The Anopheles gambiae ABC transporter family: phylogenetic analysis and tissue localisation provide clues on function and role in insecticide resistance.

Patricia Pignatelli1$, Victoria A Ingham1$, Vasileia Balabanidou2,3, John Vontas2, 4, Gareth Lycett1 and Hilary Ranson1\*

1Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK

2Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, 73100 Heraklion, Greece

3Department of Biology, University of Crete, Vassilika Vouton, 71409, Heraklion, Greece

4Faculty of Crop Science, Pesticide Science Lab, Agricultural University of Athens, 11855 Athens, Greece

$ Joint First Authors

\*Corresponding Author: Hilary.Ranson@lstmed.ac.uk

**Running Title:** Anopheles gambiae ABC transporters

Key Words: Insecticide resistance, ABC transporters, Anopheles gambiae

**Abstract**

The role of ABC transporters in conferring insecticide resistance has received much attention recently. Here we identify ABC transporters differentially expressed in insecticide resistant populations of the malaria vector, *Anopheles gambiae*. Although we found little evidence that the orthologs of the *multidrug resistance proteins* described in other species are associated with resistance in *An. gambiae* we did identify a subset of ABC proteins consistently differentially expressed in pyrethroid resistant populations from across Africa. We present information on the phylogenetic relationship, primary sites of expression and potential role of ABC transporters in mediating the mosquito’s response to insecticides. Furthermore we demonstrate that a paralogous group of eight ABCG transporters, clustered on chromosome 3R are highly enriched in the legs of *An. gambiae* mosquitoes, consistent with a proposed role for this ABC subfamily in transport of lipids to the outer surface of the cuticle. Finally, antibodies raised against one of the most highly expressed ABC transporters in adult females, ABCG7 (AGAP009850), localised this transporter to the pericardial cells. This data will help prioritise members of this gene family for further localisation and functional validation studies to identify the *in vivo* function of these transporters in the mosquito and determine whether elevated expression of members of this family contribute to insecticide resistance.

**Introduction**

The mosquito species *Anopheles gambiae* is responsible for the majority of malaria transmission in Africa and hence has been subject to extensive control efforts using insecticides. Inevitably this has led to the emergence of resistant populations, most notably to the pyrethroid insecticides that are incorporated into insecticide treated bednets (Ranson & Lissenden 2016). There are growing concerns that the increasing levels of resistance being detected in some regions in Africa could slow down or even reverse recent gains in controlling this disease (Hemingway et al. 2016). It is critically important that we understand the mechanisms responsible for this resistance to develop tools and strategies to mitigate against its potentially devastating effect. Two resistance mechanisms, alterations in the target site that reduce insecticide binding and increased rates of insecticide metabolism, have been the focus of the majority of previous studies on pyrethroid resistance in mosquitoes (Donnelly et al. 2009; Donnelly et al. 2016). A subset of detoxification genes with elevated expression in multiple resistant populations of *An. gambiae* across Africa have been identified and the ability of enzymes encoded by these genes to metabolise insecticides confirmed (Ingham et al. 2014).

Changes in the level of expression or structure of transporter proteins with affinity for insecticides (and/or their metabolites) could protect tissues from exposure and increase the rate of excretion from the organism but until recently the role of transporter proteins in conferring insecticide resistance in mosquitoes had received little attention. Multiple families of transporter proteins exist in insects but it is the ATP-binding cassette (ABC) proteins that have been primarily linked to xenobiotic transport.

ABC transporters are transmembrane ATP-dependent efflux pumps which mediate the transport of compounds out of the cell or into cellular organelles. They can transport a wide range of endogenous and exogenous compounds and some members of this family also have non-transport functions, such as control of protein biosynthesis (Dean et al. 2001). In addition to their role in transporting xenobiotics, insect ABC transporters are known to be involved in the transport of eye pigments (Mackenzie et al. 1999), development of pigmentation in the epidermis (Wang et al. 2013) and lipid transfer to the cuticle (Broehan et al. 2013).

ABC transporters contain conserved cytosolic ATP binding domains (nuclear binding domains NBDs) and more variable transmembrane domains (TMDs) that determine the substrate specificity. Half transporters contain just one ABC and one TMD and form homo or heterodimers whereas full transporters contain two copies of each domain. Arthropods typically contain between 50 and 100 ABC transporters, including both half and full transporters, which are subdivided into 8 subfamilies (A-H) (Dermauw & Van Leeuwen 2014). ABC proteins have been clearly linked to drug resistance in mammals notably the ABCB *multi drug resistant* (MDR) proteins or p-glycoproteins, ABCC *multidrug resistance associated proteins* (MRP) and the ABCG *breast cancer resistance protein* (BCRP) (Dean et al. 2001). Multiple studies in insects have identified ABC transporters either constitutively over expressed in insecticide resistant populations and/or induced by insecticide exposure (Lanning et al. 1996; Bariami et al. 2012; Jones et al. 2012). Further studies have shown that exposure to the ABC inhibitor verapamil synergises the activity of insecticides in some insect species (Lima et al. 2014; Epis et al. 2014; Figueira-Mansur et al. 2013). However, whilst these studies are supportive of the hypothesis that over expression of ABC transporters can protect insects against insecticides by protecting tissues few have directly demonstrated that insect ABC transporters can transport insecticides and only a small number of studies have considered the tissue distribution of these transporters. Notable exceptions to this are studies on the defensive mechanisms adopted by Lepidoptera. The tobacco hornworm protects itself from the toxic effects of nicotine in the diet via ABC transporters expressed in the blood brain barrier and malpighian tubules which reduce access to the targets in the central nervous system and accelerates excretion of nicotine (Murray et al. 1994; Gaertner et al. 1998). Similarly, the oleande hawk-moth protects itself from dietary cardenolides via ABC transporters in the blood brain barrier (Petschenka et al. 2013).

The *An. gambiae* ABC transporter family was originally described by Roth et al. (Roth et al. 2003) who identified 44 ABC transporters in the *An. gambiae* genome. This number was expanded to 52 in a comprehensive comparative genomics study of invertebrate ABC transporters (Dermauw & Van Leeuwen 2014). A more recent phylogenetic analysis of ABC transporters in three mosquito species identified 55 in *An. gambiae*, 69 in *Aedes aegypti* and 70 in *Culex quinquefasciatus* (Lu et al. 2016), although this gene tally includes some partial gene sequences that are likely pseudogenes.

