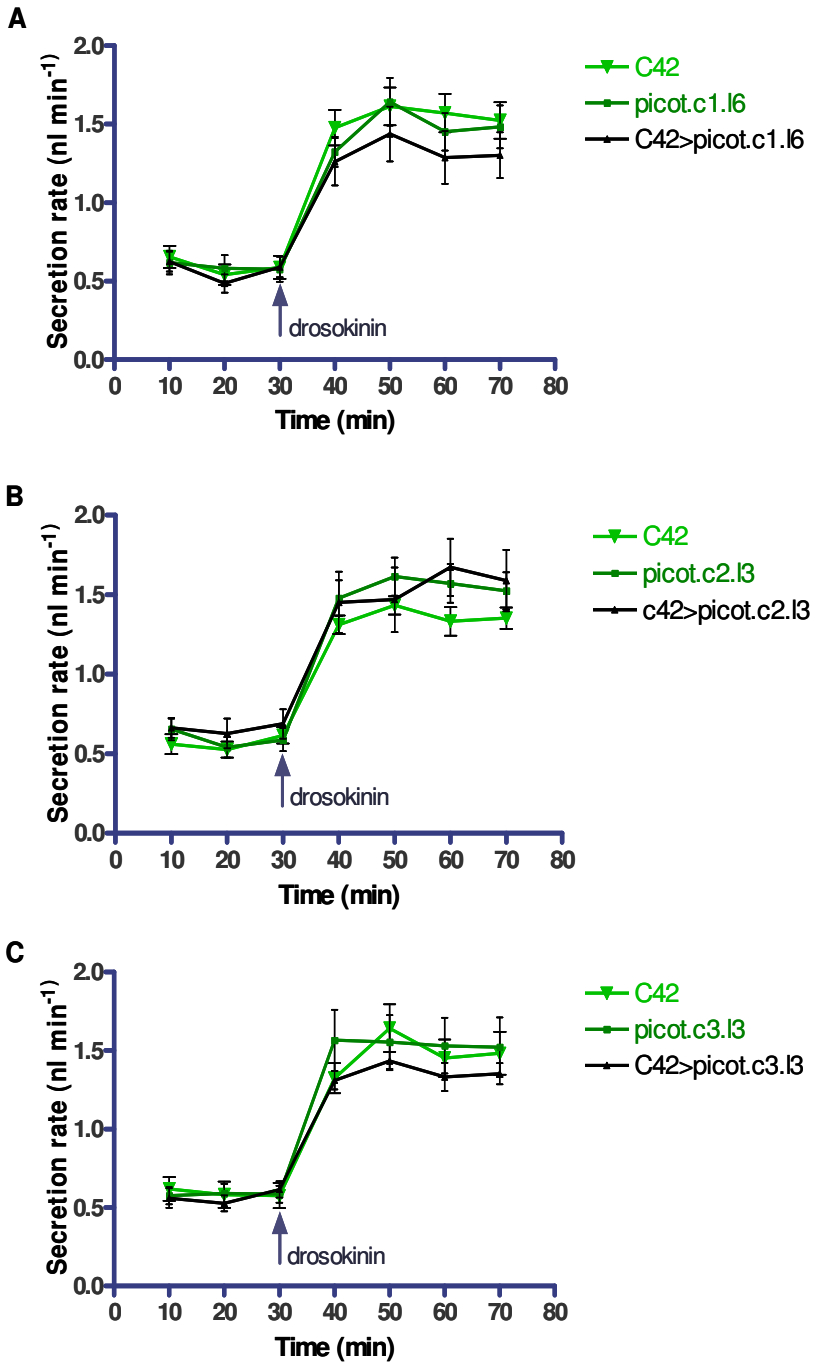


Figure 5.12 Effect of *Picot* RNAi knockdown on the rate of fluid secretion from the tubules as determined by secretion assays. Typical experimental results, showing the response of three driven RNAi lines and the appropriate RNAi parental lines to Drosokinin after 30 min of steady secretion (mean ± S.E.M., N≥10).

5.4 Discussion

The regulation, transport and storage of minerals such as phosphate and calcium are essential processes in all cells and tissues. Phosphate is accumulated in concretions in the initial segment of the anterior Malpighian tubule of *Drosophila*, and is required for the storage of vital ions such as calcium. This section discusses the expression of the putative inorganic phosphate co-transporters *AGAP012251* and *Picot* in the Malpighian tubules, and their likely function. The usefulness of *Picot* as a model for *AGAP012251*, and their likely success as insecticide targets, is also considered.

5.4.1 AGAP012251 and Picot in the Tubules

The spatial and temporal expression of *AGAP012251* and *Picot* in the tubules was investigated for two reasons; to gain further understanding of gene function, and to determine how similarly the homologues are expressed. *AGAP012251* is ~2-fold enriched in the larval tubules and ~7.5-fold enriched in the adult tubules of *Anopheles* at each life-stage. *Picot* is ~2-fold enriched in the larval tubules and ~20-fold enriched in the adult tubules of *Drosophila*. Both genes have a strong microarray signal in the whole-body samples, suggesting that their expression is not confined to the tubules. Transcription of *AGAP012251* is doubled 24 h after haematophagy, possibly to cope with the ion load associated with a human blood-meal and to promote concretion formation. The concentration of phosphorous in human blood serum is approximately 2.5 – 4.2 mg per 100 ml, and calcium approximately 10 mg per 100 ml (Farquharson and Tibbetts, 1931). It has been shown in the cave cricket, *Troglophilus neglectus*, that spherites in the Malpighian tubules provide minerals for vital processes during the dormant overwintering period of the insect (Lipovsek Delakorda *et al.*, 2009). It is not known whether the concretions can be broken down after formation in *Drosophila* or *Anopheles gambiae*, but they could be used as a store of ions for oogenesis and egg development, as has already been suggested in *Anopheles albimanus* (Martinez-Barnetche *et al.*, 2007).

In situ hybridisation indicates that *AGAP012251* is expressed in the principal cells throughout the length of the tubules in *Anopheles*, although the faint staining makes it difficult to determine whether it is also expressed in the stellate cells.

Immunocytochemistry experiments in the *Anopheles* tubules using the AGAP012251 antibody proved inconclusive, due to high levels of background auto-fluorescence (data not shown). In *Drosophila* the strongest *Picot* staining is in the initial and transitional segments of the anterior tubules. This is in agreement with the immunocytochemistry results, which show *Picot* to be expressed at the basolateral membrane of the initial and transitional segments. The precise localisation of Picot could be determined using co-labelling experiments to known plasma membrane and vesicular markers, which would also give further insight into the likely cellular function of Picot. The basolateral membrane of the initial and transitional segments is where we would expect *Picot* to be expressed if it were involved in the transport of P_i into the tubules for concretion formation.

RNAi knockdown of *Picot* using the UAS/GAL4 system was used to mimic the effect that insecticide targeting would have if the compound prevented transporter function. Knockdown of *Picot* throughout the fly using an *Actin-GAL4* driver line had a lethal or semi-lethal effect on the progeny of all nine of the lines assayed. Lethality occurred at the embryonic or early larval stages, which is unsurprising as *Picot* is known to be expressed in the embryonic salivary glands, midline glial cells, hindgut and Malpighian tubules (Laridon *et al.*, 2008), and the larval salivary gland, midgut, hindgut and tubules (Chintapalli *et al.*, 2007). In the semi-lethal lines, where reduced numbers of progeny eclosed, the progeny died when they were 4-5 days old. This corroborates the Western blotting results, which indicate that *Picot* expression in the tubules and head increases between 2-days and 7-days after eclosion. These results suggest that *Picot* is essential for early development in *Drosophila*, and also for survival of the adult fly post-eclosion.

Knockdown of *Picot* in specific domains in the tubules had little effect on fly survival, apart from in two lines driven with *c42-GAL4*, which had reduced progeny numbers. The *c42-GAL4* driver knocks down expression in the principal cells in the lower and main segments, and the bar-shaped cells of the initial and transitional segments. Specific knockdown of *Picot* in the stellate cells of the tubules and the fly nervous system had no effect on fly survival. Knockdown of *Picot* specifically in the initial segment of the tubules would help to determine

whether it is initial segment expression which is vital for *Drosophila* development and survival.

The microarray, qPCR, *in situ* hybridisation and Western blotting results are in good agreement with one another, with no notable differences in findings gained from the different techniques. There is good agreement between the data collected for *AGAP012251* and *Picot* in *Anopheles* and *Drosophila* for all five types of experiment performed. There is no evidence to suggest that *Picot* is not a reliable model for *AGAP012251* in the Malpighian tubules.

5.4.2 AGAP012251 and Picot as Inorganic Phosphate Co-transporters

The published sequences of *AGAP012251* and *Picot* contain the MFS and ACS motifs which are characteristically found in mammalian inorganic phosphate co-transporters (Hubbard *et al.*, 2009). The large number of genes which have been classed as putative inorganic phosphate co-transporters in *Anopheles* and *Drosophila* suggests that similarly to the human gene family, the transporters will not all be specific for P_i. The protein family which contains *AGAP012251* and *Picot* contains three additional *Drosophila* genes which are up-regulated in the tubules, but none with as large a microarray signal strength (Chintapalli *et al.*, 2007). As of yet no genes homologous to *AGAP012251* and *Picot* have been annotated out-with the Dipterans, which suggests a specialised form of P_i transport tailored to insects. As *AGAP012251* and *Picot* expression is not confined to the tubules, and there are no known storage concretions of P_i out-with the tubules, they may also play a more general role in P_i transport in other organs.

If *Picot* is involved in the transport of P_i into the initial and transitional segments of the anterior tubule we would not necessarily expect it to play a role in determining the rate at which fluid is secreted from the tubules. This was confirmed in the results of the secretion assays, as gene knockdown of *Picot* had no effect on the basal or stimulated secretion rate. It is also worth noting that the secretion assays were performed on flies driven with *c42-GAL4,* which were 7-days post-eclosion, and were therefore transcribing enough *Picot* for survival through to adulthood. Transport assays using mineral tracers such as phosphate and calcium could be used to determine whether *Picot* is required for phosphate

uptake and also whether *Picot* knockdown disrupts calcium uptake by the tubules. Electron microscopy and x-ray microanalysis of concretions in flies with reduced *Picot* expression could also be used to determine whether *Picot* knockdown affects the volume of concretions and their mineral composition.

5.4.3 AGAP012251 and Picot as Insecticide Targets

AGAP012251 and *Picot* appear to make good putative insecticide targets. The lethal effect of reducing *Picot* expression suggests that it is an essential gene for fly development through to adulthood. If *AGAP012251* is essential at as early a developmental stage as *Picot,* insecticide targeting could be effective at any stage in the mosquito life-cycle.

The similarity in tubule enrichment and expression between this highly homologous gene-pair also increases the likelihood of any developed insecticides being useful for multiple Diptera species. That no homologous gene to *AGAP012251* and *Picot* has been annotated in humans or any other organism outside of the insects would be beneficial, as it decreases the likelihood of an insecticide compound being dangerous to other species.

5.5 Conclusion

The results presented in Chapter 5 indicate that the putative inorganic phosphate co-transporters *AGAP012251* and *Picot* would be interesting insecticide target candidate genes. There is good agreement between the five experiments performed in both *Anopheles* and *Drosophila*, suggesting that *Picot* is a good model for studying *AGAP012251*. The enrichment of these genes in the *Anopheles* and *Drosophila* Malpighian tubules and their translation into proteins suggests that they are functionally active. The conservation of this homologous gene pair over ~150 million years of Dipteran evolution also suggests that they are functionally important in the tubules. That they contain the characteristic motifs which define mammalian phosphate transporters, but do not have any mammalian homologues, suggests a family of transporters evolved to meet the specific needs of insects. Expression of *AGAP012251* in the principal cells of the *Anopheles* tubules, and *Picot* in the initial and transitional segments of the *Drosophila* tubules, suggests that they may be involved in the import of inorganic phosphate for metallo-organic concretion formation. Import of inorganic phosphate is vital for calcium import, and therefore calcium regulation, and so it is not surprising that decreased expression of *Picot* is lethal in *Drosophila*. The lack of a secretion phenotype in the tubules when *Picot* expression is depleted confirms that the transporter is not directly involved in osmoregulation. Transport assays could be used to confirm that *Picot* transports phosphate into the tubules, and could also be used to determine whether other mineral uptake pathways also require phosphate, as has been suggested.

Chapter 6 - *AGAP009005* and *ZnT35C* as Insecticide Targets

6.1 Summary

In Chapter 3 it was established that inorganic and organic transporters are highly enriched in the *Anopheles* Malpighian tubules, and therefore may be effective targets for new insecticides. Zinc transporters are conspicuous among the genes enriched in both the *Anopheles* and *Drosophila* Malpighian tubule microarray datasets (Wang *et al.*, 2004), although the functional role of the tubules in zinc regulation has not yet been determined. The introduction to this chapter describes the known zinc transport families in Diptera and mammals, and our current knowledge of their physiological roles. The *Anopheles* gene *AGAP009005* and the *Drosophila* gene *ZnT35C* are putative zinc transporters, and are the focus of this study. The results presented in this chapter confirm the transcription of *AGAP009005* and *ZnT35C* in the tubules of *Anopheles* and *Drosophila*. *AGAP009005* and *ZnT35C* are expressed throughout the length of the Malpighian tubules in both species in the principal cells, and possibly in the stellate cells. The importance of *ZnT35C* for fly development through to adulthood is demonstrated using the UAS/GAL4 system to perform knockdown of gene expression, which is lethal to the fly. The role of *ZnT35C* in osmoregulation was also investigated, although gene knockdown of *ZnT35C* had no significant effect on the rate of fluid secretion from the tubules. Finally, the data collected for *AGAP009005* and *ZnT35C* in *Anopheles* and *Drosophila* is discussed, as is the expected function of *AGAP009005* and *ZnT35C*, and their likely success as insecticide targets.

6.2 Introduction

Metal ions such as zinc and copper are essential components of the cellular pathways responsible for transcription, respiration and growth in all forms of life. Over-accumulation of zinc and copper can also be toxic to a tissue, and so their uptake, storage and excretion must be carefully synchronized. The requirement for tight zinc regulation makes the genes involved attractive targets for the development of novel insecticides in Diptera. Two genes involved in zinc transport, *AGAP009005* and *ZnT35C*, are up-regulated in the Malpighian tubules of *Anopheles* and *Drosophila* respectively, and are investigated with regards to their usefulness as insecticide targets in this chapter.

6.2.1 The Importance of Zinc Regulation

Zinc is an essential component of many of the metalloproteins involved in cell replication, nucleic acid metabolism, and tissue repair and growth. Zinc acts as a co-factor for many enzymes, including the alcohol dehydrogenases, and fulfils both structural and catalytic roles (Auld and Bergman, 2008). Zinc is also important for control of gene transcription, as it regulates the binding of activators and repressors to gene promoter regions (Jackson *et al.*, 2008). Novel roles for zinc in the mammalian immune system are also being investigated, and preliminary studies suggest it is involved in intracellular signalling in specific immune cells (Hirano *et al.*, 2008). Zinc has also been implicated in the modulation of T-type calcium channels, which are physiologically important in the nervous and cardiovascular system of mice (Huc *et al.*, 2008).

Deficiency of dietary zinc is associated with a range of pathological conditions, and in mammals these include impaired immunity, brain development disorders, retarded growth and delayed wound healing (Murakami and Hirano, 2008). Therefore it is essential that zinc is tightly regulated at the cellular, tissue, and whole-organism levels.

6.2.2 Zinc Transport in Mammals

Tissue-specific zinc regulation in mammals is largely through buffering agents such as the metallothioneins (Kagi and Schaffer, 1988), and zinc transporters. In

higher eukaryotes two solute-linked carrier (SLC) protein families are associated with zinc transport; SLC39A and SLC30A. The SLC39A family (also known as the ZIPs) increase intracellular zinc availability by promoting uptake from extracellular fluid, and release of zinc from storage vesicles into the cytoplasm (Liuzzi and Cousins, 2004). The SLC30A family (also known as the ZnTs) reduce intracellular zinc availability by promoting both the efflux of zinc from cells, and its packaging into intracellular vesicles (Harris, 2002). The SLC39A and SLC30A families have tissue-specific expression patterns, distinct responses to fluxes in zinc availability, and a range of hormones and cytokines which act on them as physiological stimuli (Devirgiliis *et al.*, 2007). Although the mechanism of zinc transport has not yet been elucidated, both families are believed to operate via facilitated diffusion, secondary active transport or as zinc symporters (Liuzzi and Cousins, 2004).

In higher eukaryotes the physiological response to excess zinc is partly regulated by *metal-responsive-element-binding transcription factor-1* (*MTF-1*). *MTF-1* promotes the expression of numerous genes involved in zinc homeostasis and response to heavy-metal toxicity, such as the metallothioneins (Laity and Andrews, 2007). The metallothioneins are involved in the intracellular fixation of zinc and copper and regulation of their movement to specific cellular destinations, thereby regulating the intracellular concentration of free zinc and copper (Kagi and Schaffer, 1988).

6.2.3 Zinc Transport in Insects

As in higher eukaryotes, insect zinc transporters are important for zinc mobilisation under both normal physiological conditions and times of heavy metal stress. Laboratory fly-food normally contains ~0.2 mM zinc, yet *Drosophila* can survive on food containing up to 14 mM zinc (Ballan-Dufrancais, 2002). When the zinc content of food is increased from 0.2 mM to 4 mM the total body zinc content of the fly rises ~7-fold (Yepiskoposyan *et al.*, 2006). This non-proportional increase in zinc ion concentration suggests that uptake and excretion of zinc is regulated.

Microarray experiments performed in whole larval *Drosophila* to determine the transcriptomic changes associated with increased dietary zinc found that 299

genes were up-regulated and 82 genes down-regulated (Yepiskoposyan *et al.*, 2006). The gene most highly enriched in response to zinc was *CG10505*, a putative ABC transporter. The FlyAtlas microarray data shows *CG10505* to be massively enriched (~143-fold) in the Malpighian tubules of larvae on a normal diet, implying that the tubules may be where *CG10505* is most active (Chintapalli *et al.*, 2007). Other genes transcribed in response to increased zinc uptake are *GstD2* and *GstD5*, which are part of the glutathione *S*-transferase family of detoxification enzymes (Wagner *et al.*, 2002), and the putative zinc transporter *ZnT35C* (see Section 6.2.4).

