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The *Anopheles gambiae* ATP-binding cassette transporter family: phylogenetic analysis and tissue localization provide clues on function and role in insecticide resistance

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Abstract

The role of ATP-binding cassette (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 orthologues 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), localized this transporter to the pericardial cells. These data will help prioritize members of this gene family for further localization 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.

Keywords: insecticide resistance, ABC transporters, *Anopheles gambiae*.

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, 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 metabolize insecticides confirmed (Ingham *et al.*, 2014).

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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 that 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 nontransport 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) 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 eight subfamilies (A–H; Dermauw & Van Leeuwen, 2014). ABC proteins have been clearly linked to drug resistance in mammals notably the ABCB multidrug resistant (MDR) proteins or p-glycoproteins, ABCC multidrug resistance associated proteins (MRPs) and the ABCG breast cancer resistance protein (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 synergizes the activity of insecticides in some insect species (Figueira-Mansur *et al*., 2013; Epis *et al*., 2014; Lima *et al*., 2014). 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 (BBB) and Malpighian tubules that reduce access to the targets in the central nervous system and accelerate excretion of nicotine (Murray *et al*., 1994; Gaertner *et al*., 1998). Similarly, the oleander hawk-moth protects itself from dietary cardenolides via ABC transporters in the BBB (Petschenka *et al*., 2013).

The *An. gambiae* ABC transporter family was originally described by 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 probably 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 review on the ABCs in other arthropod species (Dermauw & Van Leeuwen, 2014), and published transcriptomic studies (Pitts *et al*., 2011; Gabrieli *et al*., 2014; Matthews *et al*., 2016), and by conducting quantitative PCR (qPCR) and immunolocalization experiments on a subset of this gene family, we provide new insights into the tissue localization 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 (Supporting Information 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 Fig. 1.

ABCA subfamily. The ABCA subfamily consists of nine genes in *An. gambiae* (Fig. 1). Six of these (*AGAP006379*, *AGAP006380*, *AGAP007504*, *AGAP011518*, *AGAP012155*