In this study we provide an updated catalogue of the ABC transporters in *An. gambiae* and, via a meta analysis of multiple microarray datasets comparing gene expression in pyrethroid resistant and susceptible populations of this species, identify members of this gene family that are repeatedly found up-regulated in pyrethroid resistant populations. By phylogenetic comparisons to other Dipteran ABC gene families, reference to both an excellent recent reveiw on the ABCs in other arthropod species (Dermauw & Van Leeuwen 2014), and published transcriptomic studies (Pitts et al. 2011; Matthews et al. 2016; Gabrieli et al. 2014) and by conducting RT-PCR and immunolocalisation experiments on a subset of this gene family , we provide new insights into the tissue localisation and putative functions of members of this gene family.

**Results and Discussion**

*An updated catalogue of the ABC genes in An. gambiae*

Our manual annotation of the ABC family of *An. gambiae* identified 55 putative full-length members of this gene family. We cloned and sequenced full-length cDNAs to confirm the annotation of 13 members of the ABCG family (Table S1). The number of ABC genes is similar to *Homo sapiens* (48 genes) and *Drosophila melanogaster* (56 genes) but much reduced compared to the spider mite (*Tetrancychus urticae*, 103 genes) (Dermauw & Van Leeuwen 2014) and considerably less than predicted in other mosquito species (Lu et al. 2016). The 55 *An. gambiae* ABC transporters are listed in Table S2 and their phylogenetic relationship shown in Figure 1.

**The ABCA subfamily** consists of nine genes in *An. gambiae* (Figure 1).Six of these (AGAP006379, AGAP006380, AGAP007504, AGAP011518, AGAP012155 and AGAP012156) cluster with the insect specific ABCA clade, characterised by lineage specific expansions (Dermauw & Van Leeuwen 2014). Two pairs of ABCAs appear to be the results of relatively recent duplications, being found in tandem in the genome and sharing > 84 % similarity (AGAP012155 and AGAP012156: 84.8 % similarity, 63.3% identity; AGAP006379 and AGAP006380: 84.7 % similarity, 60.9% identity) although both have 1:1 orthologs in other mosquito species so this duplication presumably occurred prior to the divergence of these lineages (Figure S1). Transcription of this insect specific clade of ABCA genes is enriched in the midgut in *An. gambiae* (Figure 2, Table S1) with the exception of AGAP0011518 which is enriched in the female carcass (MozAtlas, (Baker et al. 2011)).

The remaining three ABCA genes all have clear orthologous relationships with other insect ABCAs (Figure S1 and Figure S1 in (Dermauw & Van Leeuwen 2014)). AGAP001523 clusters with FBgn0028539 from *D. melanogaster* and an expanded cluster of ABCA genes in the spider mite *T. urticae*. AGAP010416 is most closely related to the human ABCA genes A5, A6, A8, A9 and A10 which have suggested roles in lipid transport (Tarling et al. 2013). The ortholog of this ABC in *Ae. aegypti* (AAEL004331) is highly expressed in the brain ((Matthews et al. 2016), Table S1). The final *An. gambiae* ABCA gene, AGAP010582 is more distantly related to other ABCA genes within this class but clusters with a second group of human ABCA genes (ABCA1, 2, 4, 7, 12 and 14) which have been implicated in phospho and sphingo lipid export (Tarling et al. 2013); transcripts of this gene are enriched in the carcass (Figure 2) and the *Ae. aegypti* ortholog (AAEL018040) is very highly expressed in antennae, legs and other sensory tissues ((Matthews et al. 2016) Table S1).

**The ABCB subfamily** consists of full transporters (FTs) and half transporters (HTs). *An. gambiae* (and also *Cx. quinquefasciatus* and *Ae. aegypti*, (Lu et al. 2016)) contains just a single ABCB FT, AGAP005639, in contrast to the four FTs in this subfamily in *D. melanogaster* and in *H. sapiens*. The ABCB FTs in *D. melanogaster* are known as the *multidrug transporters* (mdr49, mdr50, mdr65 and FBgn0035695) based on their similarity to the mammalian ‘p-glycoprotein’ transporters implicated in resistance to a wide range of drug (Sarkadi et al. 2006). Both Mdr49 and Mdr65 have been implicated in DDT resistance in Drosophila (Gellatly et al. 2015; Strycharz et al. 2013; Seong et al. 2016).

There are four ABCB HTs in *An. gambiae* (AGAP006273, AGAP002717, AGAP002278 and AGAP006364) with alternative splice variants of AGAP006364 further expanding the diversity in this family. Each has a clear orthologous relationship to ABCB HTs from other insects and human ABCB6, 7, 8 or 10 respectively (Figure S1 and Figure 3 in (Dermauw & Van Leeuwen 2014)). The human ABCB HTs 7, 8 and 10 are localised to the mitochondria (Kiss et al. 2012) and we detected mitochondrial tagging sequences on all the *An. gambiae* orthologs of these genes. AGAP002278 is the ortholog of hABCB6, both of which lack mitochondria tags; the mosquito transcript is enriched in the midgut (Figure 2).

**The ABCC subfamily** has been linked with drug resistance in several species and frequently works in partnership with phase II conjugating enzymes such as glutathione S transferases or UDP glyosyl transferases, exporting the conjugated forms of the drugs (Homolya et al. 2003). Both *An. gambiae*, and *D. melanogaster* have 14 ABCC transporters and all are FTs. AGAP009835 is the ortholog of the Drosophila ‘multi drug resistance protein 1’ (MRP1 FBgn0032456) (Figure S1). Although the Drosophila gene is highly enriched in the midgut and malpighian tubules (Chintapalli et al. 2007) AGAP009835 is one of the few ABCC genes in *An. gambiae* that does not show enrichment in these tissues (Figure 2) but instead shows high expression in the testes (Baker et al. 2011); the ortholog in *Ae. aegypti* (AAEL004743) is similarly enriched in reproductive tissue (Matthews et al. 2016). The ABCC family is also involved in transport of ions and a wide range of endogenous substrates (Sarkadi et al. 2006). Two *An. gambiae* ABCCs (AGAP007917 and AGAP009799) have clear orthologous relationships across arthropods with the latter being the ortholog of the human sulfonylurea receptor; this ABCC is enriched in the head in *An. gambiae* (Table S1).