In *Drosophila* ten genes have been annotated as containing a ZIP-like domain, which is required for the functioning of zinc influx transporters in mammals (Hubbard *et al.*, 2009). A number of genes containing ZIP-like domains are expressed uniformly in the adult fly (*ZIP1, CG9430, CG7816*), whereas others are up-regulated in specific tissues, such as the midgut, hindgut, salivary gland and male accessory glands (Chintapalli *et al.*, 2007). Five genes are highly enriched in the larval or adult Malpighian tubules; *CG13189, CG10006, CG4334, Zip3,* and *CG2177*. The best characterised ZIP-like gene in *Drosophila* is *fear of intimacy* (*foi*), which is functionally important for central nervous system development (Hummel *et al.*, 1999), gonad development (Howard, 1998), and cell migration (Van Doren *et al.*, 2003). *Foi* expression is relatively uniform in the adult fly, with a slight up-regulation in the female ovaries and male accessory glands (Chintapalli *et al.*, 2007). A second *Drosophila* ZIP-like gene, *Catsup,* has been shown to function as a negative regulator of tyrosine hydroxylase activity (Stathakis *et al.*, 1999). The other members of the *Drosophila* ZIP-like protein family are yet to be characterized, although *Zip3* is known to be up-regulated by the Imd immune pathway (De Gregorio *et al.*, 2002). The *Anopheles* genome contains nine genes which include a ZIP-like domain, none of which have been functionally characterised (Hubbard *et al.*, 2009).

The ZnT-like domain which is associated with zinc efflux transporters in mammals is found in seven *Drosophila* genes (Hubbard *et al.*, 2009). Five of the seven genes are expressed throughout the adult fly, with up-regulation in specific tissues such as the Malpighian tubules, midgut, hindgut, thoraccicoabdominal ganglion and male accessory gland (Chintapalli *et al.*, 2007). *ZnT35C* is the only ZnT-like gene to have been studied functionally in

Drosophila (Yepiskoposyan *et al.*, 2006), and is also the gene under investigation in this study. In *Anopheles* there are six genes annotated as containing a ZnT-like domain, none of which have been functionally characterised (Hubbard *et al.*, 2009). One of these genes, *AGAP009005*, is homologous to *ZnT35C*, and is the *Anopheles* gene investigated in this chapter.

6.2.3.1 Zinc Transport in the Malpighian Tubules

In 1976, Sohal et al observed that specific storage vacuoles in the Malpighian tubules of the housefly *Musca domestica* contained an accumulation of zinc, and it was hypothesised that the vacuoles were a mechanism of zinc detoxification (Sohal *et al.*, 1976; Maddrell, 1977). Insects occasionally favour long-term storage of small toxic ions over excretion as they can be re-absorbed in the hindgut back into the haemocoel during excretion, instead of being expelled in the urine. In *Drosophila hydei*, Zeirold and Wessing observed zinc storage in dense vacuoles in the proximal region of the adult and larval anterior tubules (Zierold and Wessing, 1990). They found that increased dietary intake of zinc resulted in increased zinc storage in the vesicles in the Malpighian tubules. These studies were furthered by Schofield *et al*, who examined zinc and copper accumulation in *Drosophila* (Schofield *et al.*, 1997). They determined that zinc is stored in vesicles in both the anterior and posterior Malpighian tubules, predominantly in the main and lower segments. The ureter contains very little zinc, while the initial and transitional segments of the tubules store some zinc, but less than the main and lower segments. The initial and transitional segments of the anterior tubule are the main storage site of calcium and inorganic phosphate in *Drosophila* in the form of metallo-organic concretions (see Chapter 5). The zinc content of the Malpighian tubules is much greater than the zinc content of any other organ in the fly, including the reproductive organs, digestive tract, fat body, epidermis and muscle (Schofield *et al.*, 1997). This suggests that zinc accumulation in the tubules is not merely a detoxification mechanism, but a storage system which ensures that there is an accessible supply of zinc for biological processes.

6.2.4 The Putative Zinc Transporters AGAP009005 and ZnT35C

AGAP009005 and *ZnT35C* are ranked as one-to-one homologues by ENSEMBL with an identity of 42% and an Expect value (e-value) of 3.4e-165 (Hubbard *et al.*, 2009). Figure 6.1 shows the partial evolutionary tree which contains *ZnT35C* (*CG3994*) and *AGAP009005*.

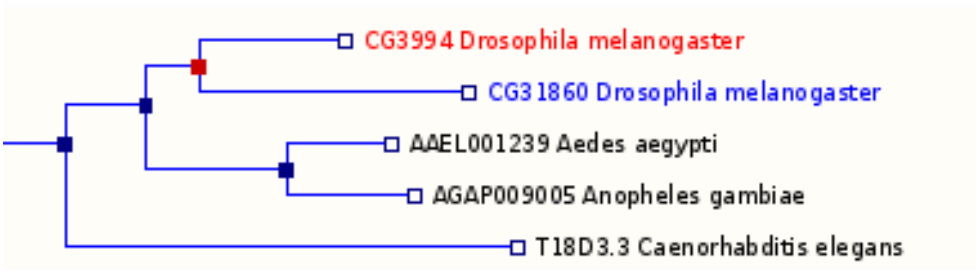


Figure 6.1 Homology tree for ZnT35C (*CG3994)* and *AGAP009005*. The tree shows a close relationship between the *Anopheles* and *Drosophila* zinc transporters. (Tree generated by Ensembl, see Section 3.4.6.2 (Hubbard *et al.*, 2009; Vilella, 2009).

Both *AGAP009005* and *ZnT35C* contain the ZnT-like domain associated with mammalian zinc efflux proteins. *ZnT35C* is highly up-regulated in the adult and larval tubules, with a slight enrichment in the adult testis (Table 6.1). The expression pattern of *AGAP009005* in *Anopheles* has not yet been determined.

Table 6.1 *ZnT35C* expression in larval and adult *Drosophila* (Chintapalli *et al.*, 2007).

Tissue	mRNA Signal	Present Call	Enrichment	Affy Call
Brain	4 ± 1	1 of 4	0.00	Down
Head	8 ± 0	1 of 4	0.00	Down
Thoracicoabdominal ganglion	8 ± 1	0 of 4	0.00	Down
Salivary gland	11 ± 4	0 of 4	0.05	Down
Crop	5 ± 1	0 of 4	0.00	Down
Midgut	9 ± 3	1 of 4	0.00	Down
Tubule	**4645 ± 86**	**4 of 4**	**22.10**	**Up**
Hindgut	59 ± 9	4 of 4	0.30	Down
Heart	8 ± 1	2 of 4	0.04	Down
Fat body	17 ± 9	4 of 4	0.08	Down
Ovary	275 ± 3	4 of 4	1.30	None
Testis	477 ± 22	4 of 4	2.30	Up
Male accessory glands	6 ± 1	0 of 4	0.00	Down
Virgin spermatheca	15 ± 1	2 of 4	0.07	Down
Mated spermatheca	11 ± 1	2 of 4	0.05	Down
Adult carcass	30 ± 3	3 of 4	0.10	Down
Larval CNS	6 ± 1	2 of 4	0.03	Down
Larval Salivary gland	7 ± 0	1 of 4	0.04	Down
Larval midgut	7 ± 1	0 of 4	0.04	Down
Larval tubule	**1960 ± 123**	**4 of 4**	**9.30**	**Up**
Larval hindgut	13 ± 1	4 of 4	0.07	Down
Larval fat body	50 ± 19	3 of 4	0.20	Down
S2 cells (growing)	6 ± 1	1 of 4	0.03	Down
Whole fly	**210 ± 24**	**4 of 4**		

ZnT35C was identified by Yepiskoposyan *et al* in a microarray screen for genes enriched by heavy metal stress in *Drosophila* (Yepiskoposyan *et al.*, 2006). *ZnT35C* is up-regulated in *Drosophila* in response to increased dietary intake of zinc or cadmium (Figure 6.2A). *ZnT35C* up-regulation in response to zinc and cadmium is via the *dMTF-1* transcription factor, which is not involved in regulating basal *ZnT35C* transcription. Expression of a ZnT35C-GFP fusion protein driven with the *ZnT35C* promoter shows that the protein is localised along the length of the apical membrane in the Malpighian tubules, and nowhere else in *Drosophila* (Figure 6.2B). The apical expression of *ZnT35C* suggests that the gene

is involved in zinc secretion from the tubules, and is therefore an efflux transporter, as suggested by its homology to the mammalian ZnTs.

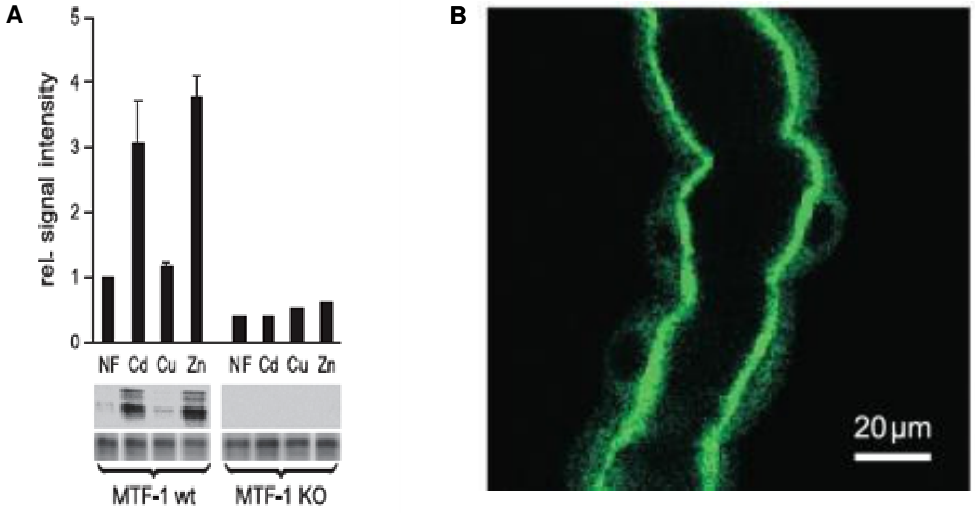


Figure 6.2 *ZnT35C* expression in whole-fly *Drosophila*. A: The transcript levels of *ZnT35C* in whole-fly measured after exposure to different foods: NF, normal food; Cd, 50 µM $CdCl_2$; Cu, 0.5 mM $CuSO_4$; Zn, 5 mM $ZnCl_2$. Results are shown for wild-type flies (MTF-1 wf) and flies in which dMTF-1 expression has been knocked down (MTF-1 KO). Representative gels are shown below the bars. B: Expression pattern of the ZnT35C-GFP fusion protein driven by the *ZnT35C* promoter in *Drosophila* larvae. A segment of Malpighian tubule from an animal fed with zinc shows strong green fluorescence on the apical membrane (Yepiskoposyan *et al.*, 2006).

ZnT35C expression is lower in the larval Malpighian tubules than in the adult tubules, and this may be a mechanism to retain zinc for growth and development during the larval and pupal stages. Ubiquitous overexpression of *ZnT35C* in *Drosophila* results in flies which are zinc super-resistant, and which have 3-fold greater survival than wild-type flies on food containing 14 mM zinc. The *Anopheles* homologue of *ZnT35C*, *AGAP009005,* is presently uncharacterised.

6.3 Results

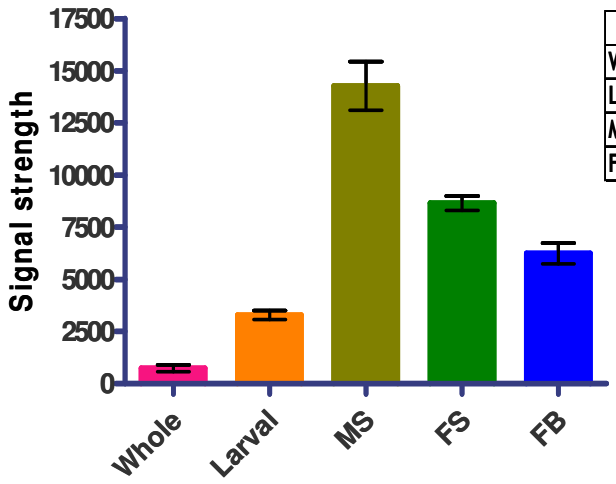
In this section the results from the investigation into the genes *AGAP009005* and *ZnT35C* as possible insecticide targets in *Anopheles* and *Drosophila* are described. The expression of the *AGAP009005* and *ZnT35C* transcripts was explored using microarrays, qPCR and *in situ* hybridisation. *ZnT35C* was further investigated in *Drosophila*, where RNAi-mediated gene knockdown was used to demonstrate that its expression is required for fly development through to adulthood. The effect of *ZnT35C* knockdown on the rate of fluid secretion from the tubules was also investigated in *Drosophila*.

6.3.1 AGAP009005 in the Tubules

The expression of the *AGAP009005* transcript in the *Anopheles* Malpighian tubules is described in this section. The *Anopheles* microarray data is presented, as is its subsequent verification by qPCR. The spatial expression of the *AGAP009005* transcript was also investigated in the *Anopheles* tubules, using RNA probe *in situ* hybridisation.

6.3.1.1 Expression of the Transcript

The putative zinc transporter *AGAP009005* was chosen for investigation from the *Anopheles* microarray due to its strong signal strength in the larval and adult Malpighian tubules in comparison to the average expression in whole mosquito (Figure 6.3). The statistical significance of the microarray data was determined using one-way ANOVA analysis and is presented in the attached table.



	FB	FS	MS	Larval
Whole	***	***	***	**
Larval	**	***	***	
MS	***	***		
FS	*			

Figure 6.3 Microarray data for *AGAP009005* expression in the *Anopheles* tubules at different life-stages. The attached table shows the statistical significance between life-stages. (N=4, mean ± S.E.M.) One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

At each of the life-stages assayed the *AGAP009005* transcript is significantly enriched in the Malpighian tubules in comparison to the average expression in the whole adult. The strongest expression of the transcript is in the tubules of adult males, where it is significantly more abundant than in sugar-fed and blood-fed females. The average *AGAP009005* signal strength in the whole mosquito is ~600, which suggests that although the transcript is highly enriched in the tubules, there is expression elsewhere in the mosquito.

Quantitative PCR was used to validate the *Anopheles* microarray data and ensure that it was representative of *AGAP009005* transcript expression (Figure 6.4A). The qPCR data was also extended to include *AGAP009005* expression in the female tubules 24 h after a blood-meal (Figure 6.4B).

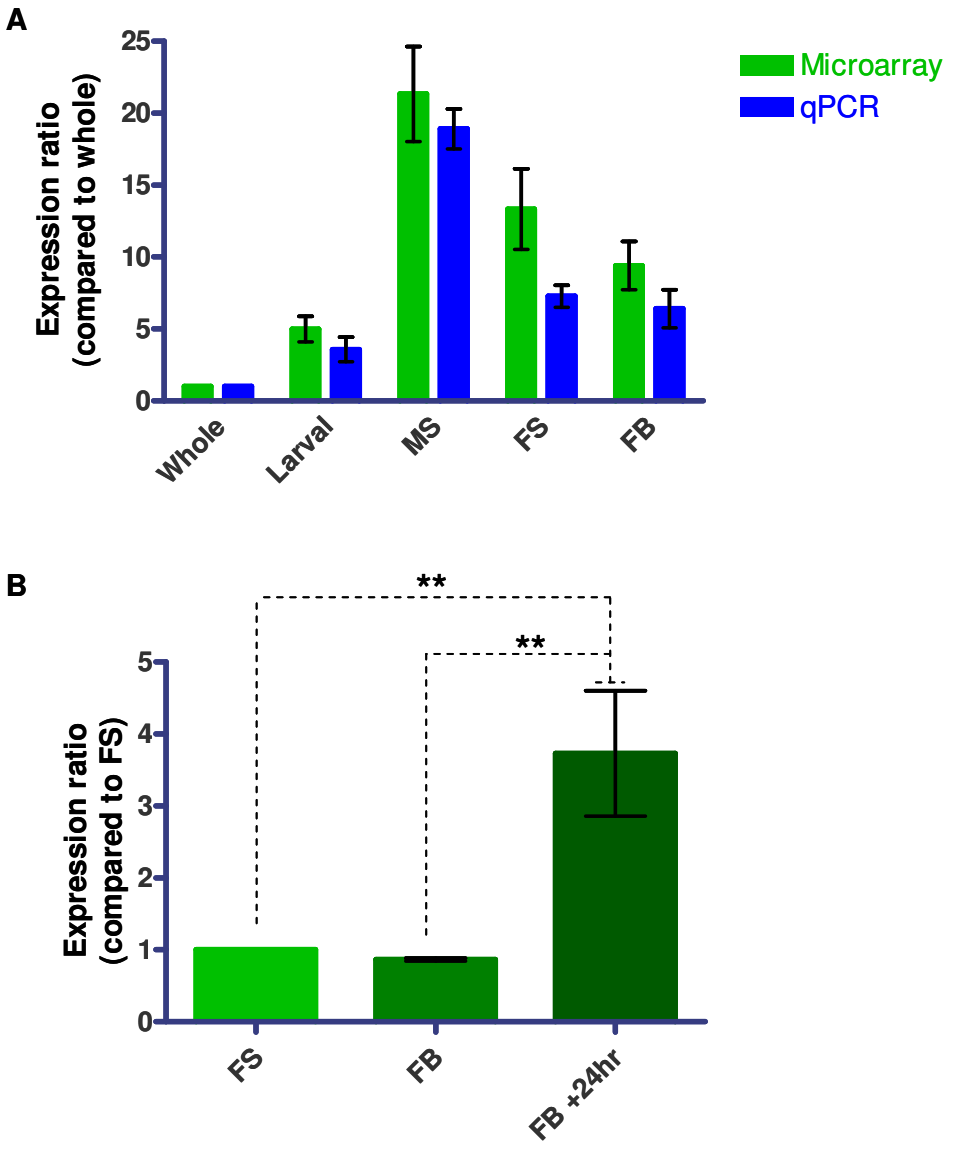


Figure 6.4 qPCR validation of *AGAP009005* expression in the *Anopheles* tubules. A: ratio of expression as calculated from microarray and qPCR data. B: ratio of expression in female tubules 3 h and 24 h after a blood-meal (N≥4, mean ± S.E.M.). One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

The validation data produced by qPCR is in good agreement with the microarray data, although it suggests that the female sugar-fed and blood-fed tubules have similar expression of the *AGAP009005* transcript. Expression of *AGAP009005* in each of the tubule samples is up-regulated by ~5-fold to ~20-fold over the whole-fly sample. The qPCR data also shows *AGAP009005* to be up-regulated 24 h after haematophagous behaviour. Expression of *AGAP009005* is enriched ~3.5-fold after a blood-meal, at a time when the mosquito is dealing with the heavy ion load found in human blood. Human blood contains approximately 8.8

μg ml^{-1} of zinc, found in the plasma, leucocytes and erythrocytes (Vallee and Gibson, 1948). It seems likely that *AGAP009005* is enriched in the tubules to excrete excess dietary zinc and prevent it from reaching a toxic level in the insect.