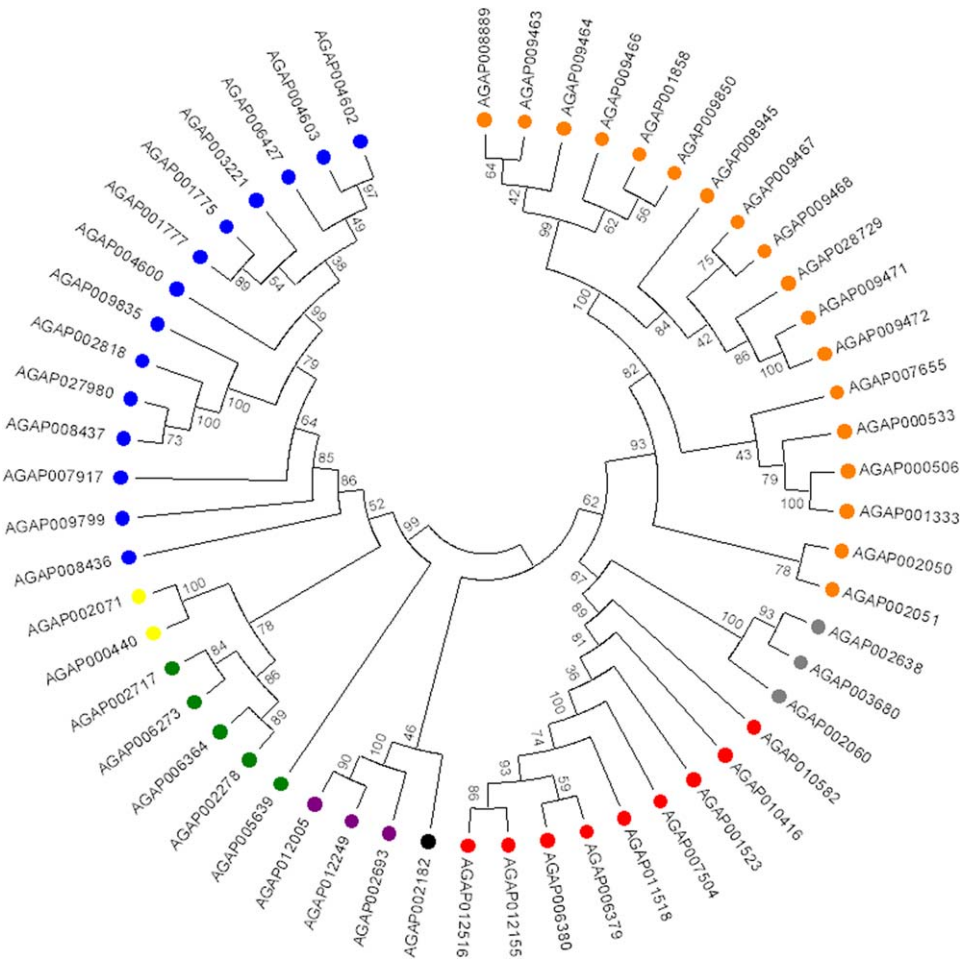

Figure 1. Unrooted phylogenetic tree of *Anopheles gambiae* ATP-binding cassette (ABC) proteins. Amino acid sequences of the 55 putative ABC proteins were aligned and the neighbour-joining method used to construct a phylogenetic tree in MEGA7 (Kumar *et al.*, 2016). Numbers at nodes represent percentage 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).

and *AGAP012156*) cluster with the insect-specific ABCA clade, characterized 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 orthologues in other mosquito species so this duplication presumably occurred prior to the divergence of these lineages (Fig. S1). Transcription of this insect-specific clade of ABCA genes is enriched in the midgut in *An. gambiae* (Fig. 2, Table S2) 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 (Fig. S1 and fig. S1 in Dermauw & Van Leeuwen, 2014). *AGAP001523* clusters with *FBgn0028539* from *D. melanogaster* and an expanded cluster of ABCA genes in the spider mite *Te. 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 orthologue of this ABC in *Ae. aegypti* (*AAEL004331*) is highly expressed in the brain (Matthews *et al.*, 2016; Table S2). 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 sphingolipid export (Tarling *et al.*, 2013); transcripts of this gene are enriched in the carcass (Fig. 2) and the *Ae. aegypti* orthologue (*AAEL018040*) is very highly expressed in antennae, legs and other sensory tissues (Matthews *et al.*, 2016; Table S1).

ABCB subfamily. The ABCB subfamily consists of full transporters (FTs) and half transporters (HTs). *An. gambiae* (and also *C. 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*

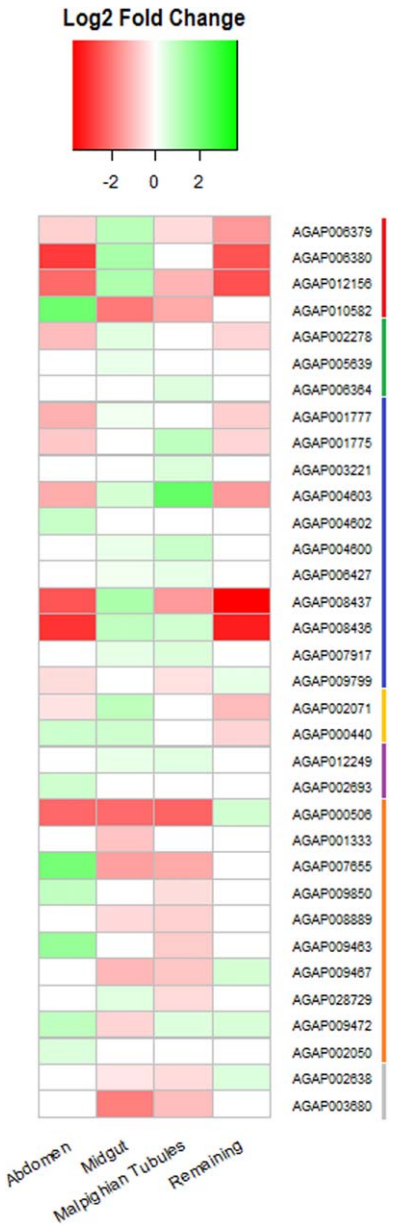

Figure 2. Tissue enrichment of ATP-binding cassette (ABC) genes in the insecticide susceptible N'Gousso strain. Genes showing significant enrichment ($P \leq 0.05$) in specific body parts from a previously published microarray experiment are shown here, clustered by ABC subfamily. The colour bar represents the ($\log_2$) 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 Fig. 1. Genes without any significant enrichment in any of the tissues are omitted from the figure.