Lineage specific expansions are evident in the ABCC subfamily and within *An. gambiae* local duplications have resulted in a cluster of three sequentially arranged ABCC genes on chromosome 2R, division 19D and a further cluster of four ABCC genes on chromosome 3R, division 30D (Table S1).

**The ABCD subfamily** contains two genes in *An. gambiae,* AGAP002071 and AGAP000440. Both are HTs and belong to highly conserved orthologous groups in insects and humans; AGAP002071 is related to human ABCD1/2 and AGAP000440 is related to hABCD3 (see Figure 5 in (Dermauw & Van Leeuwen 2014)). The human ABCD transporters are located in the peroxisome and involved in transport of long and branched acyl coA molecules (Baker et al. 2015). Both *An. gambiae* ABCD genes are highly enriched in the midgut (Figure 2).

**The ABCDE and ABCDF subfamilies** lack transmembrane domains and therefore do not play a role in transport. There is a single ABCE gene in *An. gambiae* (AGAP002182) which shares 86 % amino acid identity with its Drosophila ortholog *pixie*. The human ortholog, also has a conserved function in ribosome biogenesis and translation regulation (Chen et al. 2006). This ABC is very highly and ubiquitously expressed (ranked 2nd amongst ABC transcripts in non-blood females ((Pitts et al. 2011) Table S1), consistent with a housekeeping role. Silencing the single ABCE gene in *Tribolium castaneum* larvae or adults resulted in 100 % mortality (Broehan et al. 2013).

The ABCF proteins are also involved in regulation of translation (Kerr 2004). Three ABCFs are present in *An. gambiae*: AGAP012249 (ortholog of hABCF1), AGAP002693 (ortholog of hABCF2) and AGAP012005 (ortholog of hABCF3). All have 1:1 orthologs in other mosquito species (Figure S2). The ABCFs are amongst the most highly expressed transporter genes in *An. gambiae* females ((Pitts et al. 2011), Table S2).

**The ABCH subfamily** in *An gambiae,* in common with many insect species, contains three genes, all encoding HTs. This sub family is not found in mammals or plants. Their role in insects is poorly understood although silencing of one member of this family in Tribolium adults resulted in reduced fertility and fecundity and also a reduction in lipid deposition at the cuticle suggesting a possible role in transport of the lipids to the cuticle (Broehan et al. 2013). The mosquito ABCH genes are highly enriched in sensory tissue ((Pitts et al. 2011) Table S2).

**The ABCG subfamily** is the largest of the 8 subfamilies in *An. gambiae* containing 18 putative half transporters which can form homo or heterodimers. Insect ABCG HTs are well known for their role in transport of eye pigments (Ewart & Howells 1998). Disruption of the genes *white, scarlet and brown* leads to eye colour mutants in Drosophila but also other neural phenotypes (Borycz et al. 2008). These HTs form heterodimers with *white* dimerising with either brown or scarlet. There are clear orthologs of *white* and *brown* in *An. gambiae* (AGAP000553 and AGAP007655)and there are two mosquito paralogs with orthology to Drosophila *scarlet* (AGAP00506 and AGAP01333) (Figure S3).

One to one orthologous relationships can be detected for a further six *An. gambiae: D. melanogaster* ABCG HTs including *atet* (originally named ABC protein expressed in trachea but recently shown to be involved in ecdysone transport in the prothoracic glands (Yamanaka et al. 2015)), a highly conserved protein found in several arthropod taxa (Figure S3). Interestingly, no ortholog of the *D melanogaster* 20-OH ecdysone (20E) induced ABC transporter, E23 (FBgn0020445) is present in *An. gambiae* or other mosquito species(Hock et al. 2000) (Figure S3) but three ABCG genes (AGAP009463, 9471 and 9472) were highly up-regulated in ovaries following 20-E injection into virgin females (Gabrieli et al. 2014) (Table S2).

In addition to the conserved members of this subfamily, there is evidence of extensive lineage specific duplication of arthropod ABCGs (Qi et al. 2016; Dermauw & Van Leeuwen 2014). Within *An. gambiae* 8 genes are clustered on chromosome 3R, division 34B, the majority of which are highly expressed in the legs of the mosquito (see below). Previous microarray studies have also found enrichment of multiple ABCGs in the abdomen integument and carcass (Figure 2, Table S1 (Baker et al. 2011)).

Numerous physiological functions have been demonstrated for mammalian ABCGs with several implicated in the transport of lipids and steroids (Wang et al. 2004; Klucken et al. 2000). In insects and plants ABCG transporters have also been shown to transport lipids from the epidermis to the cuticle to prevent water loss (McFarlane et al. 2010; Broehan et al. 2013). Mining of mosquito RNAseq data revealed that several *An. gambiae* and *Ae. aegypti* ABCG genes were highly expressed in sensory tissues including the antennae, palps and legs (Pitts et al. 2011; Matthews et al. 2016) (Table S2). We were interested in exploring the expression of this subfamily further. We therefore performed qPCR on dissected tissue from adult female mosquitoes of two strains focusing on tissues associated with detoxification and excretion (midgut and malpighian tubules) and structures involved in synthesis and transport of the cuticular components (abdomen integument, including fat body, oenocytes, and cuticle) and legs (which consist primarily of muscle, nervous tissue and cuticle). Ten of the 12 ABCG genes analysed by qPCR, including all 8 of the genes sequentially arranged on 3R division 34B, were enriched in legs compared to the whole bodies (Figure 3). This enrichment was generally more pronounced in the insecticide resistant Tiassale strain and was particularly notable for AGAP009467, AGAP009471 and AGAP009472 which each showed > 30-fold higher expression in the legs versus the whole body in this strain (Figure 3). Only two of the ABCG genes (AGAP009466 and AGAP02879) were enriched in the midgut in both strains and only one (AGAP009464) was enriched in the malpighian tubules. This expression pattern varies markedly from other ABC families in *An. gambiae* which typically show enrichment in the digestive tissue (Figure 2 and Table S1).