It is of interest to determine whether *AGAP009005* is expressed throughout the Malpighian tubules, or in specific functional areas. *In situ* hybridisation was used to determine the expression pattern of *AGAP009005* in the Malpighian tubules of adult *Anopheles* (Figure 6.5).

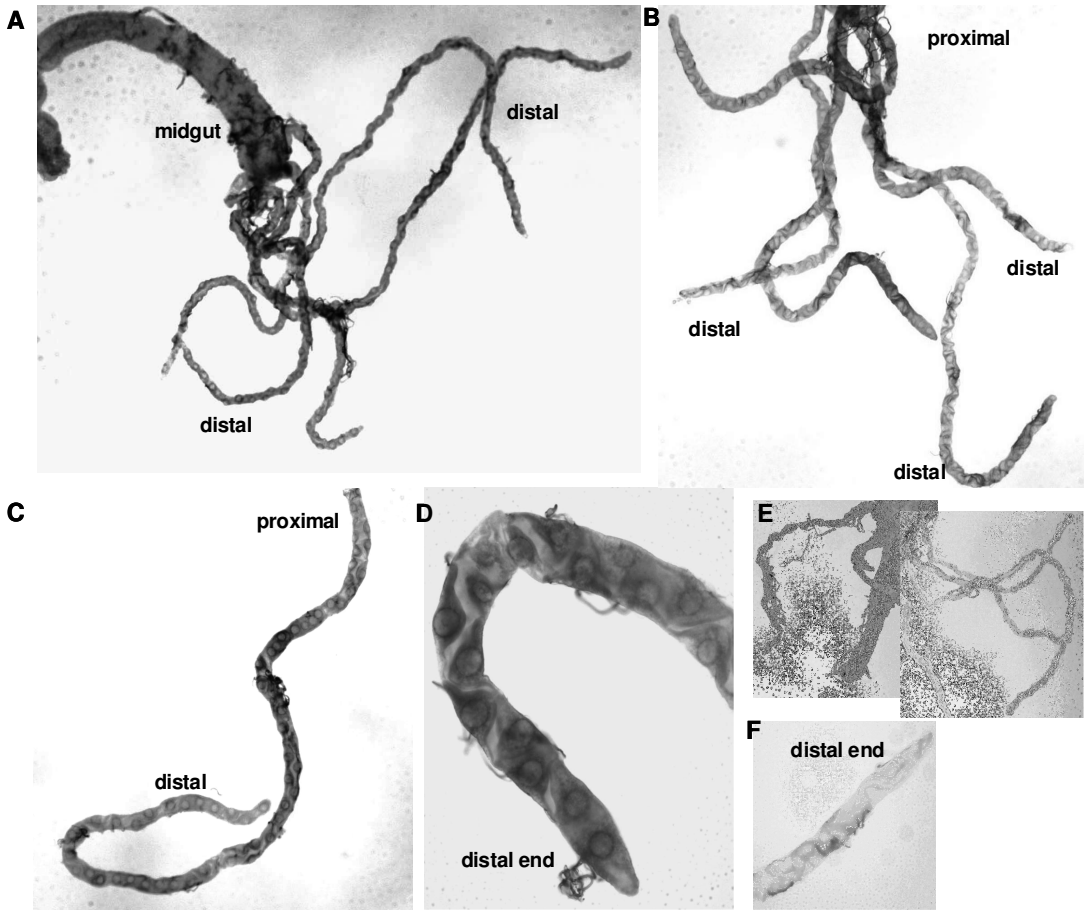


Figure 6.5 Expression of *AGAP009005* in the adult *Anopheles* tubules. A: low power view of the male tubules showing expression throughout. B: low power view of the female tubules showing expression throughout. C: high power view showing expression in the female tubule from the proximal segment to the distal end. D: high power view of the distal end of the female tubule showing strong expression in the principal cells, with lighter areas which may be stellate cells. E: low power view of male tubule stained with sense probe as a control, showing no expression. G: high power view of the female distal end stained with sense probe as a control, showing no expression.

The *in situ* hybridisation experiments confirmed that *AGAP009005* is expressed in all five tubules in adults of both genders. The transcript was abundant in all regions of the tubules, from where they attach to the gut through to the blind distal end. From the *in situ* hybridisations it was difficult to determine whether staining was in the stellate cells or solely in the principal cells in middle section of the tubules. At the distal end of the tubules the stellate cells did not stain as strongly as the principal cells. The *AGAP009005* probe also stained the mid-gut, suggesting that *AGAP009005* is transcribed in other areas of the alimentary canal. The sense control probe produced no staining in the tubules or mid-gut, although there was non-specific staining in the trachea which wrap around the tubules.

6.3.2 ZnT35C in the Tubules

The endogenous expression of the *ZnT35C* transcript in the *Drosophila* Malpighian tubules is described in this section. This includes quantification of transcript expression in the tubules by qPCR, as well as verification of a previous *Drosophila* Malpighian tubule microarray (Wang *et al.*, 2004). The spatial expression of the transcript in the tubules is also described, as determined by RNA probe *in situ* hybridisation.

6.3.2.1 Expression of the Transcript

ZnT35C was identified as a *Drosophila* homologue of *AGAP009005* from its conserved sequence homology and enriched tubule expression (Wang *et al.*, 2004; Hubbard *et al.*, 2009). Up-regulation of *ZnT35C* in the larval and adult tubules of *Drosophila* was verified by qPCR (Figure 6.6A). Expression of the transcript was also compared in the tubules of adult males and females, to determine whether it has sex-specific expression (Figure 6.6B).

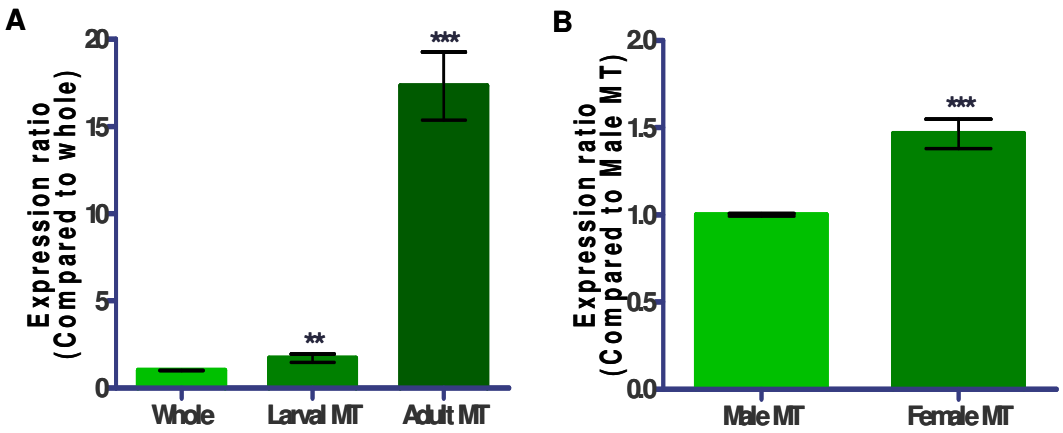


Figure 6.6 Expression of *ZnT35C* in the *Drosophila* Malpighian tubules. A: expression in the larval and adult Malpighian tubules in comparison to the average whole-fly expression. B: expression in the female tubules in comparison to the male tubules (N=4, mean ± S.E.M.). Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001.

Expression of *ZnT35C* is substantially up-regulated in the larval and adult Malpighian tubules when compared to the average expression in the whole-fly, by approximately 2-fold and 17-fold respectively. Expression is also significantly greater in the tubules of adult females than males, with an enrichment of ~1.5-fold. *Drosophila* and *Anopheles* are therefore similar in terms of tubule-enriched expression of *AGAP009005* and *ZnT35C*.

In situ hybridisation was performed on the tubules of adult *Drosophila* to determine whether transcript expression is localised to a particular domain or cell type (Figure 6.7).

The *in situ* hybridisations show that *ZnT35C* is abundantly expressed in all regions of the *Drosophila* tubules in both adult males and females. Staining is lighter in the initial and transitional segments than in the main segment, but is stronger than the staining in the control experiments. No distinction could be made between the principal and stellate cells, suggesting that the *ZnT35C* transcript may be expressed in both. From the *in situ* hybridisation data it would appear that *ZnT35C* is transcribed ubiquitously in the *Drosophila* tubule.

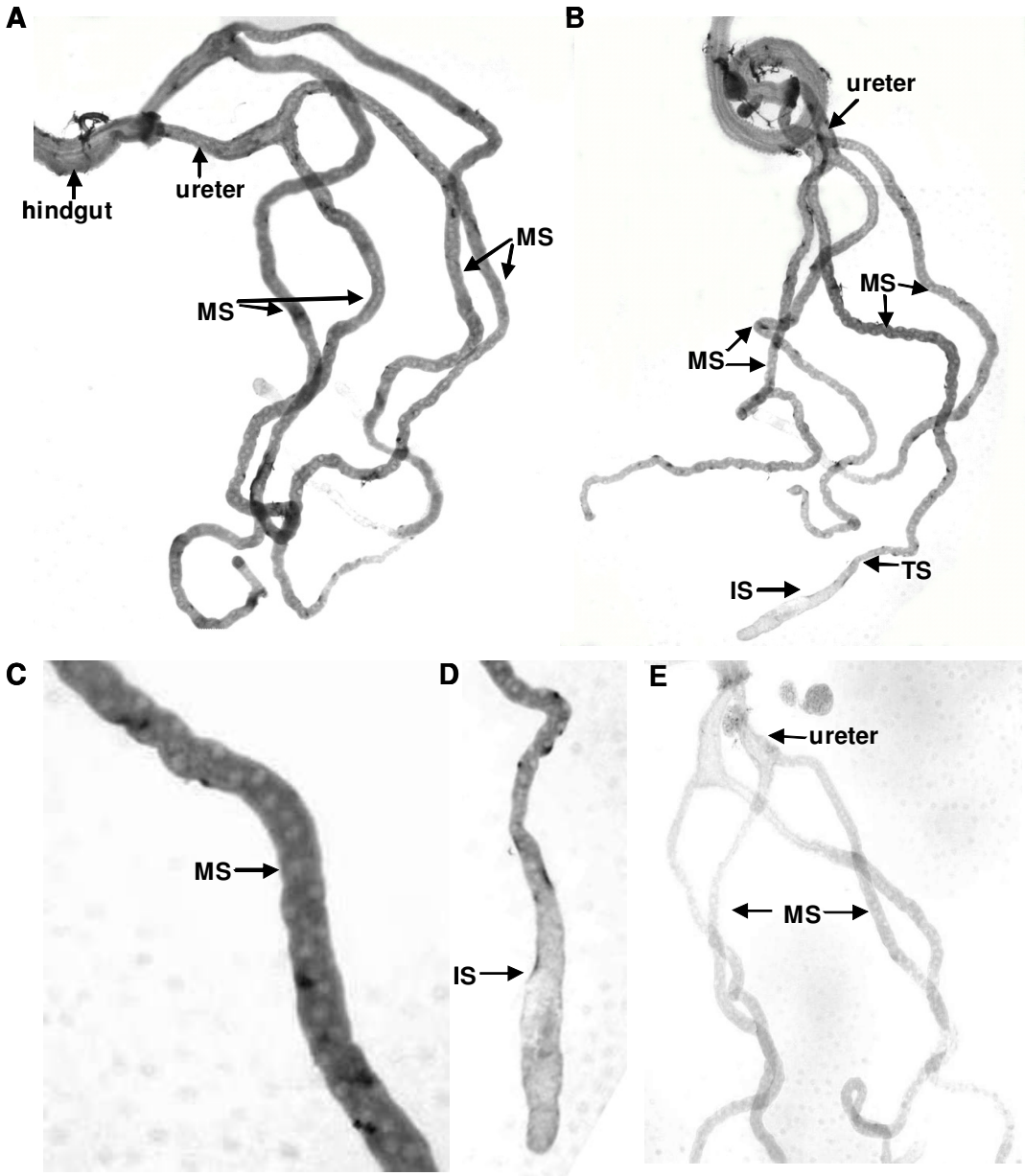


Figure 6.7 Expression of *ZnT35C* in the adult *Drosophila* tubules as determined by *in situ* hybridisation. Low power views showing expression in the ureter, main segment (MS), transitional segment (TS) and initial segment (IS) in the (A) male and (B) female tubules. C: high power shot of the main segment showing staining. D: high power shot of the anterior distal tubule showing staining in the initial segment. E: sense probe control showing no staining in the tubules.

6.3.3 RNAi Knockdown of ZnT35C

RNAi knockdown was used to determine whether *ZnT35C* is an essential gene for *Drosophila* development through to adulthood. As no RNAi lines were publicly available for targeting *ZnT35C*, the pWIZ transgene (Lee and Carthew, 2003) was used as a vector. Three constructs were cloned, each targeted to a different part of the *ZnT35C* transcript. The constructs were microinjected into w^{1118} flies

and balanced over the homozygous lethal CyO on the second chromosome and TM3,*Sb* on the third chromosome. Three balanced lines were selected for each construct, resulting in nine UAS-RNAi fly-lines in total. Further studies were performed to determine the effect of *ZnT35C* knockdown on fly survival and the rate of fluid secretion from the Malpighian tubules.

6.3.3.1 Effect on Fly Survival

For a gene to be an appropriate insecticide target it should be functionally essential to the species. The nine UAS-RNAi fly-lines under study were driven by four different GAL4 drivers (Table 6.2) to determine the effect of gene knockdown on fly survival through to adulthood.

Table 6.2 Pattern of expression of GAL4 drivers used to knock-down *ZnT35C*

Driver line	Pattern of expression
Actin-GAL4	Ubiquitous expression throughout the life-cycle
C42-GAL4	Principal cells in the main and lower segments, and bar-shaped cells in the initial and transitional segments of the tubules
Uro-GAL4	Principal cells in the main segment of the tubules
C724-GAL4	Stellate cells in the main segment and bar-shaped cells in the initial and transitional segments of the tubules

Fly survival in GAL4 driven UAS-RNAi lines was classified as normal, semi-lethal, or lethal. Normal was defined as the expected ratio of driven-RNAi to non-driven-RNAi progeny being observed in a cross between a parental RNAi line and a driver line. Semi-lethal was defined as significantly fewer RNAi-driven progeny being observed than expected (as determined by Chi-squared test). Lethal was classified as no driven-RNAi progeny emerging from a cross. The results can be found in Table 6.3.

Table 6.3 Effect of *ZnT35C* knockdown on fly survival (cross N=3, raw data is presented in Appendix 2).

Fly-line	*Actin-GAL4*	*C42-GAL4*	*Uro-GAL4*	*C724-GAL4*
CG3994.c1.l1	normal	normal	normal	normal
CG3994.c1.l3	normal	normal	normal	normal
CG3994.c1.l4	normal	normal	normal	normal
CG3994.c2.l1	**semi-lethal**	normal	normal	normal
CG3994.c2.l2	**lethal**	normal	normal	normal
CG3994.c2.l8	**lethal**	normal	normal	normal
CG3994.c3.l1	normal	normal	normal	normal
CG3994.c3.l2	**semi-lethal**	normal	normal	normal
CG3994.c3.l4	normal	normal	normal	normal

When crossed to the *Actin-GAL4* driver, four fly-lines had greatly reduced progeny numbers or no progeny which survived to adulthood. Where there was complete lethality it occurred before pupation, during embryogenesis or larval growth. In the fly-lines classified as semi-lethal, a reduced number of progeny eclosed, although those which did eclose survived as normal. The number of lines in which GAL4-driven UAS-RNAi expression results in partial or full lethality indicates that it is unlikely to be insertional effects of the transgene which are responsible. The tubule specific GAL4-drivers did not have such an obvious effect, with none of the fly-lines driven by the tubule-specific GAL4-drivers producing reduced numbers of progeny. As *Actin-GAL4* is the only driver which expresses ubiquitously in the tubule, we cannot discount *ZnT35C* knockdown in the tubules as the reason for fly lethality.

ZnT35C knockdown was quantified using qPCR for each GAL4-driven UAS-RNAi fly-line (Figure 6.8A-C). In the lines which were not lethal or semi-lethal the knockdown was quantified in the Malpighian tubules of *Actin-GAL4* driven lines. Where UAS-RNAi expression driven by *Actin-GAL4* was lethal, knockdown was quantified in *c42-GAL4* driven lines. *CG11163*, the closest homologue of *ZnT35C*, was also quantified in two driven RNAi lines to check for off-target gene silencing (Figure 6.8D).

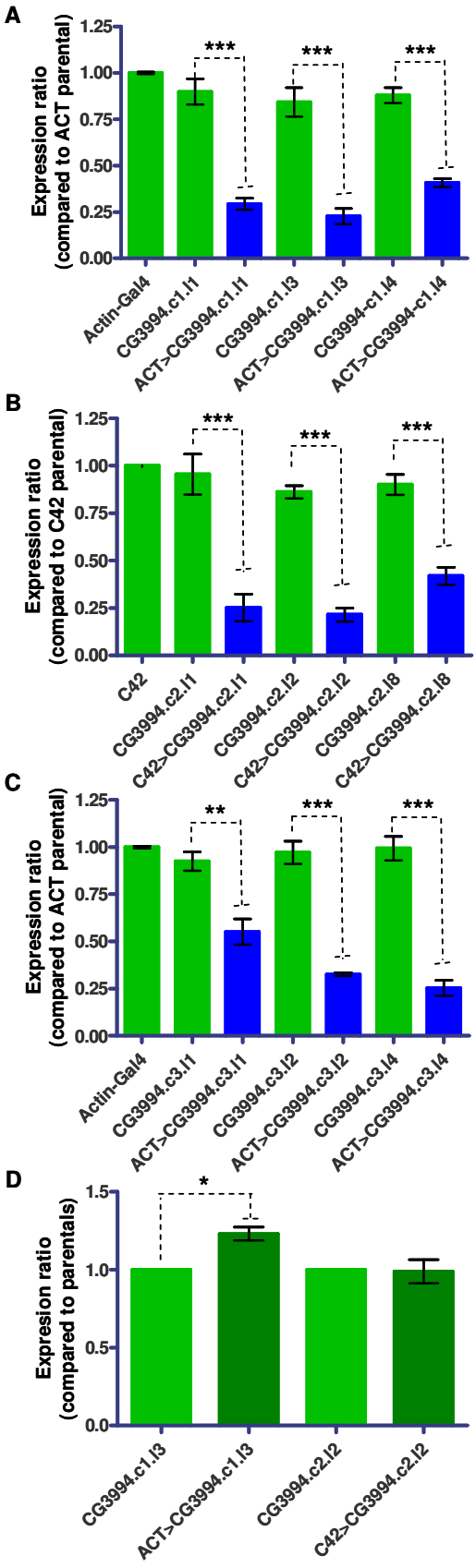


Figure 6.8 qPCR verification of *ZnT35C* knockdown in the tubules of driven RNAi lines. ZnT35C expression in A: RNAi lines from construct 1, B: RNAi lines from construct 2, and C: RNAi lines from construct 3. D: Quantification of CG11163 expression in driven RNAi lines (N=3, mean ± S.E.M.) (Student's t-test, *p<0.05, **p<0.01, ***p<0.001).