transporters (*mdr49*, *mdr50*, *mdr65* and *FBgn0035695*) based on their similarity to the mammalian 'p-glycoprotein' transporters implicated in resistance to a wide range of drugs (Sarkadi *et al.*, 2006). Both Mdr49 and Mdr65 have been implicated in DDT resistance in *Drosophila* (Strycharz *et al.*, 2013; Gellatly *et al.*, 2015; 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 (Supporting Information Fig. S1 and fig. 3 in Dermauw & Van Leeuwen, 2014). The human ABCB HTs *7*, *8* and *10* are localized to the mitochondria (Kiss *et al.*, 2012) and we detected mitochondrial tagging sequences on all the *An. gambiae* orthologues of these genes. *AGAP002278* is the orthologue of *hABCB6*, both of which lack mitochondria tags; the mosquito transcript is enriched in the midgut (Fig. 2).

ABCC subfamily. 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 Uridine diphosphate 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 orthologue of the *Drosophila* '*multidrug resistance protein 1*' (*MRP1 FBgn0032456*; Fig. 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 (Fig. 2) but instead shows high expression in the testes (Baker *et al.*, 2011); the orthologue 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 orthologue of the human sulfonylurea receptor; this ABCC is enriched in the head in *An. gambiae* (Table S2).

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 S2).

ABCD subfamily. 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 fig. 5 in Dermauw & Van Leeuwen, 2014). The human ABCD transporters are located in the peroxisome and involved in transport of long and branched acyl coenzyme A molecules (Baker *et al.*,