RNA extracted from mosquito legs would be expected to be enriched in genes expressed in cuticular tissues given the predominance of cuticle in the appendages. Thus the localisation of ABCG genes in the mosquito legs, is supportive of a role for this subfamily in transporting lipids to the mosquito cuticle as has been functionally confirmed for members of this subfamily in the beetle *T. castaneum* (Broehan et al. 2013).

We attempted to verify the tissue enrichment by immunostaining with antibodies raised against two ABCG genes. AGAP009850 is one of the most highly expressed ABC transporters in non-blood fed females ((Pitts et al. 2011), Table S1). Previous microarray studies and our own qPCR data found AGA009850 highly enriched in the abdomen integument and legs. Although we were unable to achieve reliable leg staining due to technical difficulty with cryosectioning of this body part, AGAP009850 antisera did localise to a layer of cells underlying the abdominal cuticle. Whole mount staining of the abdomen carcass suggested that these cells were pericardial cells (Figure 4), part of the reticulosystem in insects that import and export compounds to/from the hemolymph (Fife et al. 1987). This location is in agreement with the enrichment of the *Drosophila* ortholog in the heart (and fatbody) (Chintapalli et al. 2007). Immunostaining with antibodies raised against AGAP009466 was inconclusive; this gene is expressed at relatively low levels in whole adult females relative to other ABC genes ((Pitts et al. 2011), Table S1) and the only clear signal we could detect was to the nerve cord, although this requires further confirmation.

*ABC genes differentially expressed in insecticide resistance in An. gambiae*

Elevated expression of ABC genes has previously been reported in insecticide resistant strains of *An. gambiae* (Jones et al. 2012; Fossog Tene et al. 2013). Whilst recognising that differential expression in resistant populations is only a weak indicator of a role for ABC transporters in conferring the resistant phenotype, identification of particular transporters whose expression is consistently correlated with the resistance phenotype in independent experiments and populations would suggest that these genes were worthy of further investigation. Data on ABC expression levels were therefore extracted from 14 independent microarray studies, spanning four years of mosquito collections across five African countries. All the data sets compared expression in field collected pyrethroid resistant populations with matched susceptible strains; two members of the *An. gambiae* complex were included, 11 experiments with *An. coluzzii* and three experiments with *An. gambiae s.s.* Further details of the mosquito populations are provided in Table S3.

The results identify multiple ABC transporters that are significantly (p <0.05) differentially expressed in pyrethroid resistant populations (Figure 5) with 11 of the 14 populations showing differential expression of at least one third of the ABC transporters and in two populations (Tiassale 2010 and VK7 2011) 36 transporters were differentially expressed. Comparing across experiments, four ABCs were up-regulated in 9 or more of the 14 populations. The most striking result was for AGAP006364 (ABCB4) which is up-regulated in 11 populations (and down regulated in none). The ortholog of this ABC in *Ae* aegypti (AAEL006717) is also up-regulated in pyrethroid resistant populations from the Caribbean (Bariami et al. 2012). Interestingly these ABCBs are not homologues of the ABCB multidrug transporters in Drosophila but are instead both half transporters, with mitochondrial tag sequences. The three other genes most frequently up-regulated belonged to the ABCA family (AGAP011518, up-regulated in 10 populations, down-regulated in one), ABCC family (AGAP007917, up in 9, down in one) and ABCF family (AGAP002693, up in 9, down in one). The orthologs of the ABCC MRP(AGAP009835) and the ABCB MDR (AGAP005639) were up-regulated in some populations but did not stand out as being particularly strongly associated with the pyrethroid resistance phenotype (Figure 5)*.* Two genes were consistently down regulated in the pyrethroid resistant populations; the ABCA gene AGAP012156 was down regulated in 10 populations (although up-regulated in two) and the ABCC gene AGAP008436 was down regulated in nine (and up in one).

Studies on the role of ABCs in insecticide resistance have often focused on two putative modes of action: protection of the target sites in the nervous system and accelerated clearance via the excretory system. The majority of the commonly used insecticides are nerve toxins and thus must cross the blood brain barrier (BBB) to exert their toxic effect. In mammals, ABC proteins in the BBB protect the nervous tissue from xenobiotics and evidence suggests they play a similar role in insects. ABCs from the ABCB and ABCG families transporters are present in the BBB of human and insects (Miller 2015; DeSalvo et al. 2014; Murray et al. 1994; Petschenka et al. 2013) and elevated expression of ABC proteins has been observed in the BBB of an avermectin resistant strain of *D melanogaster* (Luo et al. 2013). Hence ABC proteins may play an important role in reducing exposure to insecticides. Preliminary RNAseq data from the ventral nerve cord of *An. gambiae* did detect transcripts for multiple ABC transporters belonging to the ABCA, B, D, F and G family (Pignatelli, Lycett, unpublished data) but further replicates, followed by immunolocalisation studies are needed to identify transporters that may be involved in reducing the amount of pyrethroid insecticide reaching its target site in the nerve axons.

ABC transporters may also protect against insecticide exposure by accelerating their rate of excretion from the body. Transcripts for many of the *An. gambiae* ABC genes are enriched in the malpighian tubules and midguts (Figure 2) and the four ABCB p-glycoprotein paralogs in *D melanogaster* are all found in adult midgut and/or malpighian tubules. A transcriptomic analysis of the leaf beetle *Chysomela populii* also found a large number of ABC genes, predominately belonging to the B, C and G subfamilies enriched in the malpighian tubules and or guts (Strauss et al. 2014). Two of the four ABC genes most consistently over expressed in pyrethroid resistant populations are enriched in the malpighian tubules (ABCB4: ABAP006363) or malpighian tubules plus midguts (ABCC12: AGAP007917). Nevertheless the functional significance of overexpression of ABC transporters in excretory tissues for insecticide toxicology has not been explored.