All nine of the GAL4-driven UAS-RNAi lines had significantly lower *ZnT35C* expression than their parental lines, as determined by Student's *t*-test. The knockdowns ranged from ~25 - 50% of parental transcript levels, with variation between lines resulting from insertional effects and different efficiencies of the constructs at targeting the transcript for degradation. The two fly-lines tested for off-target gene silencing showed no significant decrease in *CG11163* expression. In principal, although it is not known whether the knockdown in transcript level results in an equivalent knockdown in protein level, the reduced fly survival which results from manipulating *ZnT35C* expression suggests that it is a valid insecticide target.

6.3.3.2 Effect on Fluid Secretion from the Tubules

The effect of *ZnT35C* gene knockdown on the rate of fluid secretion from the tubules was investigated using fluid secretion assays (Dow *et al.*, 1994). Tubules dissected from the GAL4-driven UAS-RNAi line, the UAS-RNAi parent and the GAL4 driver parent were allowed to secrete steadily for 30 min before the diuretic peptide Drosokinin was added to the reservoir bubble. Three driven RNAi lines were tested; one line for each of the three RNAi constructs (Figure 6.9).

Gene knockdown of *ZnT35C* had no statistically significant effect on the rate of basal or stimulated fluid secretion from the tubules. Secretion assays performed on the driven *CG3994.c1.l3* line showed increased basal and stimulated secretion rates, but further assays on driven fly-lines from the same construct showed no increase in secretion. The increase in secretion is therefore likely to be due to insertional effects of the transgene rather than *ZnT35C* knockdown. This result confirms the need to use multiple RNAi constructs, and test multiple insertional lines for each construct, to exclude phenotypes which are the result of transgene insertion or off-target silencing. After discounting the increased rate of secretion in the driven *CG3994.c1.l3* line, there is no secretion phenotype associated with GAL4-driven UAS-RNAi silencing of *ZnT35C*. This is not surprising if the role of *ZnT35C* is the excretion of zinc from the tubules, as the bathing medium used for tubule secretion assays does not contain zinc. Further Ramsay assays with increasing peritubular zinc concentrations could determine whether

the rate of fluid secretion is affected by an increase in zinc uptake from the haemolymph, or increased zinc content in the tubules.

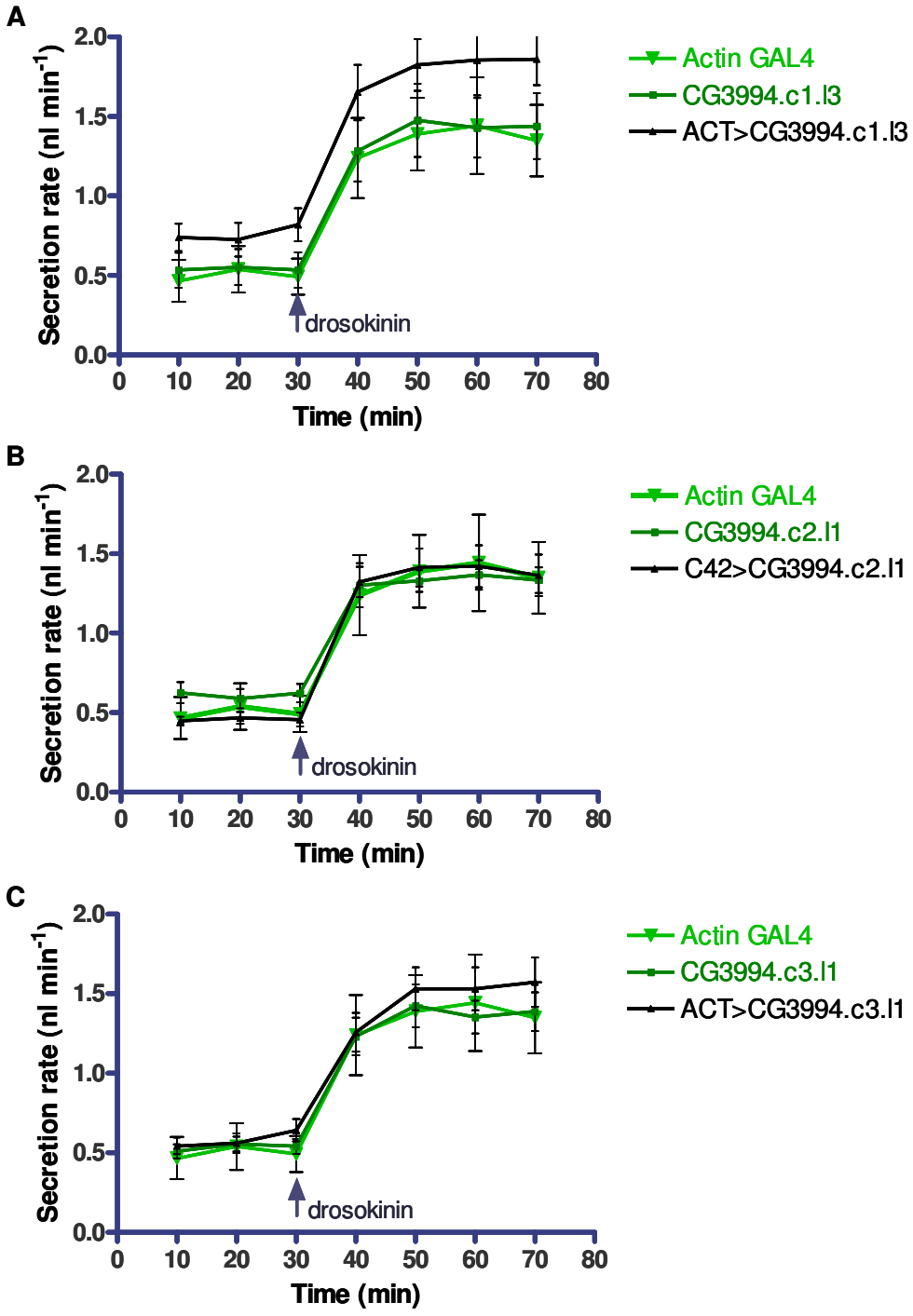


Figure 6.9 Effect of *ZnT35C* knockdown on the rate of fluid secretion from the tubules. Typical experimental results, showing the response of three GAL4-driven UAS-RNAi lines and the appropriate UAS-RNAi and GAL4 driver parental lines to Drosokinin after 30 min of steady secretion (mean ± S.E.M., N≥10).

6.4 Discussion

Zinc is an essential element, required for cell replication, nucleic acid metabolism, tissue repair and growth, and as a co-factor for many vital enzymes. Both deficiency and over-accumulation of zinc can be detrimental to a tissue, and so its transport, storage and excretion must be carefully regulated. Zinc is stored in specific vacuoles in the lower and main segment of both the anterior and posterior Malpighian tubules of *Drosophila,* and may also be stored in principal cell concretions in *Anopheles*. It is not understood whether zinc is accumulated as a detoxification mechanism, or as a storage reservoir for future use. This section discusses the expression of the zinc transporters *AGAP009005* and *ZnT35C* in the Malpighian tubules of *Anopheles* and *Drosophila*, and their likely functional roles. The usefulness of *ZnT35C* as a model of *AGAP009005*, and their likely success as insecticide targets, is also considered.

6.4.1 AGAP009005 and ZnT35C in the Tubules

The spatial and temporal expression of *AGAP009005* and *ZnT35C* in the Malpighian tubules was investigated for two reasons; to gain further understanding of gene function, and to determine how similar gene expression of the homologues is. *AGAP009005* is ~5-fold enriched in the larval tubules and ~10-fold to 20-fold enriched in the adult *Anopheles* tubules at each life-stage. Similarly, *ZnT35C* is ~2-fold enriched in the larval tubules and ~17-fold enriched in the adult tubules. A strong expression signal in both the *Anopheles* and *Drosophila* whole-body microarrays suggests that transcription of *AGAP009005* and *ZnT35C* is not confined to the Malpighian tubules.

Interestingly, expression of *AGAP009005* in the tubules quadruples 24 h after a blood-meal. Human blood plasma contains approximately 90-150 mcg/dl of zinc (Pohit *et al.*, 1981). This suggests that *AGAP009005* is enriched to deal with excretion of zinc from the tubules when the mosquito is digesting a blood-meal, and her fertilised eggs are developing (Marinotti *et al.*, 2006). Many genes involved in the early stages of zygote development are regulated by zinc-finger motifs (Staudt *et al.*, 2006; Liang *et al.*, 2008), and zinc is required for cell proliferation. In both *Anopheles* and *Drosophila* the zinc storage vacuoles may provide a store of ions for wound healing, egg development in the female, and

other cellular processes. The increased expression of *AGAP009005* after a blood-meal suggests that not all the ingested zinc is stored in the tubules for future use, but that a portion of it is secreted.

The *in situ* hybridisations indicate that both *AGAP009005* and *ZnT35C* are expressed throughout the length of the tubules in the principal cells, and possibly in the stellate cells. This is surprising as many processes in the tubules are confined to one cell-type, but is in agreement with the findings of Yepiskoposyan *et al*, who showed *ZnT35C* to be expressed at the apical membrane along the length of the *Drosophila* tubules (Yepiskoposyan *et al.*, 2006). Localisation of *ZnT35C* to the apical membrane suggests that it is involved in the secretion of zinc from the Malpighian tubules into the hindgut.

Knockdown of *ZnT35C* using the UAS/GAL4 system was used to mimic the effect that insecticide targeting would have if the compound prevented transporter function. Knockdown of *ZnT35C* using an *Actin-GAL4* driver line had a lethal or semi-lethal effect on the progeny of four of the nine lines tested. Lethality occurred before pupae formation, suggesting that *ZnT35C* is essential in *Drosophila* during early development. If *ZnT35C* is involved in zinc excretion as suggested, lethality may be due to a toxic accumulation of zinc in the larva. Knockdown of *ZnT35C* in specific segments of the tubules using *c42-GAL4* and *UrO-GAL4* did not decrease fly survival.

The microarray, qPCR and *in situ* hybridisation data are in good agreement with one another for both species, with no notable differences in findings gained from different techniques. There is also a high level of similarity in the expression patterns of *AGAP009005* and *ZnT35C* in *Anopheles* and *Drosophila* for all three types of experiment performed. None of the data collected suggests that *ZnT35C* is not a good model for *AGAP009005,* or that *Drosophila* is not a good model for *Anopheles* for zinc transporter genes.

6.4.2 AGAP009005 and ZnT35C as Zinc Transporters

AGAP009005 and *ZnT35C* contain the ZnT motif associated with mammalian zinc-efflux proteins. Of the *Drosophila* genes which contain a ZnT motif, *ZnT35C* has the largest signal strength in the Malpighian tubules, in both larvae and adult. The tubules are likely to contain influx and efflux zinc transporters, as well as

proteins required for the formation of zinc vacuoles. *AGAP009005* and *ZnT35C* have homologues in all of the species which have had their genomes sequenced (Hubbard *et al.*, 2009), suggesting an ancient and essential function.

The work of Yepiskoposyan *et al* suggests that *ZnT35C* is involved in zinc transport, and is likely to be an efflux transporter due to its position on the apical membrane of the Malpighian tubules. As such, we would not expect gene knockdown of *ZnT35C* to have an immediate effect on the rate of fluid secretion from the tubules, particularly under tolerable zinc conditions. Further assays, measuring the rate of secretion when the zinc concentration of the tubule bathing fluid is increased in *ZnT35C*-knockdown flies, could determine whether zinc toxicity inhibits secretion. Transport assays using a zinc isotope could also be used to determine whether *ZnT35C* knockdown reduces zinc excretion by the tubules in *Drosophila*.

6.4.3 AGAP009005 and ZnT35C as Insecticide Targets

The data collected in this thesis and published by Yepiskoyan *et al* suggests that *AGAP009005* and *ZnT35C* would make effective insecticide targets. The lethal effect of reducing *ZnT35C* expression suggests that it is an essential gene for *Drosophila* development through to adulthood. This is most likely due to an over-accumulation of zinc in the tubules when expression of *ZnT35C* is decreased. If *AGAP009005* is essential at as early a developmental stage as *ZnT35C* it could be targeted by an insecticide at any point in the *Anopheles* life-cycle. As *AGAP009005* expression increases after a blood-feed, an indoor insecticide which targets the mosquito while it is resting after a blood-meal could also be highly effective.

The high degree of similarity in tubule enrichment and expression of *AGAP009005* and *ZnT35C* between *Anopheles* and *Drosophila* also increases the likelihood of any developed insecticides being useful for multiple Diptera species. The downside of targeting *AGAP009005* and *ZnT35C* is that they have homologues in many other species including humans, and therefore could be dangerous to species out-with Diptera.

6.5 Conclusion

The results presented in Chapter 6 indicate that the zinc transporters *AGAP009005* and *ZnT35C* could be effective candidate genes for insecticide targeting. The large up-regulation of *AGAP009005* and *ZnT35C* in the *Anopheles* and *Drosophila* Malpighian tubules, and their expression throughout the length of the tubules, suggests that they are functionally important. The placement of *ZnT35C* on the apical membrane of the Malpighian tubules, and the increased zinc resistance in *Drosophila* which correlates with *ZnT35C* over-expression (Yepiskoposyan *et al.*, 2006), suggests that they are likely to be efflux transporters, similarly to the mammalian ZnT-like genes. Gene knockdown of *ZnT35C* has no effect on the rate at which the Malpighian tubules are able to secrete fluid, but is lethal to *Drosophila* early-on in development, suggesting that it is an essential gene. Transport assays using a zinc isotope would confirm whether *ZnT35C* is involved in zinc efflux from the tubules. The agreement between the microarrays, qPCR and *in situ* hybridisations performed in both *Anopheles* and *Drosophila* is encouraging, as the expression of the two genes is very similar. Functional studies, such as in *Xenopus* oocyte, would confirm whether the physiological properties of *AGAP009005* and *ZnT35C* are also homologous.

Chapter 7 - *AGAP002587* and *CG8028* as Insecticide Targets

7.1 Summary

Numerous genes which are putatively involved in the transport of monocarboxylates are highly transcribed in the *Anopheles* and *Drosophila* Malpighian tubules (Wang *et al.*, 2004). Although the functional role of these genes has not yet been determined, the up-regulation of multiple genes annotated as monocarboxylate transporters in the tubules in each species suggests that they are likely to be important. The introduction to this chapter describes the monocarboxylate transporters which have been characterised in mammals, and the importance of cellular regulation of monocarboxylates such as pyruvate and lactate. The *Anopheles* gene *AGAP002587* and *Drosophila* gene *CG8028* are putative monocarboxylate transporters, and are the focus of this study. Analysis of microarray data suggests that they are highly enriched in the Malpighian tubules of *Anopheles* and *Drosophila*, and the results presented in this chapter confirm their transcription in the tubules during every life-stage tested. *In situ* hybridisation shows both genes to be expressed throughout the length of the adult tubules in both genders of their respective species. The importance of *CG8028* for fly development through to adulthood was tested using the UAS/GAL4 system to perform gene knockdown, which had no significant effect on fly survival, perhaps reflecting functional redundancy in this large gene family. The role of *CG8028* in osmoregulation in the Malpighian tubules was also investigated, and knockdown of *CG8028* expression was found to have no significant effect on the rate at which fluid is secreted from the tubules. Finally, the *Anopheles* and *Drosophila* data collected for *AGAP002587* and *CG8028* is discussed, as is the expected function of *AGAP002587* and *CG8028*, and their likely success as insecticide targets.

7.2 Introduction

Monocarboxylates such as lactate and pyruvate are essential components of the processes which energise life. Lactate is the end product of glycolysis, but can also be oxidised in the human brain and red skeletal muscles to fuel cellular respiration (Juel and Halestrap, 1999). Lactate can inhibit glycolysis if it is allowed to over-accumulate in a cell, and control of cellular efflux and influx of lactate and other monocarboxylates is essential for optimal cellular function (Merezhinskaya and Fishbein, 2009). Therefore, it is highly plausible that monocarboxylate transporters could make effective targets for the development of new insecticides in Diptera. The putative monocarboxylate transporters *AGAP002587* and *CG8028* are up-regulated in the Malpighian tubules of *Anopheles* and *Drosophila* respectively, and are investigated with regards to their usefulness as insecticide targets in this chapter.

7.2.1 The Importance of Monocarboxylic Acids

The monocarboxylic acids are a family of organic acids which contain a single carboxylic group. Monocarboxylic acids can have a ring structure (benzoic acid, nicotinic acid, gentisic acid, salicylic acid) or a short-chain structure (butyric acid, lactic acid, pyruvic acid, propionic acid). Monocarboxylates such as lactate and pyruvate are by-products of glycolysis, the essential cellular process by which ATP is produced. Glycolysis produces large amounts of lactic acid, which must be exported from the cell to allow glycolysis to continue at a high rate. Although monocarboxylates are often considered waste products, many tissues such as white skeletal muscle and red blood cells utilise them as energy substrates when glucose is scarce (Juel and Halestrap, 1999; Morris and Felmlee, 2008). The process by which cells switch to using monocarboxylates as a fuel source is not yet understood, but the import of monocarboxylates may provide an additional source of fuel during times of heavy energy stress. Other monocarboxylates such as pyruvate, acetoacetate and butyrate are just as important for cellular function (Morris and Felmlee, 2008), and are carefully regulated. For example in humans, butyrate is produced by colonic bacteria during fermentation of dietary carbohydrates, and is then used as the main respiratory fuel for colonic epithelial cells (Cuff *et al.*, 2005). Monocarboxylate transporters regulate the cellular influx and efflux of monocarboxylates at the

plasma membrane, and are therefore pivotal for optimal cell physiology (Pellerin, 2003; Pierre and Pellerin, 2005).

7.2.2 Monocarboxylate Transport in Mammals

Monocarboxylates are transported by two gene families in mammals, the proton-coupled SLC16 monocarboxylate transporters (MCTs), and the sodium-coupled SLC5 monocarboxylate transporters (SMCTs). Fourteen human MCT genes have been identified from protein sequence homology (Halestrap and Price, 1999), and seven of them functionally characterized. Although their sequences are highly similar, the mammalian MCTs transport a wide variety of endogenous and exogenous substrates. *MCT1-4* are proton-dependent monocarboxylate transporters, while *MCT6*, *MCT8* and *MCT10* transport diuretics, thyroid hormones and aromatic amino acids respectively (Halestrap and Price, 1999; Juel and Halestrap, 1999; Kim *et al.*, 2001; Friesema *et al.*, 2003; Murakami *et al.*, 2005). MCT expression is widespread in mammalian tissues, although each MCT has its own tissue-specific expression pattern (Halestrap and Meredith, 2004). The movement of monocarboxylates across the plasma membrane is directed by the concentration of the transported metabolites and protons (Merezhinskaya and Fishbein, 2009).