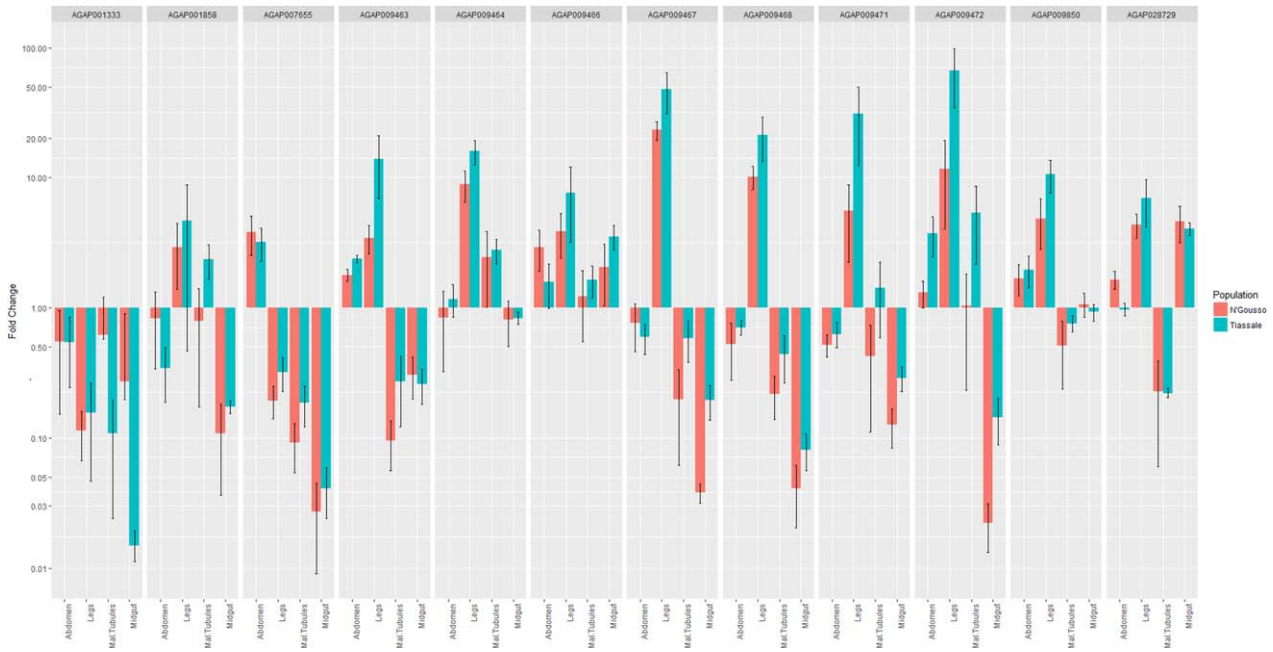

Figure 3. Tissue enrichment of ATP-binding cassette subfamily G (ABCG) transcripts in two *Anopheles gambiae* strains: resistant (Tiassale strain, shown in blue) and susceptible (N'Gousso strain in orange) to pyrethroids. Results show normalized expression in each body part relative to whole female mosquitoes. Error bars show SDs.

2015). Both *An. gambiae* ABCD genes are highly enriched in the midgut (Fig. 2).

ABCDE and ABCDF subfamilies. 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 *D. melanogaster* orthologue *pixie*. The human orthologue also has a conserved function in ribosome biogenesis and translation regulation (Chen *et al*., 2006). This ABC is very highly and ubiquitously expressed (ranked second amongst ABC transcripts in non-bloodfed 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* (orthologue of *hABCF1*), *AGAP002693* (orthologue of *hABCF2*) and *AGAP012005* (orthologue of *hABCF3*). All have 1:1 orthologues in other mosquito species (Fig. S2). The ABCFs are amongst the most highly expressed transporter genes in *An. gambiae* females (Pitts *et al*., 2011; Table S2).

ABCH subfamily. The ABCH subfamily in *An. gambiae*, in common with many insect species, contains three genes, all encoding HTs. This subfamily 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).

ABCG subfamily. The ABCG subfamily is the largest of the eight subfamilies in *An. gambiae*, containing 18 putative HTs, 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 not only to eye colour mutants in *D. melanogaster* but also other neural phenotypes (Borycz *et al*., 2008). These HTs form heterodimers, with *white* dimerizing with either *brown* or *scarlet*. There are clear orthologues of *white* and *brown* in *An. gambiae* (*AGAP000553* and *AGAP007655*) and there are two mosquito paralogues with orthology to *D. melanogaster* (*AGAP00506* and *AGAP01333*; Fig. 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 (Fig. S3). Interestingly, no

orthologue of the *D. melanogaster* 20-hydroxyecdysone (20-E) induced ABC transporter, E23 (*FBgn0020445*) is present in *An. gambiae* or other mosquito species (Hock *et al*., 2000; Fig. 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 (Dermauw & Van Leeuwen, 2014; Qi *et al*., 2016). Within *An. gambiae* eight 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 (Fig. 2, Table S2; Baker *et al*., 2011).

Numerous physiological functions have been demonstrated for mammalian ABCGs with several implicated in the transport of lipids and steroids (Klucken *et al*., 2000; Wang *et al*., 2004). 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 RNA sequencing (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 eight of the genes sequentially arranged on 3R division 34B, were enriched in legs compared to the whole bodies (Fig. 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 vs. the whole body in this strain (Fig. 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 (Fig. 2, Table S2).

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 localization 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 *Tr. 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-bloodfed females ((Pitts *et al*., 2011; Table S2). Previous microarray studies and our own qPCR data found *AGA009850* to be highly enriched in the abdomen integument and legs. Although we were unable to achieve reliable leg staining owing to technical difficulty with cryosectioning of this body part, *AGAP009850* antisera did localize to a layer of cells underlying the abdominal cuticle. Whole mount staining of the abdomen carcass suggested that these cells were pericardial cells (Fig. 4), part of the reticulosystem in insects that import and export compounds to/from the haemolymph (Fife *et al*., 1987). This location is in agreement with the enrichment of the *D. melanogaster* orthologue in the heart (and fat body; 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 S2) 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 recognizing 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 4 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 *Anopheles coluzzii* and three experiments with *An. gambiae s.s*. Further details of the mosquito populations are provided in Table S3.