ABC transporters may also act in less direct ways to protect insects from the toxic effects of insecticides. In plants and the flour beetle ABCG transporters have been shown to play an important role in delivering cuticular lipids to epidermis (Broehan et al. 2013; McFarlane et al. 2010). Elevated expression of ABCG genes with similar functions in the mosquito legs and/or abdominal cuticle could conceivably play a role in modifying the permeability of the insect cuticle to insecticides. Pyrethroid resistant populations of *An. gambiae* have been shown to have elevated levels of cuticular hydrocarbons (CHC) ((Balabanidou et al. 2016) and V Balabanidou unpublished data). Furthermore recent microarray data from a highly pyrethroid resistant population from Tiefora in Burkina Faso has identified the CHC synthesis pathway as one of the key pathways elevated in resistant strains (N Grisales, V Ingham and H Ranson, unpublished data); interestingly several of the ABCG genes that we show to be enriched in legs in this study are also overexpressed in this population (Figure 5). Could these ABC transporters be contributing to resistance by accelerating the transport of lipids to the cuticular surface thus impeding penetration of insecticides? Or do they perhaps play a more direct role in insecticide transport by preventing insecticide uptake by the epidermal cells underlying the cuticle? Alternatively the high expression in the legs could be indicative of expression in the nervous system, not the cuticle, and the ABCG transporters may be protecting the neuronal cells from contact with the nerve toxins. Providing further evidence to support any of these hypotheses will require more detailed studies such as immunolocalisation studies and/or functional characterisation of the transporter proteins. Unfortunately the ability of ABCG HTs to form homo or heterodimers adds an extra layer of complexity to studies on this subfamily of ABC transporters.

**Conclusion**

Although much has been written about the role of ABC transporters in conferring resistance to insecticides, in many cases the evidence is largely limited to correlations with whole body gene expression, which may miss significant hits and moreover gives little insight into the mechanisms by which these transporters might contribute to the resistance phenotype. In this manuscript, we have narrowed down the tissue specific expression of a subset of ABC transporters in *An. gambiae* mosquitoes. The majority of insecticides used in public health are contact nerve poisons and so must cross the cuticle and neuronal blood brain barriers to exert their affect. Therefore efflux mechanisms conferring resistance may be most potent when expressed in these compartments and the endogenous substrates of ABC transporters expressed in the these tissues, and their ability to transport insecticides warrants further investigation.

**Experimental Procedures**

*Annotation and phylogeny of ABC transporters*

Members of the ABC transporter family were identified in the *An. gambiae* genome by using the BLAST function on VectorBase with each of the ABC genes previously identified in this species (Roth et al. 2003) as queries. The retrieved sequences were manually annotated where necessary to correct automated predictions of transcripts in earlier versions of VectorBase. These errors have now been corrected in release P4.6 and the ABC nomenclature adopted in this manuscript matches the VectorBase annotation (with the exception of AGAP010582 which is named ABCE2 in VectorBase but our analysis, and that of (Dermauw & Van Leeuwen 2014), assigns it to the ABCA subfamily). Derived amino acid sequences for each full-length transcript are given in Supplementary File S1. These were aligned using CLUSTAL Omega (Sievers & Higgins 2014) and phylogenetic trees generated using the neighbour-joining algorithm in MEGA7 (Kumar et al. 2016). *An. gambiae* protein sequences were aligned with sequences from *D melanogaster*, *Ae aegypti* and *Culex quinguefasciatus* and neighbour joining trees generated as described above. Further putative orthologs of the *An. gambiae* genes were identified by reference to previous phylogenetic analyses (; Dermauw & Van Leeuwen 2014)

*Identification of ABC genes differentially expressed in pyrethroid resistant populations.*

Fourteen data sets from microarray experiments comparing the transcriptome of pyrethroid resistant and susceptible populations were extracted from Array Express. The resistant mosquitoes were collected from Cote d’Ivoire, Burkina Faso, Equatorial Guinea, Togo and Zambia between 2010 and 2014. All hybridisations were made to the Agilent whole genome 8 x 15k *An. gambiae* array (ArrayExpress accession number A-MEXP-2211). Further information on these data sets is provided in Table S2. All arrays were analysed using the R package limma, which fits linear models to normalised data (Smyth 2004). Within-array normalisation was carried out by Loess and between array normalisation by Aquantile (Smyth & Speed 2003). ABC transporter expression was extracted and the non-significant probes excluded from further analysis. The resultant probe expression was averaged for each transcript in the case of multiple probes, leaving one fold change data point per unique transcript.

*Expression profiling of ABC genes*

Initially, microarray data from a previously published study comparing gene expression across mosquito body parts (Ingham et al. 2014) was used to identify ABC transcripts that were enriched in either the midgut, malpighian tubules, abdomen integument or remaining body structures in the insecticide susceptible Ngousso strain of *An. gambiae.* Subsequently, qPCR was used to further explore the tissue enrichment of 12 members of the ABCG subfamily. The body parts were dissected as described previously (Ingham et al. 2014) with the exception that RNA was also extracted from the mosquito legs (the earlier study had included legs in ‘remaining body structures’). First the legs were removed from adult females and then the digestive and reproductive tract removed and the midgut and malpighian tubules were isolated; the abdomen integument consisted of the cuticle and underlying cells including the fat body. The rest of the body was discarded. Matched age (3-5 day old) whole females were used as a control.

Total RNA was extracted from adult females and isolated body parts from the Ngousso and Tiassale strain of *An gambiae* using the PicoPure RNA isolation kit (Applied Biosystems). The Tiassale strain originates from Cote d’Ivoire and is resistant to a wide range of insecticides (see (Edi et al. 2012) for further details). The insecticide susceptible Ngousso strain is from Cameroon (Harris et al. 2010). Extractions were performed on whole mosquitoes (7 mosquitoes per replicate) or dissected body parts: midguts and malpighian tubules (25 mosquitoes), abdomen integument (10 mosquitoes) and legs (35 mosquitoes). Four biological replicates were prepared for each body part. cDNA was synthesised using SuperScript III Reverse Transcriptase (Invitrogen).

Primers were designed using Primer- BLAST tool (NCBI: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) against the *An. gambiae* PEST sequence (Table S3). The efficiency and specificity of the primer sets were assessed by running a standard curve over a five-fold serial dilution. Between 200 and 1000ng of RNA was reverse transcribed as described above. All four biological replicates for each body part were run in triplicate using 2ng of input cDNA, 2xSYBR Brilliant III (Agilent Technologies) and 300 nM of the forward and reverse primer on the Mx3005P qPCR system (Agilent Technologies). The thermal profile for each reaction was 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 10 s. The qPCR data were analysed according to the ddCt method relative to the average of two housekeeping genes encoding S7 (AGAP010592), and elongation factor (AGAP005128). The qPCR efficiency of each primer set was incorporated into the reaction. ∆∆Ct was calculated using standard protocol (Schmittgen & Livak 2008).