The SMCT family was annotated recently in humans, and currently contains only two members, *SLC5A8* and *SLC5A12* (Coady *et al.*, 2004; Gopal *et al.*, 2004). *SLC5A8* and *SLC5A12* transport short-chain monocarboxylates and sodium ions, primarily in the kidneys and intestine (Srinivas *et al.*, 2005). Unlike the MCT family, the SMCTs are not known to transport exogenous substrates such as drug compounds (Morris and Felmlee, 2008).

7.2.3 Monocarboxylate Transport in Diptera

Insects are thought to use monocarboxylate transporters for the same purposes as higher eukaryotes; to control the cellular influx and efflux of these vital compounds. In *Drosophila*, eighteen genes have been annotated as putative monocarboxylate transporters through sequence homology to the mammalian MCTs (Filippi and Alessi, 2008). The majority of these transporters have a close homologue in *Anopheles,* none of which have been characterised. In *Drosophila*

two MCT-like genes have been investigated in recent years, *Silnoon* and *outsider*.

In 2008, Jang *et al* determined that the signalling network regulated by the tumour suppressor *LKB1* controls trafficking of the monocarboxylate transporter *Silnoon* (*CG8271*) to the plasma membrane (Jang *et al.*, 2008). *Silnoon* is principally expressed in the *Drosophila* nervous system (Chintapalli *et al.*, 2007), and has a close homologue in *Anopheles*, *AGAP010881 (Hubbard et al., 2009)*. The authors hypothesise that overexpression of *Silnoon* in the *Drosophila* wing discs results in the increased uptake of monocarboxylates, which induces apoptosis and results in a phenotype of narrow eyes and small curved wings. The mechanism by which increased uptake of monocarboxylates induces apoptosis is not yet understood, but it may be through inhibition of histone deacetylase by butyrate, or activation of p53-dependent apoptosis. In the wing disc *LKB1* is thought to control re-localization of *Silnoon* from the basolateral to the apical membrane.

The *Drosophila* MCT-like transporter *outsider* (*CG8062*) was recently characterised as being involved in *p53*-triggered apoptosis during development, primarily in the primordial germ cells (Yamada *et al.*, 2008). Functional mutants of *outsider* show abnormal germ cell death but not abnormal migration, and *outsider* is thought to operate in the same apoptotic pathway as *p53*. The transport capacity and specificity of *outsider* is yet to be determined.

7.2.4 The Putative Monocarboxylate Transporters AGAP002587 and CG8028

AGAP002587 and *CG8028* are classed as homologues by ENSEMBL with an identity of 42% and an Expect value (e-value) of 2e-85 (Hubbard *et al.*, 2009).

AGAP002587 and *CG8028* both contain the conserved protein motif which defines the mammalian MCT gene family, although neither gene has been functionally characterised as a monocarboxylate transporter. Their purpose in the tubules is not yet understood, but they could be performing a multitude of functional roles. The monocarboxylate transporters may be transporting monocarboxylates to provide fuel for energy production, as the tubules are an extremely active

tissue which requires a constant energy source for survival (Berridge, 1966). *AGAP002587* and *CG8028* could also be involved in the clearance of excess monocarboxylates from the haemolymph, or they may be transporting an unexpected set of compounds, such as the diuretics and hormones transported by human *MCT6* and *MCT8*. As little is understood about the role of monocarboxylate transporters in Diptera it is difficult to predict their functional role in specific tissues.

Three other genes annotated as monocarboxylate transporters, *CG8468*, *CG8389* and *CG12286,* are also highly expressed in the *Drosophila* tubules, although none show as high enrichment or as specific an expression pattern in the adult tubules as *CG8028* (Chintapalli *et al.*, 2007). The expression pattern of *CG8028* in larval and adult *Drosophila* tissues can be found in Table 7.1. *CG8028* is highly transcribed in two of the tissues assayed, the larval and adult Malpighian tubules. The expression pattern of *AGAP002587* in *Anopheles* is not yet known.

Table 7.1 *CG8028* expression in larval and adult *Drosophila* (Chintapalli *et al.*, 2007).

Tissue	mRNA Signal	Present Call	Enrichment	Affy Call
Brain	6 ± 0	3 of 4	0.20	Down
Head	5 ± 1	0 of 4	0.20	Down
Thoracicoabdominal ganglion	4 ± 1	0 of 4	0.10	Down
Salivary gland	19 ± 4	3 of 4	0.56	Down
Crop	7 ± 2	0 of 4	0.20	Down
Midgut	9 ± 2	3 of 4	0.30	Down
Tubule	**2008 ± 231**	**4 of 4**	**59.00**	**Up**
Hindgut	8 ± 1	2 of 4	0.30	Down
Heart	5 ± 0	0 of 4	0.16	Down
Fat body	14 ± 6	4 of 4	0.41	Down
Ovary	4 ± 1	0 of 4	0.10	Down
Testis	6 ± 0	0 of 4	0.20	Down
Male accessory glands	10 ± 1	3 of 4	0.30	Down
Virgin spermatheca	10 ± 1	3 of 4	0.33	Down
Mated spermatheca	11 ± 2	4 of 4	0.30	Down
Adult carcass	13 ± 5	2 of 4	0.40	Down
Larval CNS	5 ± 0	4 of 4	0.16	Down
Larval Salivary gland	6 ± 2	1 of 4	0.19	Down
Larval midgut	10 ± 1	4 of 4	0.31	Down
Larval tubule	**626 ± 54**	**4 of 4**	**18.4**	**Up**
Larval hindgut	11 ± 2	4 of 4	0.35	Down
Larval fat body	9 ± 0	1 of 4	0.30	Down
S2 cell (growing)	13 ± 1	4 of 4	0.39	Down
Whole fly	34 ± 4	**4 of 4**		

7.3 Results

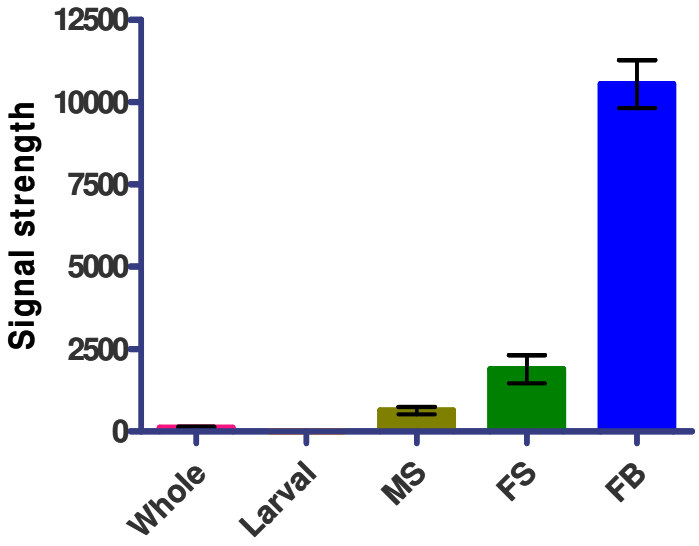
In this section the results from the investigation into the genes *AGAP002587* and *CG8028* as possible insecticide targets in *Anopheles* and *Drosophila* are described. The expression of the *AGAP002587* and *CG8028* transcripts was explored using microarrays, qPCR and *in situ* hybridisation. *CG8028* was further investigated in *Drosophila*, where RNAi-mediated gene knockdown was used to determine whether *CG8028* expression is required for fly development through to adulthood. The effect of *CG8028* knockdown on the rate of fluid secretion from the *Drosophila* Malpighian tubules was also investigated using secretion assays.

7.3.1 AGAP002587 in the Tubules

The expression of the *AGAP002587* transcript in the *Anopheles* Malpighian tubules is described in this section. This includes presentation of the relevant microarray data, and its subsequent verification by qPCR. The spatial expression of the transcript in the *Anopheles* adult tubules is also described, as defined by RNA probe *in situ* hybridisation.

7.3.1.1 Expression of the Transcript

The putative monocarboxylate transporter *AGAP002587* was chosen for investigation from the *Anopheles* microarray due to its strong signal strength in the adult female Malpighian tubules in comparison to the average expression in the whole mosquito (Figure 7.1). The statistical significance of the microarray data was determined using one-way ANOVA analysis and is presented in the attached table.



	FB	FS	MS	Larval
Whole	***	*	-	-
Larval	***	*	-	
MS	***	*		
FS	***			

Figure 7.1 Microarray data for *AGAP002587* expression in the *Anopheles* tubules at different life-stages. The attached table shows the statistical significance between life-stages. (N=4, mean ± S.E.M.). One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

The *AGAP002587* transcript is significantly enriched in the adult female Malpighian tubules, and its expression is further increased after a blood-meal. Expression of *AGAP002587* in the larval and male *Anopheles* tubules is not significantly greater than the average expression in the whole mosquito. The average microarray signal strength for the whole mosquito is very low, suggesting that expression of *AGAP002587* is not widespread, and may even be confined to the tubules.

Quantitative PCR (qPCR) was used to validate the microarray data and ensure it was representative of *AGAP002587* transcript expression (Figure 7.2A). The qPCR data was also extended to include *AGAP002587* expression in the female tubules 24 h after a blood-meal (Figure 7.2B).

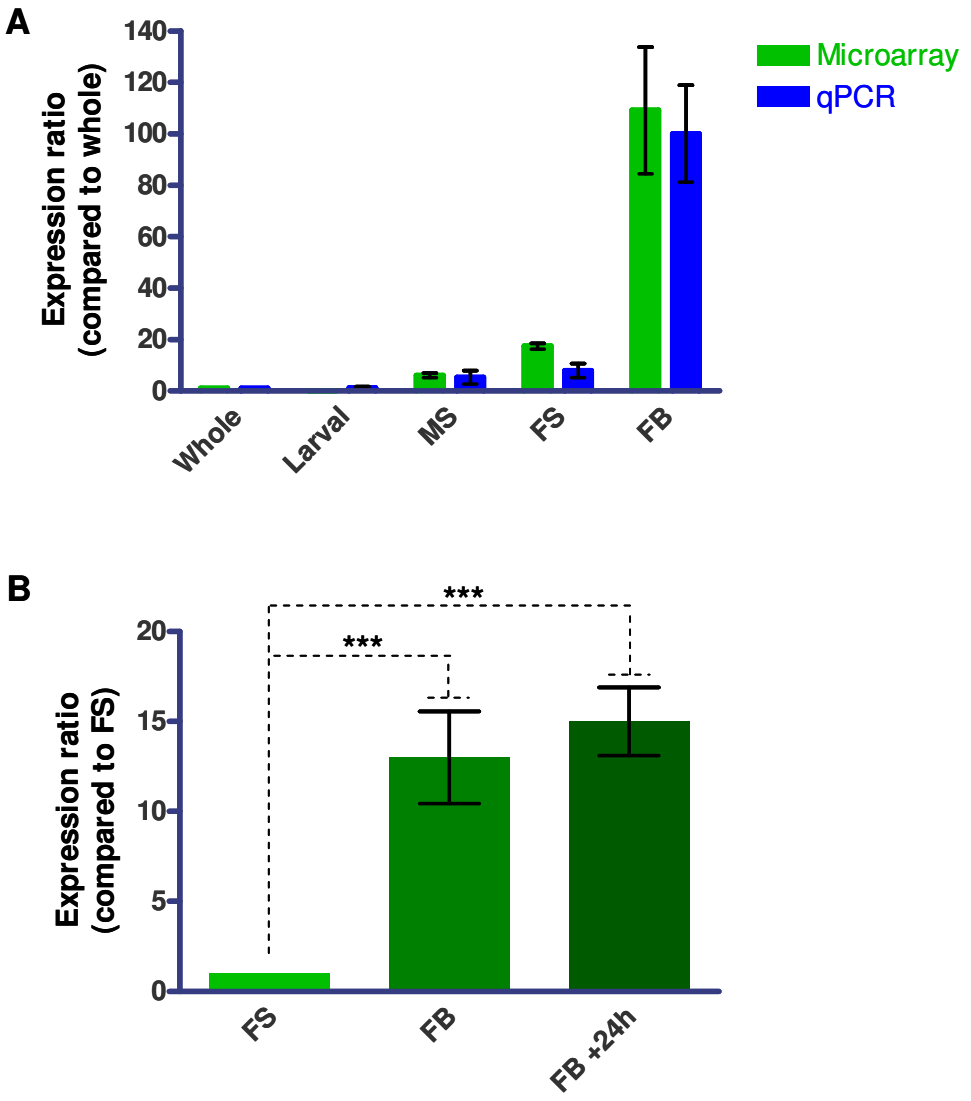


Figure 7.2 qPCR validation of *AGAP002587* expression in *Anopheles*. A: ratio of expression as calculated from microarray and qPCR data. B: ratio of expression in female tubules 3 h and 24 h after a blood-meal (N≥4, mean ± S.E.M.). One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

The qPCR data is in agreement with the microarray data, showing *AGAP002587* to be enriched in the female tubules before and after a blood-meal, by ~15-fold and ~110-fold respectively. *AGAP002587* expression does not alter significantly between 3 h and 24 h after haematophagous behaviour, when the Malpighian tubules and midgut are prioritising diuresis and digestion of the blood meal. After a blood-feed the tubules are highly active, and *AGAP002587* may be involved in the transport of monocarboxylates into the tubules as a fuel source, or the transport of by-products of blood digestion for excretion.

It is of interest to determine whether *AGAP002587* is expressed ubiquitously in the tubules, or in specific functional domains. *In situ* hybridisation was used to determine the expression pattern of the *AGAP002587* transcript in the adult *Anopheles* tubules (Figure 7.3).

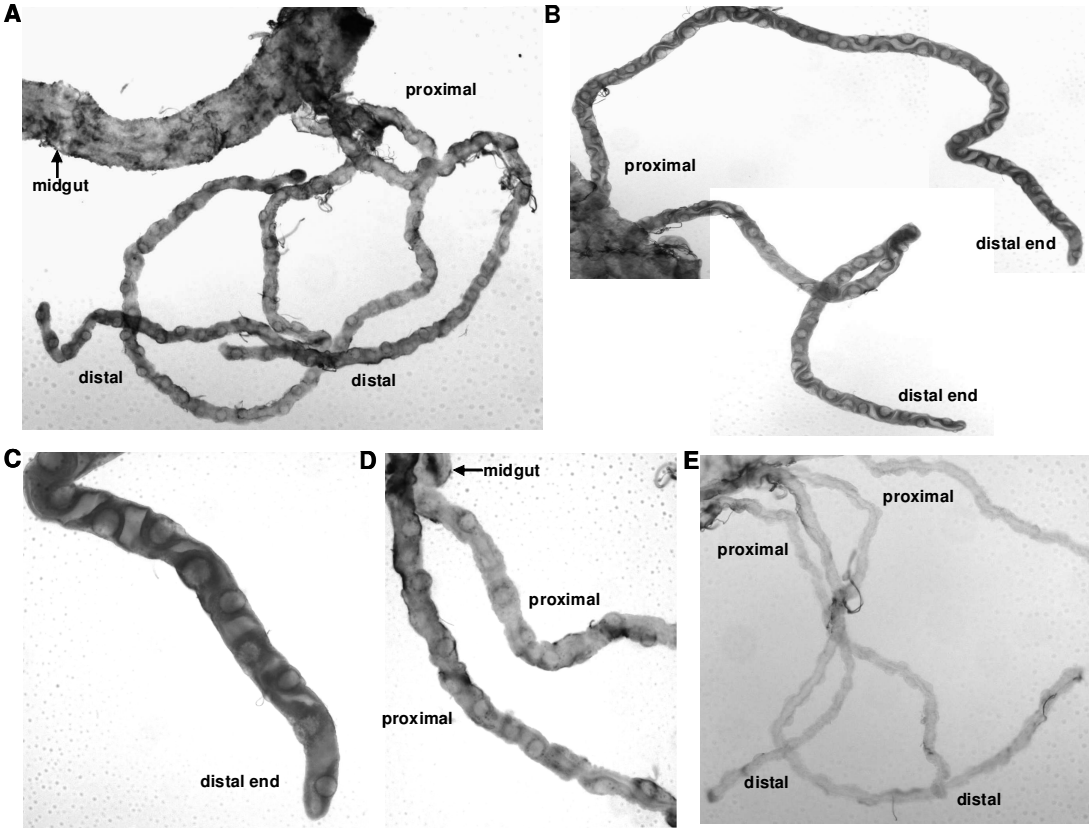


Figure 7.3 Expression of *AGAP002587* in the adult *Anopheles* tubules as determined by *in situ* hybridisation. A: low power view of the male tubules showing expression throughout. B: low power view of the female tubules showing expression throughout. C: high power view showing expression in the female distal end. D: high power view of the female tubule where it joins the midgut showing expression. E: low power view of male tubule stained with sense probe as a control, showing no expression.

In situ hybridisation confirmed that *AGAP002587* is expressed in all five tubules in adult *Anopheles* of both genders. The transcript appears to be abundant in all regions of the tubules, although the staining is slightly fainter at the proximal end of the tubule than at the distal end. The staining appears to be darker in the principal cells than the stellate cells, suggesting that the *AGAP002587* transcript has higher expression in the principal cells. The *AGAP002587* probe also stained the mid-gut, and appears to be transcribed in other areas of the alimentary

canal. The control probe produced no staining in the tubules or mid-gut, although there was non-specific staining of the trachea which wrap around the tubules.

7.3.2 CG8028 in the Tubules

The endogenous expression of the *CG8028* transcript in the *Drosophila* Malpighian tubules is described in this section. This includes quantification of transcript expression in the tubules by qPCR, as well as verification of a previous *Drosophila* tubule microarray (Wang *et al.*, 2004). The spatial expression of the transcript in the tubules is also described, as determined by RNA probe *in situ* hybridisation.

7.3.2.1 Expression of the Transcript

CG8028 was identified as a *Drosophila* homologue of *AGAP002587* due to their conserved sequence homology and enriched tubule expression (Wang *et al.*, 2004; Hubbard *et al.*, 2009). Enrichment of *CG8028* in the larval and adult tubules of *Drosophila* was verified by qPCR (Figure 7.4A). Expression of the *CG8028* transcript was also compared in the tubules of adult males and females using qPCR, to determine whether it is expressed in a sex-specific manner (Figure 7.4B).