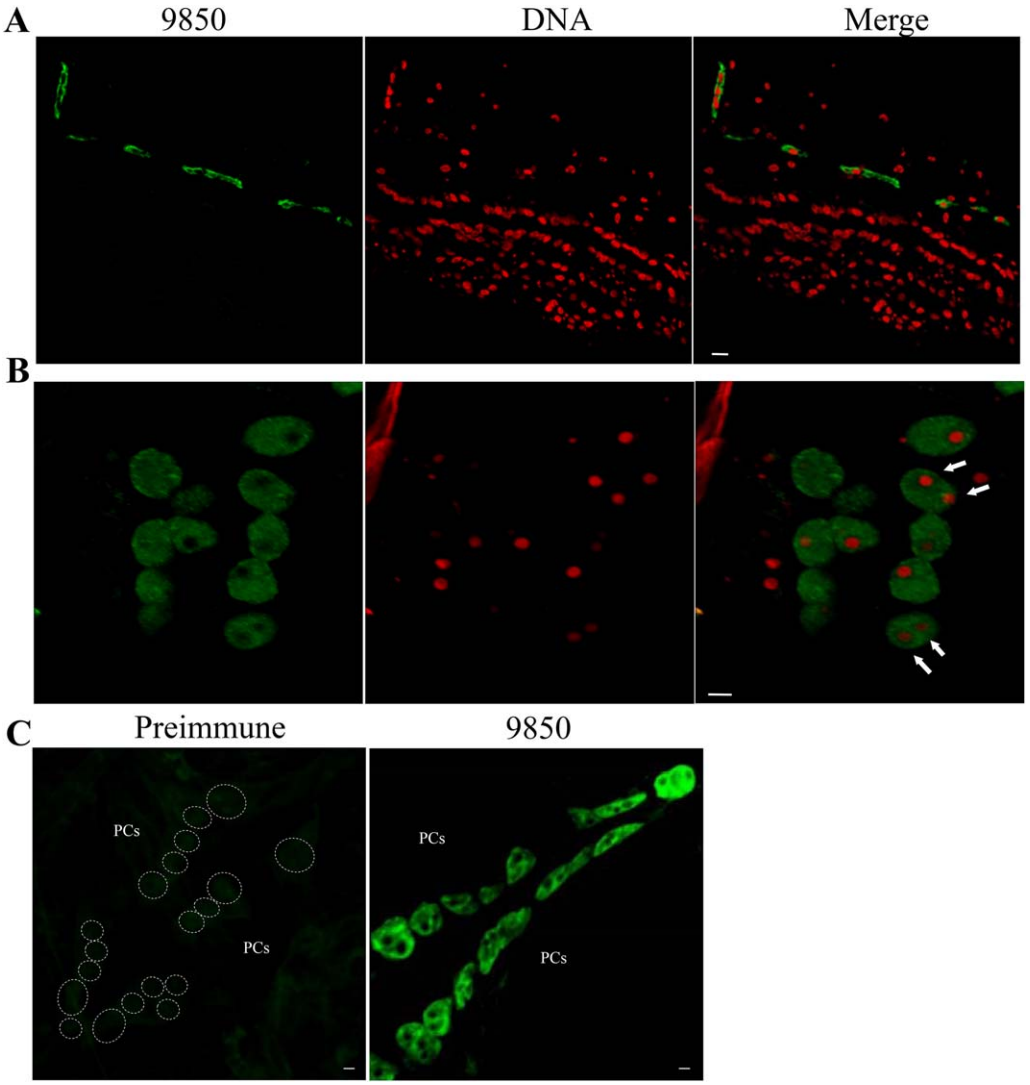

Figure 4. Immuno-localization of *Anopheles gambiae* ABCG transporter AGAP009850. (A) Longitudinal cryo-sections from mosquito specimens were immunostained with a-9850 specific antibodies (left column, green color). Cell nuclei were stained red with TOPRO (middle column). Merged immunohistochemical images (9850 and nuclei) appear in the right column. Scale bars: 10 mm. (B) Whole mount staining using a-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 mm. (C) Control pre-immune serum was tested in parallel with a-9850 on abdominal walls. No backround staining was detected and boundaries of PCs have been outlined with white dotted lines, when the preimmune serum was used. Scale bars: 10 mm.