**Raising of antibodies**

Rabbit polyclonal antibodies targeting a recombinant peptide of AGAP009850 were made and affinity purified by Davids Biotechnologie GmbH (Germany). The sequence encoding the peptide: HTRKLVNAINGKKDIRSELDFPAQKNKKNENAANGNANLKVNYERMNGGANKYADNLNLGGNGLLPPSMVNDIAKETETIKIAVEPDREPEVNSALLPVEGATDIDHSPERYPTSEFH was cloned on pET16b vector as an NdeI/BamHI fragment, under an IPTG-inducible promoter, using the following primers (Forward: 5’-CATATGCATACGCGCAAGCTGGTGAACGCGATCG-3’ and Reverse: 5’-GGATCCTCAATGGAACTCCGACGTCGGATATCGCTCC-3’). The resultant peptide was expressed in bacteria with a 6xHis tag at the N-terminus and affinity purified to homogeneity by Ni-NTA2+ affinity chromatography. The purified peptide was used as an antigen to develop antibodies in rabbits.

**Immunofluorescence and confocal microscopy**

Three to five days old sugar fed female mosquitoes from the Ngousso strain were fixed in cold solution of 4% formaldehyde (methanol free, Thermo scientific) in phosphate-buffered saline (PBS) for 4 h, cryo-protected in 30% sucrose/PBS at 4o C for 12 h, immobilised in Optimal Cutting Temperature O.C.T. (Tissue-Tek, SAKURA) and stored at -80o C until use. Immunofluorescence analysis, followed by confocal microscopy, was performed on longitudinal sections of the frozen pre-fixed mosquito specimens as described in Ingham *et al* 2014. The anti-9850 was used in 1/500 dilution. For the whole mount abdomen immunostaining, dissected abdominal walls from female mosquitoes were fixed for 30 min at room temperature in 4% formaldehyde (methanol free, Thermo scientific) in phosphate-buffered saline (PBS), supplemented with 2 mM MgSO4 and 1 mM EGTA, washed for 5 min with PBS, followed by a methanol wash, strictly for 2 min. After methanol, the tissues were washed again with PBS and then blocked for 2 h in blocking solution (1% BSA, 0.1 % TRITON X-100 in PBS). Then the tissues were stained with rabbit primary antibody in 1/500 dilution in the blocking serum, followed by goat anti-rabbit (Alexa 488, Molecular probes, 1/1000), that gave the green color. Finally, nucleic acid was stained using TO-PRO-3 Iodide (Molecular Probes 1/1000). ictures Images  were obtained on a Leica TCS-NT Laser Scanning microscope with LAS AF Lite(Leica-TCS MP5) software.

**Acknowledgements**

The research leading to these results was supported by the European Union Seventh Framework Programme FP7 (2007–2013) under grant agreement no 265660 AvecNet.

**References**

Baker, A. et al., 2015. Peroxisomal ABC transporters: functions and mechanism. *Biochemical Society Transactions*, 43(5), pp.959–965.

Baker, D.A. et al., 2011. A comprehensive gene expression atlas of sex- and tissue-specificity in the malaria vector, Anopheles gambiae. *BMC Genomics*, 12(1), p.296.

Balabanidou, V. et al., 2016. Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, 113(33), pp.9268–9273.

Bariami, V. et al., 2012. Gene amplification, abc transporters and cytochrome p450s: Unraveling the molecular basis of pyrethroid resistance in the dengue vector, aedes aegypti. *PLoS Neglected Tropical Diseases*, 6(6).

Borycz, J. et al., 2008. 1999lDrosophila ABC transporter mutants white, brown and scarlet have altered contents and distribution of biogenic amines in the brain. *Journal of Experimental Biology*, 211(21), pp.3454–3466.

Broehan, G. et al., 2013. Functional analysis of the ATP-binding cassette ( ABC ) transporter gene family of Tribolium castaneum Functional analysis of the ATP-binding cassette ( ABC ) transporter gene family of Tribolium castaneum.

Chen, Z.Q. et al., 2006. The essential vertebrate ABCE1 protein interacts with eukaryotic initiation factors. *Journal of Biological Chemistry*, 281(11), pp.7452–7457.

Chintapalli, V.R., Wang, J. & Dow, J.A.T., 2007. Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nature Genetics*, 39(6), pp.715–720.

Dean, M., Rzhetsky, A. & Allikmets, R., 2001. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Research*, 11(7), pp.1156–1166.

Dermauw, W. & Van Leeuwen, T., 2014. The ABC gene family in arthropods: Comparative genomics and role ininsecticide transport and resistance. *Insect Biochemistry and Molecular Biology*, 45(1), pp.89–110.

DeSalvo, M.K. et al., 2014. The Drosophila surface glia transcriptome: Evolutionary conserved blood-brain barrier processes. *Frontiers in Neuroscience*, 8(OCT).

Donnelly, M.J. et al., 2009. Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? *Trends in Parasitology*, 25(5), pp.213–219.

Donnelly, M.J., Isaacs, A.T. & Weetman, D., 2016. Identification, Validation, and Application of Molecular Diagnostics for Insecticide Resistance in Malaria Vectors. *Trends in Parasitology*, 32(3), pp.197–206.

Edi, C.V.A. et al., 2012. Multiple-insecticide resistance in Anopheles gambiae mosquitoes, Southern Cote d’Ivoire. *Emerging Infectious Diseases*, 18(9), pp.1508–1512.

Epis, S. et al., 2014. ABC transporters are involved in defense against permethrin insecticide in the malaria vector Anopheles stephensi. *Parasites & Vectors*, 7(1), p.349.

Ewart, G.D. & Howells, A.J., 1998. ABC Transporters: Biochemical, Cellular, and Molecular Aspects. *Methods in Enzymology*, 292(1980), pp.213–224.

Fife, H.G., Palli, S.R. & Locke, M., 1987. A function for pericardial cells in an insect. *Insect Biochemistry*, 17(6), pp.829–840.