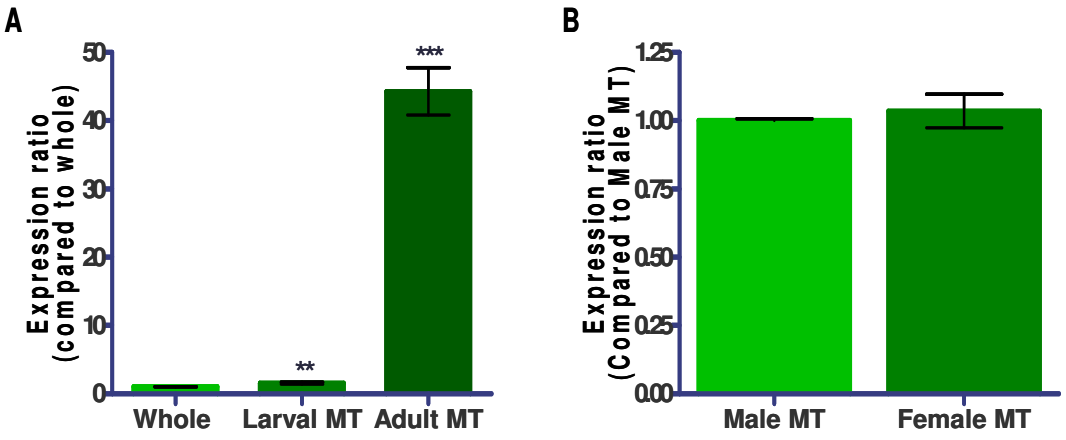


Figure 7.4 Expression of *CG8028* in the *Drosophila* Malpighian tubules as determined by qPCR. A: expression in the larval and adult Malpighian tubules in comparison to whole-fly. B: expression in the female tubules in comparison to the male tubules (N=4, mean ± S.E.M.). Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001.

Expression of *CG8028* is substantially up-regulated in both the larval and adult tubules when compared to the average expression in the whole fly, by ~2-fold and ~45-fold respectively. Unlike in *Anopheles*, there is no significant difference in the amount of *CG8028* transcript found in the tubules of female and male *Drosophila*.

In situ hybridisation was performed on adult *Drosophila* Malpighian tubules to determine whether expression of the *CG8028* transcript is localised to a particular domain or cell type (Figure 7.5).

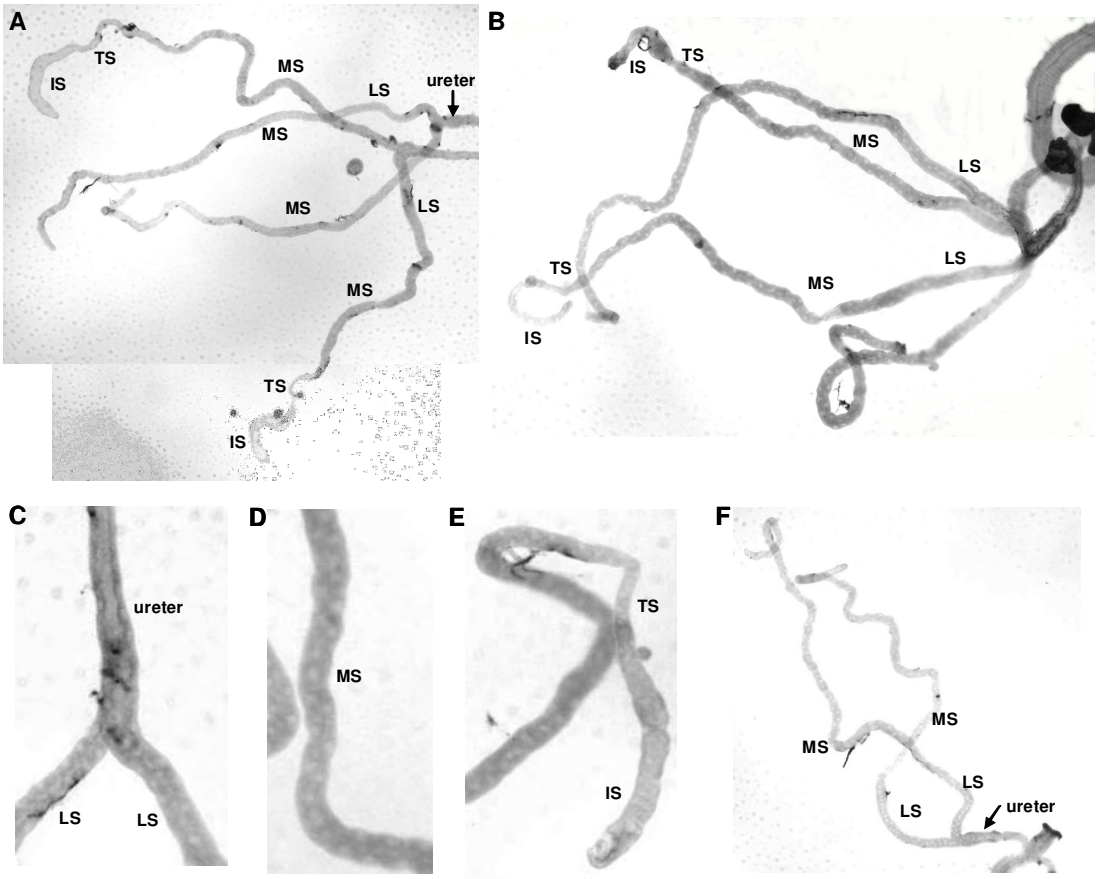


Figure 7.5 Expression of *CG8028* in adult *Drosophila* tubules as determined by *in situ* hybridisation. Low power views showing expression in the ureter, lower segment (LS), main segment (MS), transitional segment (TS) and initial segment (IS) in the male (A) and female (B) tubules. C: high power view of the ureter and lower segment showing staining. D: high power view of the main segment showing staining. E: high power view of the anterior initial and transitional segments showing staining. F: sense control showing no staining in the tubules.

CG8028 is transcribed in all regions of the *Drosophila* tubules in both males and females. The anti-sense *CG8028* probe staining is constant throughout the length of the tubules, and is darker than the staining produced by the sense control

probe. No distinction can be made between the principal and stellate cells, suggesting that *CG8028* is expressed in both. From the *in situ* hybridisation data it would appear that the *CG8028* transcript is expressed in every domain of the *Drosophila* Malpighian tubules, from the ureter through to the initial segment.

7.3.3 RNAi Knockdown of CG8028

As no RNAi lines were publicly available for targeting *CG8028*, the pWIZ transgene (Lee and Carthew, 2003) was used as a vector. Three constructs were cloned, each targeted to a different part of the *CG8028* transcript. The constructs were microinjected into w^{1118} flies and balanced over the homozygous lethal CyO marker on the second chromosome, and the TM3,*Sb* marker on the third chromosome. Three balanced lines were selected for each construct, resulting in nine RNAi fly-lines in total. Further studies were performed to determine the effect of *CG8028* knockdown on fly survival and fluid secretion in the Malpighian tubules.

7.3.3.1 Effect on Fly Survival

For a gene to be an appropriate insecticide target it should be functionally essential to the species. The nine UAS-RNAi fly-lines under study were driven by four different GAL4 drivers (Table 7.2) to determine the effect of gene knockdown on fly development through to adulthood.

Table 7.2 Pattern of expression of GAL4 drivers used in this study.

GAL4 Driver line	Pattern of expression
Actin-GAL4	Ubiquitous expression throughout the life-cycle
C42-GAL4	Principal cells in the main and lower segments, and bar-shaped cells in the initial and transitional segments of the tubules
ELAV-GAL4	Nervous tissues
C724-GAL4	Stellate cells in the main segment and bar-shaped cells in the initial and transitional segments of the tubules

Fly survival in GAL4-driven UAS-RNAi lines was classified as normal, semi-lethal, or lethal. Normal was defined as the expected ratio of driven-RNAi to non-

driven-RNAi progeny being observed in a cross between a parental UAS-RNAi line and a GAL4-driver line. Semi-lethal was defined as significantly fewer RNAi-driven progeny being observed than expected (as determined by Chi-squared test). Lethal was classified as no driven-RNAi progeny emerging from a cross. The results of the survival crosses can be found in Table 7.3.

Table 7.3 Effect of *CG8028* knockdown on fly survival (cross N=3, raw data is presented in Appendix 2).

Fly-line	*Actin-GAL4*	*C42-GAL4*	*ELAV-GAL4*	*C724-GAL4*
CG8028.c1.l1	normal	normal	normal	normal
CG8028.c1.l3	normal	normal	normal	normal
CG8028.c1.l4	normal	normal	normal	normal
CG8028.c2.l1	normal	normal	normal	normal
CG8028.c2.l2	normal	normal	normal	normal
CG8028.c2.l4	normal	normal	normal	normal
CG8028.c3.l1	normal	normal	normal	normal
CG8028.c3.l4	normal	normal	normal	normal
CG8028.c3.l6	normal	normal	normal	normal

Driving the nine UAS-RNAi lines with the four GAL4-driver lines did not affect the number of progeny which resulted from each cross. This suggests that *CG8028* is either not essential for fly development through to adulthood, or that the RNAi constructs were not effective in decreasing transcript expression. *CG8028* knockdown was quantified to determine whether there were decreased transcript levels in the GAL4-driven UAS-RNAi fly-lines (Figure 7.6A-C). *CG8028* knockdown was quantified in the tubules of progeny from UAS-RNAi lines driven with *Actin-GAL4*. *CG8034,* the closest homologue of *CG8028*, was quantified in RNAi-driven lines to check for a decrease in its expression caused by off-target gene silencing (Figure 7.6D).

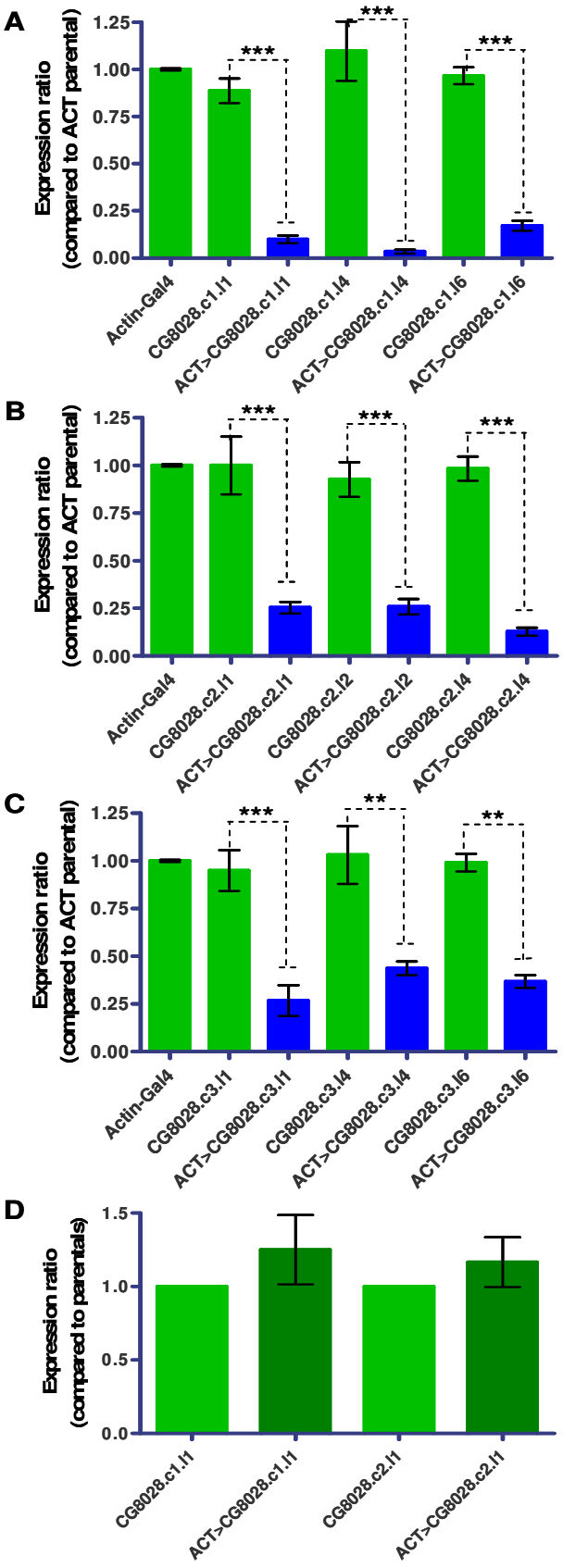


Figure 7.6 qPCR verification of *CG8028* RNAi knockdown in the tubules. *CG8028* expression in A: RNAi lines from construct 1, B: RNAi lines from construct 2, and C: RNAi lines from construct 3. D: Quantification of *CG8034* expression in driven RNAi lines (N=3, mean ± S.E.M.) (Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001).

All nine of the GAL4-driven UAS-RNAi fly-lines had significantly lower *CG8028* expression than their parental lines, as determined by Student's *t*-test. The knockdowns ranged from ~5% - 45% of the parental transcript levels, with variation between lines resulting from insertional effects and different efficiencies of the constructs at targeting the transcript for degradation. The two lines tested for off-target effects showed no significant decrease in *CG8034* expression, suggesting that the RNAi constructs are specific for *CG8028*. As the knockdown of *CG8028* had no effect on fly viability it may not be an appropriate gene for insecticide targeting, as an insecticide which causes the loss-of-function of *CG8028* is unlikely to kill the insect. The enrichment of multiple monocarboxylate transporters in the Malpighian tubules suggests that they may be better targeted as a family rather than individually.

7.3.3.2 Effect on Fluid Secretion from the Tubules

The effect of knocking down *CG8028* expression on the rate of fluid secretion from the Malpighian tubules was investigated using secretion assays (Dow *et al.*, 1994). Tubules dissected from the GAL4-driven UAS-RNAi line, the UAS-RNAi parent and the GAL4 driver parent were allowed to secrete steadily for 30 min before the diuretic peptide Drosokinin was added to the bathing medium. Three GAL4-driven UAS-RNAi lines were tested; one line for each of the three RNAi constructs (Figure 7.7).

Knockdown of *CG8028* expression had no significant effect on the rate of basal or stimulated fluid secretion from the tubules. This suggests that *CG8028* does not have a direct role in controlling the rate of fluid secretion from the Malpighian tubules. This may be because the functional role of *CG8028* is not related to osmoregulation, or may be due to the redundancy of having multiple monocarboxylate transporters which are highly expressed in the tubules.

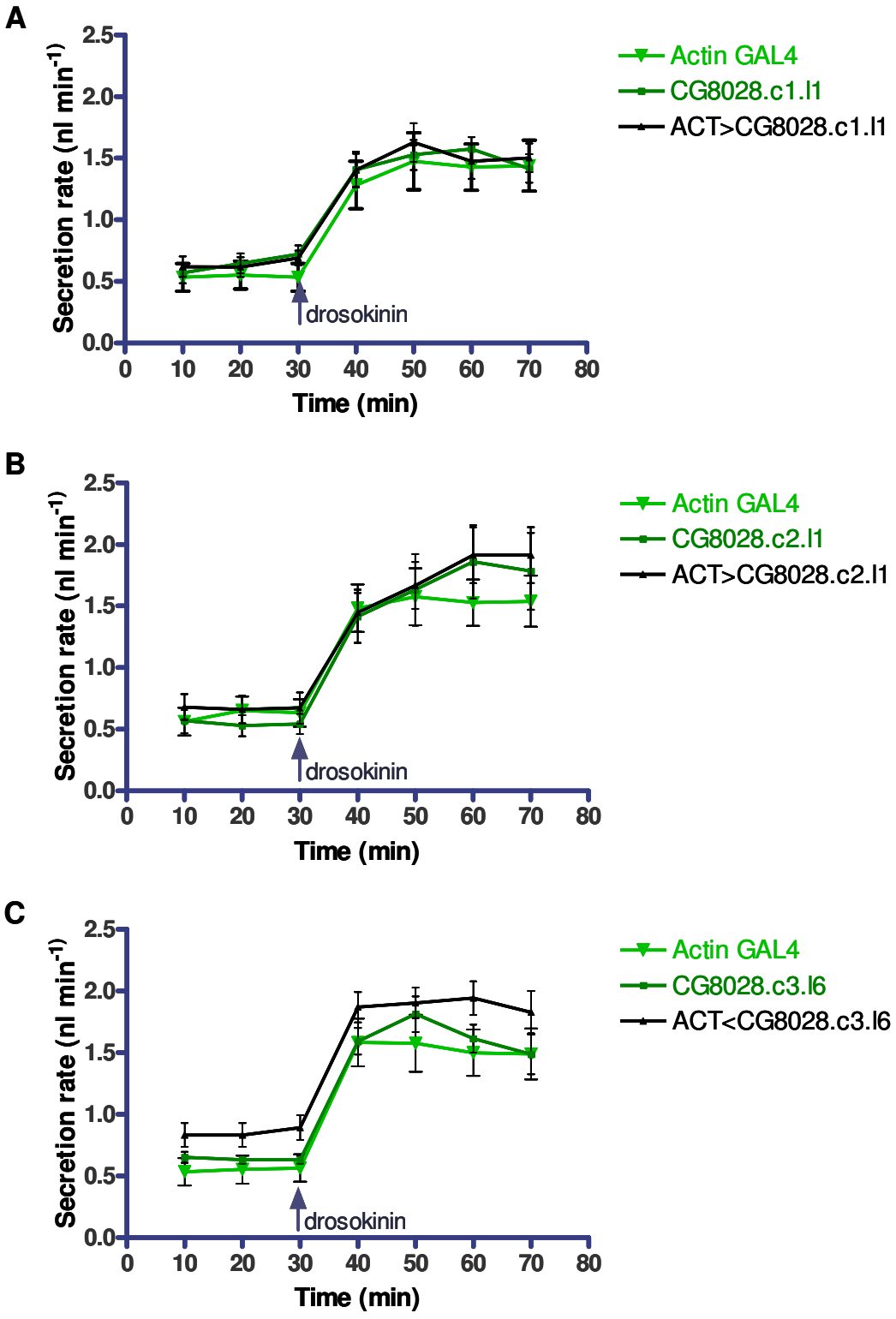


Figure 7.7 Effect of *CG8028* knockdown on the rate of fluid secretion from the tubules. Typical experimental results, showing the response of three driven RNAi lines and the appropriate RNAi parental lines to Drosokinin after 30 min of steady secretion (mean ± S.E.M., N≥10).

7.4 Discussion

The monocarboxylates are essential components of many of the processes which energise cells. Compounds such as lactate, pyruvate and butyrate are not only by-products of glycolysis, but can fuel cellular respiration when required in specific tissues. The monocarboxylates may also have roles in development and apoptosis in *Drosophila*, suggesting that the eighteen putative MCTs may have very diverse functions. Both deficiency and over-accumulation of monocarboxylates can be detrimental to a tissue, and so their transport must be carefully regulated. Little is known about the role of monocarboxylates in the Malpighian tubules of insects, but the enrichment of multiple putative monocarboxylate transporters in the tubules of *Anopheles* and *Drosophila* implies that they may be important. This section discusses the expression of the putative monocarboxylate transporter *AGAP002587* and its homologue *CG8028* in the Malpighian tubules of *Anopheles* and *Drosophila*, and their likely functional roles. The usefulness of *CG8028* as a model for *AGAP002587*, and their likely success as insecticide targets, is also considered.