The results identify multiple ABC transporters that are significantly ($P < 0.05$) differentially expressed in pyrethroid-resistant populations (Fig. 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 nine 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 orthologue 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 multidurg transporters in *Drosophila* but are instead both HTs, 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 nine, down in one) and ABCF family (*AGAP002693*, up in nine, down in one). The orthologues of the ABCC MRP (*AGAP009835*) and the ABCB MDR (*AGAP005639*) were up-regulated in some

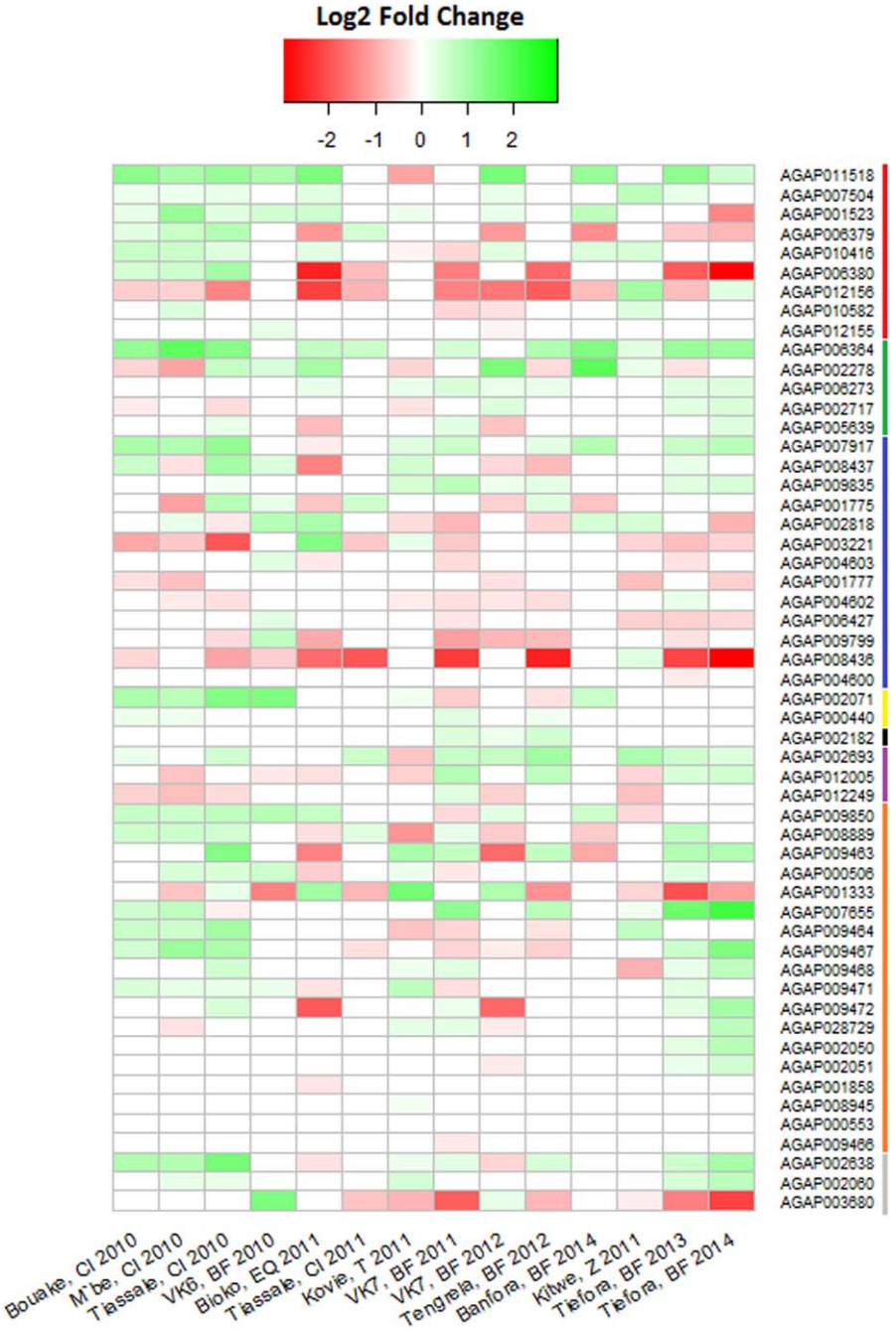

Figure 5. Heatmap showing differential expression of ATP-binding cassette (ABC) genes between insecticide resistant and susceptible strains of *Anopheles gambiae s.l.* Genes expressed at significantly ($P < 0.05$) higher levels in the resistant populations are shown in green, those higher in the susceptible strains are shown in red. ABC subfamilies are represented by the same colours as in Fig. 1. Further details of the data sets, including ArrayExpress accession numbers, are included in Table S3.

populations but did not stand out as being particularly strongly associated with the pyrethroid resistance phenotype (Fig. 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 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 humans and insects (Murray *et al.*, 1994; Petschenka *et al.*, 2013; DeSalvo *et al.*, 2014; Miller, 2015) 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 (P. Pignatelli & G. Lycett, unpubl. data) but further replicates followed by immunolocalization 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 (Fig. 2) and the four ABCB p-glycoprotein paralogues in *D. melanogaster* are all found in the 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 overexpressed 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 the epidermis (McFarlane *et al*., 2010; Broehan *et al*., 2013). 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 (CHCs; Balabanidou *et al*., 2016; V. Balabanidou, unpubl. data). Furthermore recent microarray data from a highly pyrethroid resistant population from Tiefora in Burkina Faso have identified the CHC synthesis pathway as one of the key pathways elevated in resistant strains (N. Grisales, V. Ingham & H. Ranson, unpubl. data); interestingly several of the ABCG genes that we show to be enriched in legs in this study are also overexpressed in this population (Fig. 