Figueira-Mansur, J. et al., 2013. Silencing of P-glycoprotein increases mortality in temephos-treated Aedes aegypti larvae. *Insect Molecular Biology*, 22(6), pp.648–658.

Fossog Tene, B. et al., 2013. Resistance to DDT in an Urban Setting: Common Mechanisms Implicated in Both M and S Forms of Anopheles gambiae in the City of Yaoundé Cameroon. *PLoS ONE*, 8(4).

Gabrieli, P. et al., 2014. Sexual transfer of the steroid hormone 20E induces the postmating switch in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, 111(46), pp.16353–16358.

Gaertner, L.S., Murray, C.L. & Morris, C.E., 1998. Transepithelial transport of nicotine and vinblastine in isolated malpighian tubules of the tobacco hornworm (Manduca sexta) suggests a P-glycoprotein-like mechanism. *The Journal of experimental biology*, 201(Pt 18), pp.2637–45.

Gellatly, K.J. et al., 2015. RNAi validation of resistance genes and their interactions in the highly DDT-resistant 91-R strain of Drosophila melanogaster. *Pesticide Biochemistry and Physiology*, 121, pp.107–115.

Harris, C. et al., 2010. Polymorphisms in Anopheles gambiae immune genes associated with natural resistance to plasmodium falciparum. *PLoS Pathogens*, 6(9).

Hemingway, J. et al., 2016. Averting a malaria disaster: Will insecticide resistance derail malaria control? *The Lancet*, 387(10029), pp.1785–1788.

Hock, T. et al., 2000. The E23 early gene of Drosophila encodes an ecdysone-inducible ATP-binding cassette transporter capable of repressing ecdysone-mediated gene activation. *Proceedings of the National Academy of Sciences of the United States of America*, 97(17), pp.9519–9524.

Homolya, L., Váradi, A. & Sarkadi, B., 2003. Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate. *BioFactors (Oxford, England)*, 17(1–4), pp.103–114.

Ingham, V.A. et al., 2014. Dissecting the organ specificity of insecticide resistance candidate genes in Anopheles gambiae: known and novel candidate genes. *BMC genomics*, 15(1), p.1018.

Jones, C.M. et al., 2012. Additional Selection for Insecticide Resistance in Urban Malaria Vectors: DDT Resistance in Anopheles arabiensis from Bobo-Dioulasso, Burkina Faso. *PLoS ONE*, 7(9).

Kerr, I.D., 2004. Sequence analysis of twin ATP binding cassette proteins involved in translational control, antibiotic resistance, and ribonuclease L inhibition. *Biochemical and Biophysical Research Communications*, 315(1), pp.166–173.

Kiss, K. et al., 2012. Shifting the paradigm: The putative mitochondrial protein ABCB6 resides in the lysosomes of cells and in the plasma membrane of erythrocytes. *PLoS ONE*, 7(5).

Klucken, J. et al., 2000. ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proceedings of the National Academy of Sciences of the United States of America*, 97(2), pp.817–22.

Kumar, S., Stecher, G. & Tamura, K., 2016. MEGA7 : Molecular Evolutionary Genetics Analysis Version 7 . 0 for Bigger Datasets Brief communication. , 33(7), pp.1870–1874.

Lanning, C.L., Ayad, H.M. & Abou-Donia, M.B., 1996. P-glycoprotein involvement in cuticular penetration of [14C]thiodicarb in resistant tobacco budworms. *Toxicology Letters*, 85(3), pp.127–133.

Lima, E.P., Goulart, M.O. & Rolim Neto, M.L., 2014. Evaluation of the role of ATP-binding cassette transporters as a defence mechanism against temephos in populations of Aedes aegypti. *Mem Inst Oswaldo Cruz*, 109(7), pp.964–966.

Lu, H., Xu, Y. & Cui, F., 2016. Phylogenetic analysis of the ATP-binding cassette transporter family in three mosquito species. *Pesticide Biochemistry and Physiology*, 132(July 2016), pp.118–124.

Luo, L., Sun, Y.J. & Wu, Y.J., 2013. Abamectin resistance in drosophila is related to increased expression of P-glycoprotein via the dEGFR and dAkt pathways. *Insect Biochemistry and Molecular Biology*, 43(8), pp.627–634.

Mackenzie, S.M. et al., 1999. Mutations in the white gene of Drosophila melanogaster affecting ABC transporters that determine eye colouration. *Biochimica et Biophysica Acta - Biomembranes*, 1419(2), pp.173–185.

Matthews, B.J. et al., 2016. The neurotranscriptome of the Aedes aegypti mosquito. *BMC Genomics*, pp.1–20.

McFarlane, H.E. et al., 2010. *Arabidopsis* ABCG Transporters, Which Are Required for Export of Diverse Cuticular Lipids, Dimerize in Different Combinations. *The Plant Cell*, 22(9), pp.3066–3075.

Miller, D.S., 2015. Regulation of ABC transporters at the blood-brain barrier. *Clinical pharmacology and therapeutics*, 97(4), pp.395–403.

Murray, C.L. et al., 1994. A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. *Journal of Neurobiology*, 25(1), pp.23–34.

Petschenka, G. et al., 2013. Functional evidence for physiological mechanisms to circumvent neurotoxicity of cardenolides in an adapted and a non-adapted hawk-moth species. *Proceedings of the Royal Society B: Biological Sciences*, 280(1759), p.20123089.

Pitts, R.J. et al., 2011. Transcriptome profiling of chemosensory appendages in the malaria vector Anopheles gambiae reveals tissue- and sex-specific signatures of odor coding. *BMC Genomics*, 12(1), p.271.

Qi, W. et al., 2016. Characterization and expression profiling of ATP-binding cassette transporter genes in the diamondback moth , Plutella xylostella ( L .). *BMC Genomics*, pp.1–18.

Ranson, H. & Lissenden, N., 2016. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends in Parasitology*, 32(3), pp.187–196.

Roth, C.W. et al., 2003. Identification of the Anopheles gambiae ATP-binding cassette transporter superfamily genes. *Molecules and cells*, 15(2), pp.150–8.

Sarkadi, B. et al., 2006. Human Multidrug Resistance ABCB and ABCG Transporters: Participation in a Chemoimmunity Defense System. *Physiological Reviews*, 86(4), pp.1179–1236.