7.4.1 AGAP002587 and CG8028 in the Tubules

The spatial and temporal expression of *AGAP002587* and *CG8028* in the tubules was investigated for two reasons; to gain further understanding of gene function, and to determine how similar gene expression of the homologues is. In *Anopheles*, *AGAP002587* is ~15-fold enriched in adult female tubules and ~100-fold enriched in the tubules of adult females 3 h after a blood-meal. Expression of *AGAP002587* is similarly enriched 24 h after a blood-meal, suggesting that the gene is important during the initial natriuresis and subsequent diuresis. In *Drosophila*, *CG8028* is ~2-fold enriched in the larval tubules and ~45-fold enriched in the adult tubules. Unlike in *Anopheles*, there is no sex-specific enrichment of *CG8028* in the *Drosophila* tubules. Low expression of the homologues in the *Anopheles* and *Drosophila* whole-body samples suggests that their transcription is not widespread, and may even be confined to the tubules.

The *in situ* hybridisations indicate that both *AGAP002587* and *CG8028* are expressed throughout the length of the tubules in both males and females. In *Anopheles* the stellate cells appear to have much lighter staining, suggesting

that *AGAP002587* is principal cell specific. In the *Drosophila* tubules it is difficult to differentiate between the principal and stellate cells from the staining, meaning that *CG8028* may be transcribed in both.

Knockdown of *CG8028* expression using the UAS/GAL4 system was used to mimic the effect that insecticide targeting would have if the compound prevented transporter function. Knockdown of *CG8028* using an *Actin-GAL4* driver line had no lethal or semi-lethal effect on the progeny. Knockdown of *CG8028* in specific segments of the tubules using *c42-GAL4* and *UrO-GAL4* also had no effect on fly survival. As some of the fly-lines tested contained only ~5% of the parental transcript level, it would appear that *CG8028* expression is not essential for *Drosophila* development through to adulthood, or survival thereafter. It is possible that *CG8028* could be highly important when the fly is under certain life-style pressures, such as during starvation or metal toxicity, although this was not tested during this investigation.

The microarray, qPCR and *in situ* hybridisation data are in good agreement with one another for both species, with no notable differences in findings gained from different techniques. The most significant difference is that *CG8028* is not expressed sex-specifically in the tubules, whereas *AGAP002587* is highly enriched in the tubules of females in comparison to males, particularly after a blood-meal. This implies that *AGAP002587* is primarily functional while the mosquito tubules are highly active, and at a stage for which there is no obvious parallel in *Drosophila*. *Drosophila* may therefore not be a suitable model for *Anopheles* genes which are highly up-regulated in response to a blood-meal, as it is difficult to model their function in *Drosophila* tubules which are not under a similar pressure. *AGAP002587* could be involved in a range of transport activities, such as the excretion of waste by-products of haematophagy, or the uptake of monocarboxylates as a fuel source, during a period of high activity for the tubules.

7.4.2 AGAP002587 and CG8028 as Monocarboxylate Transporters

AGAP002587 and *CG8028* contain the MCT-like motif associated with the mammalian monocarboxylate transport families. Of the *Drosophila* genes which contain an MCT-like motif, *CG8028* has the largest signal strength in the adult

Malpighian tubules. The role of monocarboxylate transporters in the tubules is not understood, and so it is not known if there are efflux as well as influx proteins, and which compounds they transport. *AGAP002587* and *CG8028* have homologues in *Aedes aegypti*, but none in the other organisms which have had their genome sequenced (Hubbard *et al.*, 2009). This suggests an ancient, but insect-specific function for *AGAP002587* and *CG8028*.

If *CG8028* is only one of several genes involved in the import or export of waste monocarboxylates, or the import of monocarboxylates as a fuel source, we would not expect gene knockdown to have an immediate effect on the rate of fluid secretion from the tubules. This was reflected in the results of the secretion assays, which showed no basal or stimulated fluid secretion phenotype associated with decreased expression of *CG8028*. With such an extensive range of putative substrates, it would be useful to express *CG8028* and *AGAP002587* in a system such as *Xenopus* oocytes, where the transport of a large range of compounds could be characterised.

7.4.3 AGAP002587 and CG8028 as Insecticide Targets

The experiments performed during this investigation have produced no evidence that *AGAP002587* and *CG8028* would make effective insecticide targets. The gene knockdown of *CG8028* does not have a lethal effect on *Drosophila* survival, suggesting that it is not an essential gene for development through to adulthood. This may be because of a redundancy of monocarboxylate transporters in the *Drosophila* Malpighian tubules. Whether *AGAP002587* is an essential gene for development and survival in *Anopheles* is not tested in this investigation. If *AGAP002587* was to prove essential for survival in females which had taken a blood-meal, it would make a very attractive insecticide target.

The difference in expression of *AGAP002587* and *CG8028* between *Anopheles* and *Drosophila* suggests that the two genes may either have different functions, or have the same function but be important to the insects under different physiological circumstances. *CG8028* is strongly expressed in the tubules of male and females throughout adulthood, whereas expression of *AGAP002587* is linked to haematophagous behaviour. Further investigation of *AGAP002587* could be undertaken in *Aedes aegypti*, which is haematophagous, and easily maintained in

the laboratory. *Aedes* may be a more appropriate model for studying *Anopheles* genes involved in or induced by blood-feeding, as it is closer in life-style to *Anopheles*, and has a more advanced molecular toolbox than *Anopheles*. The four *Anopheles* genes studied during this investigation all have a good homologue in *Aedes,* (Hubbard *et al.*, 2009) although it is not yet known whether they are expressed in the Malpighian tubules.

As there are a large number of uncharacterised monocarboxylate transporters in the tubules, designing an insecticide to target them as a family, rather than individually, could be more effective. This may also increase the likelihood of the insecticide being harmful to species out-with Diptera, and so would require extensive testing.

7.5 Conclusion

The results presented in Chapter 7 indicate that the putative monocarboxylate transporters *AGAP002587* and *CG8028* are unlikely to be effective candidate genes for the insecticide targeting of Diptera species. Gene knockdown of *CG8028* has no effect on the rate at which the Malpighian tubules are able to secrete fluid, and is not lethal to *Drosophila* at any stage in development, suggesting it is not an essential gene under the life-style conditions tested.

The large up-regulation of *AGAP002587* and *CG8028* in the *Anopheles* and *Drosophila* Malpighian tubules, and their expression throughout the length of the tubules, suggests that they may still be functionally important, although that function is yet to be defined. The microarrays and qPCR data suggests that *Anopheles* genes which are highly up-regulated after haematophagous behaviour may not be appropriate for functional studies in *Drosophila,* and an alternative model such as *Aedes aegypti* could prove more reliable. *AGAP002587* may be essential for survival if it was further characterised in *Anopheles*. Extensive studies using an expression system such as *Xenopus* oocytes could be utilised to characterize the affinity of *CG8028* and *AGAP002587* for a range of monocarboxylates.

Chapter 8 - Conclusions and Further Work

8.1 Summary

The main conclusions identified during this investigation are described in this chapter. These include a summary of results of the *Anopheles* Malpighian tubule microarray, and the follow-up functional work in *Anopheles* and *Drosophila*. The usefulness of *Drosophila* as a model for *Anopheles* is also evaluated, as is the effectiveness of the genes studied as putative insecticide targets. Interesting lines of enquiry for future work are also described.

8.2 Conclusions

Dipteran species such as *Anopheles* and *Aedes* have a large impact on human and veterinary health, as well as the economy, of countries across the world. A greater understanding of mosquito physiology, as well as a revised strategy for insecticide use, is required to regain control of pest populations. Equally important is the development of new insecticides and insecticide targets, without which large areas of the world will continue to be plagued by diseases which are ultimately controllable.

8.2.1 Tissue-specific Microarrays are Advantageous for Tissue-specific Studies

The *Anopheles* Malpighian tubule data-set identified a list of highly expressed and enriched genes which are likely to be functionally interesting and important in the tubules. By comparing the *Anopheles* Malpighian tubule data to the whole-*Anopheles* data-set generated by Marinotti *et al* it is apparent that many interesting tissue-specific expression patterns are not detectable in whole-organism studies (Marinotti *et al.*, 2006). A large increase in the expression of a gene in a specific tissue will often not significantly alter the overall quantity of the gene in the whole-organism (Wang *et al.*, 2004). This reflects what has already been found in fruit-fly, where a comparison of whole-*Drosophila* gene expression to tissue-specific gene expression has found organ-specific microarrays to be advantageous for determining the tissue in which it is best to study a gene (Wang *et al.*, 2004; Chintapalli *et al.*, 2007). Table 8.1 shows data collected from tissue-specific microarrays performed in *Drosophila*, and

identifies numerous genes which have predominately been characterised in tissues which are not the primary site of their expression.

Table 8.1 Genes which are predominately expressed in unexpected tissues (Chintapalli *et al.*, 2007).

		Mean signal levels							
Gene	Described in	Brain	Head	Midgut	Tubule	Hindgut	Ovary	Testis	Accessory gland
cry	Circadian behavior	279	575	267	**1,972**	868	7	25	205
fas2	Neuronal fasciculation	129	66	49	**1,676**	78	5	9	53
obp56d	Olfaction	71	4,045	1	1	**5,663**	1	106	5
kelch	Nurse cell	181	**185**	22	16	22	5	6	6
rpk	Sensory neurons	1	0	1	7	0	**904**	47	0
toe	Eye, thorax	10	68	7	8	14	8	13	**3,725**
vnd	Embryonic CNS	6	4	**289**	5	6	3	2	8
dsx	Sex determination	21	119	89	**140**	106	9	1	8

Boldface indicates the maximum signal for each gene.

The vast majority of genes identified from the *Anopheles* Malpighian tubule microarray data-set are uncharacterised, confirming the need for tissue-specific rather than whole-organism microarray studies. The *Anopheles* microarray data-set also demonstrates the importance of determining the correct life-stage at which to perform a microarray, as there is significant variation in the expression of thousands of genes in the tubules between each stage in the life-cycle. It is vital that microarray studies are carefully planned, to maximise the power of the experimental output.

8.2.2 Transporters are Highly Expressed in the Malpighian Tubules of Dipterans

Transcriptomic analysis of the *Drosophila* Malpighian tubules shows that the most highly enriched genes are broad-specificity transporters, for organic cations, anions, monocarboxylic acids, and inorganic anions such as phosphate and iodide (Wang *et al.*, 2004). Analysis of the *Anopheles* Malpighian tubule transcriptome shows highly similar results. Almost every class of transporter is represented by at least one gene, with many being highly enriched in the tubules, as well as highly abundant. This suggests that similarly to the *Drosophila* tubules, the *Anopheles* tubules are likely to play an important role in ion regulation and detoxification.

By comparing the renal transcriptome of the two species it is possible to determine pathways which have been conserved, as well as the processes which have diverged or expanded. This data could be used to identify genes which are involved in the response to haematophagous behaviour. Genes involved in heamatophagy could be effective targets for an insecticide which is mosquito-specific rather than Diptera-wide. This data could also be used to study the gender identity of the tubules, and the mechanisms which control whether the tissue is male or female. The transcription factors and peptides which control gender identity in the Malpighian tubules are yet to be determined.

8.2.3 Drosophila is a Good Model for the Anopheles Malpighian Tubules

Previous findings from the *Drosophila* Malpighian tubules which have been tested in the *Anopheles* tubules have shown conservation of function. The primary roles of the tubules, osmoregulation and ion balance, appear to be similarly controlled in the two species. The data collected during this study shows good conservation of the expression profile of three of the homologous gene-pairs tested. Three levels of homology are apparent; conservation of protein sequence, increased expression in the tubules, and similarities in the spatial expression pattern. The gene-pair which has the least homology is *AGAP002587* and *CG8028*, as *AGAP002587* is highly specific to the female tubules, whereas *CG8028* is transcribed similarly in males and females. This suggests that genes which are highly enriched in *Anopheles* after a blood-feed may respond to physiological factors which are not present in *Drosophila*, and therefore may not show the same level of conservation. This does not mean that their primary function has also diverged, and *Drosophila* may still prove to be a valid model for the characterisation of genes transcribed in response to haematophagous behaviour.

8.2.4 Cross-species Studies are an Effective Strategy for Identifying Essential Genes

Model organisms have been used successfully for many decades, both to study other species, and to understand the underlying principles of life. By studying two species in tandem, it is possible to utilise the experimental advantages of

both organisms. *Anopheles* is a highly important species for its impact on human health, but is difficult to perform high-throughput studies on due to a lack of natural or transgenic resources. Unlike *Anopheles*, *Drosophila* does not spread fatal diseases, and has a highly annotated genome with reliable and easily accessible transgenics. In this case, *Drosophila* can be used to perform transgenic studies which can help increase understanding of mosquito physiology.

By comparing the transcriptome of an organ in two species which diverged 150-250 million years ago it becomes easier to identify genes which are likely to be essential for survival. This study demonstrates that genes which have a highly conserved protein sequence and expression profile across species are often important. Three of the four homologous gene-pairs studied are essential in *Drosophila*, both for development and survival as an adult. The monocarboxylate transporter *CG8028* is the only gene which is not essential in *Drosophila*, and also has the least conserved expression profile. By publishing microarrays as a publicly available searchable database it increases the power and ease of cross-species studies, and promotes the use of model organisms such as *Drosophila*.

8.2.5 Limitations of the Study

The successful characterisation of the four transporters in *Drosophila* was restricted by the investigative techniques utilised. RNAi is a useful molecular tool when combined with the UAS/GAL4 system, but it also has shortcomings. Although every effort is made to produce RNAi constructs which are gene specific they may still cause an off-target silencing effect on other genes when inserted into the *Drosophila* genome and expressed. During this investigation off-target effects were assessed by quantifying the closest homologue of each targeted gene, none of which showed any significant knockdown. The results of these experiments do not exclude the effects of off-target silencing on other genes, which could be assessed by further microarray studies on each UAS-RNAi fly-line.

The knockdown of the *CG15406* and *Picot* transcripts by RNAi was not proven to result in decreased protein expression in the tubules. This may be due to failings in the techniques used to assay protein expression, or as a result of indirect

correlation between transcript and protein levels. The lethal phenotype which resulted from driving three of the UAS-RNAi gene constructs with *Actin-GAL4* meant that alternative GAL4-drivers which did not have the optimum spatial and temporal expression pattern had to be utilised. Constructing GAL4 lines with the temporal and spatial expression required can be difficult and time-consuming, and a GAL4-driver which ubiquitously expresses solely in the Malpighian tubules of *Drosophila* would be incredibly useful.

Other approaches to gene knockdown, such as partial deletion of the gene from the genome using homologous recombination, could also be utilised (Rong and Golic, 2000). Although homologous recombination should only disrupt expression of the target gene-of-interest, the ubiquitous nature of the genomic alteration leaves no scope for spatial or temporal manipulation. An alternative method of controlling protein function is the blocking of transporter function using pharmacological agents. The four gene-types investigated in this thesis have been successfully targeted by pharmacological agents in mammals. Whether these mammalian pharmacological agents block homologous genes in *Drosophila* is not yet known, but they could prove to be a useful way of controlling the efficacy of transporter function without interfering with transcription or translation.

The antibodies designed and used during immunolocalisation and Western blotting were not specific to the genes against which they were targeted. The antibodies were designed against an antigenic region of each protein which was predicted to be available for antibody binding and not part of the membrane spanning regions of the protein. The putative antigenic areas were then checked for homology to other known proteins in the *Anopheles* or *Drosophila* genome, to minimise the likelihood of cross-reactivity with other proteins. The antigenic region which was most likely to produce a specific, working antibody was then used to produce the four antibodies utilised in this investigation. The four antigenic sequences chosen had at least some homology to other proteins within the same species, which has resulted in extra protein bands in the Western blots. Non-specific binding was minimised by using the lowest antibody concentration possible, and by using antibody solutions which had been pre-absorbed on other tissues, which had little effect on the number of proteins identified during Western blotting. Had time permitted, it would have been

useful to express the *CG15406* and *Picot* genes in *Drosophila* with an additional tag, such as tetra-HIS, which would have allowed immunolocalisation using a highly-specific, commercially available antibody.

The Ramsay secretion assay was also used with limited success during this investigation. The secretion assay is an excellent technique for finding proteins which are essential for epithelial fluid transport, but the rate of secretion may not be directly affected by minor solutes or solutes which are metabolised by the tubules. It is also unlikely that every gene which is important for tubule function is directly involved in fluid secretion, as the tubules are involved in a wide range of processes, such as detoxification and immune response (McGettigan *et al.*, 2005; Yang *et al.*, 2007). Transport assays using radioactively-labelled substrates can be effective, although only if the function of the gene-of-interest can be distinguished from that of other genes. The redundancy of many gene families, such as those investigated during this study, can make it difficult to unmask the function of a specific protein. This can be circumvented by expressing genes in an alternative system such as *Xenopus oocyte*, where they can be characterised in isolation.

During this study experimentation on the *Anopheles* Malpighian tubule was restricted, as the *Anopheles* colony was maintained by an external research group, and the availability of mosquitoes was limited. This was largely due to the size of the mosquito colony dropping rapidly on several occasions, and for no apparent reason. This resulted in difficulty optimising *Anopheles* experiments, such as the immunolocalization of AGAP007752 and AGAP012251 in the Malpighian tubules, and Western blotting for the AGAP007752 and AGAP012251 proteins.

The preliminary physiological studies performed during this investigation were largely inconclusive, and some of the experiments which could be performed to further characterise each gene-pair are described in the following section.

8.3 Future Work

Many aspects of the work described in this thesis would benefit from further investigation. These studies could not only help find new ways of controlling populations of Diptera, but increase our understanding of the function of the insect Malpighian tubules. Possible ways of extending the data include further characterisation of the homologous gene-pairs in both *Anopheles* and *Drosophila*, and assays to determine whether the genes investigated would be successful insecticide targets.

The *Anopheles* and *Drosophila* Malpighian tubule microarray data-sets allowed identification of gene homologues which were highly enriched in both species. It would be interesting to extend this work to other tissues, to identify organs which are highly similar or dissimilar between the insects. This would further assess how appropriate a model *Drosophila* is for *Anopheles*. By extending the data to other invertebrates and vertebrates it may also be possible to determine the best model organisms in which to study particular physiological processes.

The *Anopheles* Malpighian tubule microarray data-set could also be further investigated to determine how the tubules alter in response to changes in life-style. Thousands of genes are differentially expressed between the adult male and female tubules, and little is known about the control or effect of this. Analysis of the promoter regions of these genes could identify common control regions, and lead to the identification of the transcription factors involved in determining the gender of the tubules.

The functional analysis of the *Anopheles* and *Drosophila* transporters investigated during this study could also be extended. The putative function of each gene-pair could be determined by expressing them in *Xenopus laevis* oocytes, and characterising the compounds which they are able to transport. This would determine whether each *Anopheles* gene has the same function as its *Drosophila* counterpart. This system could also be used to test the efficiency of novel insecticides at preventing gene function. As transgenic capabilities improve in mosquito it may also be possible to manipulate gene expression in *Anopheles*, and determine whether the four genes are essential for development or survival.

Transgenic knockdown of multiple genes in *Drosophila* could be used to determine whether the monocarboxylate transporters could be effectively targeted by an insecticide as a family. Targeting gene families rather than single proteins may increase efficacy while decreasing the likelihood of insecticide resistance developing.

Many other genes enriched in the *Anopheles* and *Drosophila* data-sets are likely to have important and interesting functions in the tubules. Further investigation of genes which could be involved in transporting substrates such as pesticides and other drug compounds could provide new insight into insecticide resistance. Other roles which have been characterised in the *Drosophila* tubules, such as immune function and heavy metal detoxification, could also be further studied in the mosquitoes.

Appendix 1

Quantitative PCR Primers for *Drosophila melanogaster*

Primer Name	Primer Purpose	Primer Sequence
PicotqF	Forward qPCR primer for *Picot*	TCAACCACTTGGACCTCACACC
PicotqR	Reverse qPCR primer for *Picot*	TGACGCACGACTACGGCTAATAC
CG3994qF	Forward qPCR primer for *CG3994*	GCAATGGCAACAACAATCACC
CG3994qR	Reverse qPCR primer for *CG3994*	GAAATCCGTTAGCAAATGAGCG
CG15406qF	Forward qPCR primer for *CG15406*	GCATAATGGTGGGCTTTGTGG
CG15406qR	Reverse qPCR primer for *CG15406*	AGTTGTTGAAGGCGAAGGTGC
CG8028qF	Forward qPCR primer for *CG8028*	TCCCTTCCTCCCACTAATCTCAC
CG8028qR	Reverse qPCR primer for *CG8028*	TACGCCTCCGAAACTTGACG
CG4330F	Forward qPCR primer for *CG4330*	GGAATCCACGAAACCAAATAGAGC
CG4330R	Reverse qPCR primer for *CG4330*	TAGACCACGGCGAATCCCAG
CG11163F	Forward qPCR primer for *CG11163*	AATCTGGTTCGTAACTACCCTGCTC
CG11163R	Reverse qPCR primer for *CG11163*	GCTTTGACTTCCAATGGTCGTAAG
CG8051F	Forward qPCR primer for *CG8051*	ACAACCACAACAACAACAGACGAC
CG8051R	Reverse qPCR primer for *CG8051*	CCATTATCGGTCATCTTGGAACTG
CG15408F	Forward qPCR primer for *CG15408*	CCATACCACATTCGTGCTTTTGG
CG15408R	Reverse qPCR primer for *CG15408*	CCACCAGGATTCCCACATTGAC

In situ hybridisation primers for *Drosophila melanogaster*

Primer name	Primer purpose	Primer sequence
CG15406situF	Forward *CG15406 in situ* probe primer	CCTTCATCATCTTCATTGCCAACG
CG15406situR	Reverse *CG15406 in situ* probe primer	CATCAGTGGAAAAGTCTTCAGCG
PicotsituF	Forward *Picot in situ* probe primer	TCAACCACTTGGACCTCACACC
PicotsituR	Reverse *Picot in situ* probe primer	TGACGCACGACTACGGCTAATAC
CG3994situF	Forward *CG3994 in situ* probe primer	ACATCGGCGGTTCATTTGC
CG3994situR	Reverse *CG3994 in situ* probe primer	TAGACGGGGGTGTCACTCTTATCG
CG8028situF	Forward *CG8028 situ* probe primer	CTGGTCTTTGCCGACTACTTGC
CG8028situR	Reverse *CG8028 in situ* probe primer	CGATTTTGTTACGCCTCCGAAAC

Quantitative PCR primers for *Anopheles gambiae*

Primer Name	Primer Purpose	Primer Sequence
AGAP007752F	Forward qPCR primer for *AGAP007752*	CGGCGGTGGAATAATCGTAGTC
AGAP007752R	Reverse qPCR primer for *AGAP007752*	AAGGGCGACGGTATGATAGGACAG
AGAP012251F	Forward qPCR primer for *AGAP012251*	TGATGGCGTTCACCAACTGCTC
AGAP012251R	Reverse qPCR primer for *AGAP012251*	TTCTGCGGTTCGTCGTTCTC
AGAP009005F	Forward qPCR primer for *AGAP009005*	CACGAGGAGCAAAACATCAACG
AGAP009005R	Reverse qPCR primer for *AGAP009005*	AGCACCAGCACCGAAAACAG
AGAP002587F	Forward qPCR primer for *AGAP002587*	TTCGTTCGCCGTCTTTCAAG
AGAP002587R	Reverse qPCR primer for *AGAP002587*	TCATCATCACCACCCGCTTCTC
AGAP002858F	Forward qPCR primer for *AGAP002858*	TGGTCTGAGTCACGCAAAAGC
AGAP002858R	Reverse qPCR primer for *AGAP002858*	TGAAACACAGGATGGCACCG
AGAP010892F	Forward qPCR primer for *AGAP010892*	CCCACATCTCCCCTCAAAATG
AGAP010892R	Reverse qPCR primer for *AGAP010892*	TCTTTATCCCCCCTGGTTACCC
AGAP007495F	Forward qPCR primer for *AGAP007495*	GAACGATGCAAAAGTTTCTGG
AGAP007495R	Reverse qPCR primer for *AGAP007495*	GCGACGATTGTTTGTGAGAGC
AGAP008386F	Forward qPCR primer for *AGAP008386*	TAACGGCAACCCACATCTCG
AGAP008386R	Reverse qPCR primer for *AGAP008386*	CGGTCTGGAGTTCGTGAAAAAC

In situ hybridisation primers for *Anopheles gambiae*

Primer name	Primer purpose	Primer sequence
AGAP007752situF	Forward *AGAP007752 in situ* probe primer	CCCAATACATCCGCCATCATC
AGAP007752situR	Reverse *AGAP007752 in situ* probe primer	CTGGTAACACGAAGAAAGGAATGC
AGAP012251situF	Forward *AGAP012251 in situ* probe primer	ATCGTGTTCGTGATAGCCGC
AGAP012251situR	Reverse *AGAP012251 in situ* probe primer	ATTGTTACCGTTCGCCTCGCTGTG
AGAP009005situF	Forward *AGAP009005 in situ* probe primer	TGTGGTTCTTATGGAGGGCACC
AGAP09005situR	Reverse *AGAP009005 in situ* probe primer	CCGTTTCCGTGGCTTTATTCAG
AGAP002587situF	Forward *AGAP002587 in situ* probe primer	TATGGGCTTTCTACGGACCTGG
AGAP002587situR	Reverse *AGAP002587 in situ* probe primer	AACCACGGTATCACGCACAGTG

Primers for *Drosophila melanogaster* pWIZ RNAi constructs

Primer Name	**Primer Purpose**	**Primer Sequence**
CG15406RNAiF	Forward primer for *CG15406* RNAi insert	GCATTCTAGAGAAATCGCCGACAATAGCATTC
CG15406RNAiR	Reverse primer for *CG15406* RNAi insert	GCATTCTAGACCACCATCGTCACCGAGATAAC
CG15406RNAi2F	Forward primer for *CG15406* RNAi insert	GCATTCTAGACGCCACTCTCCTTTTACATTGACG
CG15406RNAiR	Reverse primer for *CG15406* RNAi insert	GCATTCTAGAGTGTCGTTGTTTTGCTGTTGGATG
CG15406RNAi3F	Forward primer for *CG15406* RNAi insert	GCATTCTAGATGTCCGATTTCCTCACCAAGC
CG15406RNAi3R	Reverse primer for *CG15406* RNAi insert	GCATTCTAGACAGGCAGACCACCACATAGAACAG
PicotRNAiF	Forward primer for *Picot* RNAi insert	GCATTCTAGAAAATGGATTCCGCCCAACG
PicotRNAiR	Reverse primer for *Picot* RNAi insert	GCATTCTAGATAAGACCCACTCCGATGGTGAG
PicotRNAi2F	Forward primer for *Picot* RNAi insert	GCATTCTAGAGCAGGGCTACATCTTGTCGTCC
PicotRNAi2R	Reverse primer for *Picot* RNAi insert	GCATTCTAGATGAAGGGAATGGGAGGGCTC
PicotRNAi3F	Forward primer for *Picot* RNAi insert	GCATTCTAGAAATGGATTCCGCCCAACG
PicotRNAi3R	Reverse primer for *Picot* RNAi insert	GCATTCTAGAAAGACCCACTCCGATGGTGAG
CG3994RNAiF	Forward primer for *CG3994* RNAi insert	GCATTCTAGACGACTACGAGGTGAATGCGAAG
CG3994RNAiR	Reverse primer for *CG3994* RNAi insert	GCATTCTAGACGGCGTAGTGCATATAGTTGG
CG3994RNAi2F	Forward primer for *CG3994* RNAi insert	GCATTCTAGAAGGGCGTGGATGTGAAGGC
CG3994RNAi2R	Reverse primer for *CG3994* RNAi insert	GCATTCTAGACAGCGGCGACAAAAACTCC
CG3994RNAi3F	Forward primer for *CG3994* RNAi insert	GCATTCTAGACAGCGTATTGAGGGCGGTG
CG3994RNAi3R	Reverse primer for *CG3994* RNAi insert	GCATTCTAGAACGGGGGTGTCACTCTTATCG
CG8028RNAiF	Forward primer for *CG8028* RNAi insert	GCATTCTAGACCAACGGTGGTGATTAGAGTGC
CG8028RNAiR	Reverse primer for *CG8028* RNAi insert	GCATTCTAGATTGAAGGCTGTGGGAAAATGAG
CG8028RNAi2F	Forward primer for *CG8028* RNAi insert	GCATTCTAGACGAAATCCTACACACTGGAGGC
CG8028RNAi2R	Reverse primer for *CG8028* RNAi insert	GCATTCTAGAAAGGCGAATCCCTGAACGAC
CG8028RNAi3F	Forward primer for *CG8028* RNAi insert	GCATTCTAGACCTCTACTCCGACATCACCTTCTTC
CG8028RNAi3R	Reverse primer for *CG8028* RNAi insert	GCATTCTAGACCCTGGTTATCAACATCCTCGTG

Appendix 2

The following table contains the number of driven *CG15406*-RNAi flies which eclosed out of the total number of fly eclosures for each of the crosses listed (n=3). Crosses which were lethal or semi-lethal (as determined by *Chi*-squared analysis) are annotated with an asterisk.

Fly-line	*Actin-GAL4*	*C42-GAL4*	*Uro-GAL4*	*C724-GAL4*
CG15406.c1.l1	0/191*	120/295*	52/109	94/174
	0/212*	99/272*	63/116	97/201
	0/165*	103/261*	77/147	82/175
CG15406.c1.l4	0/224*	65/171*	81/180	76/159
	0/151*	54/142*	74/162	79/151
	0/172*	71/191*	59/128	70/142
CG15406.c1.l9	0/298*	86/169	61/135	64/130
	0/225*	78/163	75/159	96/205
	0/231*	99/206	74/152	87/194
CG15406.c2.l4	69/415*	103/199	65/139	57/120
	50/424*	115/219	68/134	69/139
	37/381*	79/164	79/163	73/138
CG15406.c2.l7	186/600	78/187	101/208	83/161
	167/552	93/178	87/161	94/188
	142/441	52/114	85/156	82/157
CG15406.c2.l8	154/509	67/145	57/110	97/195
	160/507	81/153	68/124	85/163
	182/598	94/175	70/146	58/127
CG15406.c3.l3	0/162*	82/175	45/97	79/152
	0/178*	59/131	62/141	84/169
	0/193*	58/135	80/152	85/172
CG15406.c3.l4	0/179*	147/273	59/111	66/135
	0/148*	94/201	73/135	69/131
	0/188*	83/154	64/129	78/150
CG15406.c3.l5	16/247*	73/166	76/147	88/169
	8/215*	88/161	81/174	78/145
	13/228*	105/202	96/203	99/191

Red font: ¼ of eclosed flies should be GAL4-driven UAS-RNAi.
Blue font: ⅓ of eclosed flies should be GAL4-driven UAS-RNAi.
Black font: ½ of eclosed flies should be GAL4-driven UAS-RNAi.

The following table contains the number of driven *Picot*-RNAi flies which eclosed out of the total number of fly eclosures for each of the crosses listed (n=3). Crosses which were lethal or semi-lethal (as determined by *Chi*-square analysis) are annotated with an asterisk.

Fly-line	*Actin-GAL4*	*C42-GAL4*	*C724-GAL4*	*ELAV-GAL4*
Picot.c1.l3	0/184*	72/191*	63/119	84/164
	0/190*	97/282*	74/156	87/181
	0/214*	50/151*	89/197	72/138
Picot.c1.l5	0/232*	51/185*	84/170	76/149
	0/178*	57/202*	94/203	89/171
	0/174*	73/199*	68/128	76/162
Picot.c1.l6	0/254*	89/179	70/148	65/130
	0/214*	71/152	80/159	100/205
	0/222*	94/200	73/152	84/179
Picot.c2.l3.	18/285*	54/131	75/159	57/126
	24/215*	68/148	86/184	69/139
	36/237*	85/189	85/173	85/178
Picot.c2.l5	26/298*	74/157	107/218	81/169
	17/197*	93/188	77/151	94/198
	25/278*	82/164	73/149	88/186
Picot.c2.l6	0/209*	75/144	55/111	99/207
	0/157*	73/133	68/124	84/159
	0/198*	84/175	100/206	55/117
Picot.c3.l2	15/182*	82/165	75/157	69/155
	19/176*	55/121	67/141	83/159
	20/190*	58/125	70/160	74/157
Picot.c3.l3	15/170*	142/293	58/121	65/135
	32/248*	84/171	71/144	70/130
	26/198*	80/174	64/129	80/152
Picot.c3.l5	0/227*	78/166	98/207	69/149
	0/193*	81/161	88/180	87/175
	0/176*	96/206	94/202	103/199

Red font: ¼ of eclosed flies should be GAL4-driven UAS-RNAi.
Blue font: ⅓ of eclosed flies should be GAL4-driven UAS-RNAi.
Black font: ½ of eclosed flies should be GAL4-driven UAS-RNAi.

The following table contains the number of driven *CG3994*-RNAi flies which eclosed out of the total number of fly eclosures for each of the crosses listed (n=3). Crosses which were lethal or semi-lethal (as determined by *Chi*-square analysis) are annotated with an asterisk.

Fly-line	*Actin-GAL4*	*C42-GAL4*	*Uro-GAL4*	*C724-GAL4*
CG3994.c1.l1	71/299	73/161	61/129	75/161
	53/199	94/181	78/156	89/189
	66/258	70/151	79/157	73/148
CG3994.c1.l3	140/438	56/125	85/177	76/149
	72/222	67/152	100/203	89/171
	65/212	95/189	56/103	76/162
CG3994.c1.l4	76/240	89/179	73/149	56/114
	59/188	51/110	80/152	74/150
	64/201	92/179	76/158	54/112
CG3994.c2.l1	0/204*	126/261	79/150	57/126
	0/187*	170/352	86/161	101/220
	0/193*	120/263	72/155	82/178
CG3994.c2.l2	0/250*	62/120	102/196	83/156
	0/197*	101/213	78/159	92/197
	0/278*	85/174	48/95	78/166
CG3994.c2.l8	0/206*	75/156	52/112	59/107
	0/159*	83/163	76/157	74/149
	0/194*	94/185	109/210	65/137
CG3994.c3.l1	137/404	92/175	74/158	67/135
	61/177	59/121	64/143	82/169
	62/199	77/155	90/178	78/151
CG3994.c3.l2	89/329*	112/243	78/160	75/145
	61/248*	173/349	76/155	72/138
	50/198*	79/154	54/113	88/172
CG3994.c3.l4	54/229	79/144	63/128	79/169
	43/181	46/92	85/160	81/171
	61/256	91/194	94/201	105/199

Red font: ¼ of eclosed flies should be GAL4-driven UAS-RNAi.
Blue font: ⅓ of eclosed flies should be GAL4-driven UAS-RNAi.
Black font: ½ of eclosed flies should be GAL4-driven UAS-RNAi.

The following table contains the number of driven *CG8028*-RNAi flies which eclosed out of the total number of fly eclosures for each of the crosses listed (n=3). Crosses which were lethal or semi-lethal (as determined by *Chi*-square analysis) are annotated with an asterisk.

Fly-line	*Actin-GAL4*	*C42-GAL4*	*ELAV-GAL4*	*C724-GAL4*
CG8028.c1.l1	83/250	79/164	66/137	105/211
	59/219	91/183	76/146	79/159
	69/298	75/150	89/187	78/158
CG8028.c1.l3	79/233	66/135	80/171	74/139
	72/272	87/172	104/205	90/184
	63/232	101/199	72/143	72/152
CG8028.c1.l4	95/210	69/149	79/159	66/134
	69/198	86/170	83/162	91/180
	61/195	95/189	56/108	84/162
CG8028.c2.l1	76/227	135/263	67/130	87/166
	83/239	145/292	76/151	111/225
	91/279	130/253	82/169	72/148
CG8028.c2.l2	59/176	79/1650	105/206	86/176
	86/261	109/210	88/179	102/199
	85/259	75/154	68/135	88/166
CG8028.c2.l4	52/205	85/166	92/182	89/188
	63/259	87/173	84/177	77/150
	45/171	92/189	119/232	75/157
CG8028.c3.l1	74/304	82/165	77/157	74/142
	79/327	69/141	74/149	86/179
	67/269	79/163	80/170	84/161
CG8028.c3.l4	88/270	101/205	98/200	81/155
	94/298	93/189	78/159	73/145
	69/218	77/155	73/154	85/167
CG8028.c3.l6	74/228	89/184	83/168	92/188
	63/190	74/144	95/199	81/168
	81/246	90/186	92/192	94/179

Red font: ¼ of eclosed flies should be GAL4-driven UAS-RNAi.
Blue font: ⅓ of eclosed flies should be GAL4-driven UAS-RNAi.
Black font: ½ of eclosed flies should be GAL4-driven UAS-RNAi.

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