Schmittgen, T.D. & Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, 3(6), pp.1101–1108.

Seong, K.M. et al., 2016. Splice form variant and amino acid changes in MDR49 confers DDT resistance in transgenic Drosophila. *Scientific Reports*, 6, p.23355.

Sievers, F. & Higgins, D.G., 2014. Clustal omega, accurate alignment of very large numbers of sequences. *Methods in Molecular Biology*, 1079, pp.105–116.

Smyth, G.K., 2004. Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments. *Statistical Applications in Genetics and Molecular Biology*, 3(1), pp.1–25.

Smyth, G.K. & Speed, T., 2003. Normalization of cDNA microarray data. *Methods (San Diego, Calif.)*, 31(4), pp.265–273.

Strauss, A.S. et al., 2014. Tissue-specific transcript profiling for ABC transporters in the sequestering larvae of the phytophagous leaf beetle Chrysomela populi. *PLoS ONE*, 9(6).

Strycharz, J.P. et al., 2013. Resistance in the highly DDT-resistant 91-R strain of Drosophila melanogaster involves decreased penetration, increased metabolism, and direct excretion. *Pesticide Biochemistry and Physiology*, 107(2), pp.207–217.

Tarling, E.J., de Aguiar Vallim, T.Q. & Edwards, P.A., 2013. Role of ABC transporters in lipid transport and human disease. *Trends Endocrinol Metab*, 24(7), pp.342–50.

Wang, L. et al., 2013. Mutation of a novel ABC transporter gene is responsible for the failure to incorporate uric acid in the epidermis of ok mutants of the silkworm, bombyx mori. *Insect Biochemistry and Molecular Biology*, 43(7), pp.562–571.

Wang, N. et al., 2004. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proceedings of the National Academy of Sciences*, 101(26), pp.9774–9779.

Yamanaka, N., Marque, G. & Connor, M.B.O., 2015. Vesicle-Mediated Steroid Hormone Secretion in Drosophila melanogaster Article Vesicle-Mediated Steroid Hormone Secretion in Drosophila melanogaster. , pp.907–919.

Figure Legends

Figure 1: Unrooted phylogenetic tree of *Anopheles* *gambiae* ABC proteins. Amino acid sequences of the 55 putative ABC proteins were aligned and the Neighbour-Joining method used to construct a phylogenetic tree in MEGA7 (Kumar et al. 2016). Numbers at nodes represent percentage bootstrap support from 500 replicates. The ABC classes are shaded in different colours (ABCA red, ABCB green, ABCC blue, ABCD black, ABCE yellow, ABCF purple, ABCG orange, ABCH grey).

Figure 2: Tissue enrichment of ABC genes in the insecticide susceptible Ngousso strain. Genes showing significant enrichment (p ≤ 0.05) in specific body parts from a previously published microarray experiment are shown here, clustered by ABC subfamily. The colour bar represents the (log2) fold change compared to the whole organism. See (Ingham et al. 2014) for further details of the experimental design. ABC subfamilies are represented by the same colours as in Figure 1. Genes without any significant enrichment in any of the tissues are omitted from the figure.

Figure 3: Tissue enrichment of ABCG transcripts in two *Anopheles gambiae* strains:resistant *(*Tiassale strain, shown in blue) and susceptible (Ngousso strain in orange) to pyrethroids. Results show normalised expression in each body part relative to whole female mosquitoes. Error bars show standard deviations.

Figure 4: Figure 4: Immuno-localization of *Anopheles gambiae* ABCG transporter AGAP009850. A) Longitudinal cryo-sections from mosquito specimens were immunostained with -9850 specific antibodies (left column, green color). Cell nuclei were stained red with TOPRO (middle column). Merged immunohistochemical images (9850 and nuclei) appear in the right column. Scale bars: 10 m. B) Whole mount staining using -9850 (green color) and ToPRO (red color), in the dorsal side of abdominal walls, showing localization of AGAP009850 to the characteristic Pericardial Cells (PC). Binucleate PCs are marked with 2 white arrows. Scale bars: 10 m. **C.** Control pre-immune serum was tested in parallel with -9850 on abdominal walls and confocal images are presented. PCs are depicted with white arrows (OR the boundaries of PCs are drawn with dotted lines) and no fluorescent signal is detected. Scale bars: 10 m.

Figure 5: Heatmap showing differential expression of ABC genes between insecticide resistant and susceptible strains of *An gambiae* sl. Genes expressed at significantly (p= <0.05) higher levels in the resistant populations are shown in green, those higher in the susceptible strains are shown in red. Further details of the datasets, including ArrayExpress accession numbers are included in Table S2.

**Supplementary Figures and Tables**

Table S1: Amino acid sequences of Anopheles gambiae ABC transporters. Sequences shown in blue were obtained from sequencing cDNA from the Kisumu strain. The remaining sequences were downloaded from VectorBase and are included here for ease of reference.

Table S2: Summary of the *Anopheles gambiae* ABC gene family including chromosomal location, predicted length of translated protein and expression data in different mosquito tissues (Pitts et al. 2011; Gabrieli et al. 2014; Ingham et al. 2014; Baker et al. 2011). The *Aedes aegypti* orthologs (from (Lu et al. 2016) are also included along with RNAseq data on the expression of these genes in sensory tissue (Matthews et al. 2016)

Table S3: Details of the microarray experiments used to generate Figure 5.

Table S4: Primer sequences used in qPCR of *Anopheles gambiae* ABCG subfamily.

Figure S1: Phylogenetic Tree of the ABCA, ABCB and ABCC subfamilies in mosquitoes. Amino acid sequences of ABCG genes from *Anopheles gambiae* (circles), *Aedes aegypti* (triangles)*, Culex quinquefasciatus* (squares)and *Drosophila melanogaster* (diamonds) were aligned using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (50 replicates) are shown next to the branches. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016) .

Figure S2: Phylogenetic Tree of ABCD, ABCE and ABCF subfamilies in mosquitoes. See legend to Figure S1 for further details.

Figure S3: Phylogenetic Tree of the ABCG subfamily in mosquitoes. See legend to Figure S1 for further details. Note that exons from CPIJ002198 and CPIJ002199 were merged to form a full length ABCG protein (labelled CPIJ020198\_9\*).