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 immunolocalization studies and/or functional characterization 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 BBBs 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 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.org 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 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 *C. quinquefasciatus* and neighbour-joining trees generated as described above. Further putative orthologues 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 ArrayExpress (www.ebi.ac.uk/arrayexpress). The resistant mosquitoes were collected from Ivory Coast, Burkina Faso, Equatorial Guinea, Togo and Zambia between 2010 and 2014. All hybridizations were made to the Agilent whole genome 8 x 15k *An. gambiae* array (ArrayExpress accession number A-MEXP-2211; Agilent Technologies, Palo Alto, CA, USA). Further information on these data sets is provided in Table S3. All arrays were analysed using the R package limma (R Development Core Team, 2013), which fits linear models to normalized data (Smyth, 2004). Within-array normalization was carried out by Loess and between-array normalization by Aquantile (Smyth & Speed, 2003). ABC transporter expression was extracted and the nonsignificant 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) were used to identify ABC transcripts that were enriched in either the midgut, Malpighian tubules, abdomen integument or remaining body structures in the insecticide susceptible N'Gousso 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 N'Gousso and Tiassale strains of *An. gambiae* using a PicoPure RNA isolation kit (Applied Biosystems, Foster City, CA, USA). The Tiassale strain originates from Ivory Coast and is resistant to a wide range of insecticides (see Edi *et al.*, 2012, for further details). The insecticide-susceptible N'Gousso strain is from Cameroon (Harris *et al.*, 2010). Extractions were performed on whole mosquitoes (seven 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 synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

Primers were designed using the Primer-BLAST tool (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) against the *An. gambiae* Pink Eye Standard sequence (Table S4). The efficiency and specificity of the primer sets were assessed by running a standard curve over a fivefold serial dilution. Between 200 and 1000 ng of RNA was reverse-transcribed as described above. All four biological replicates for each body part were run in triplicate using 2 ng 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 Delta Delta ($\Delta\Delta$)Ct 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. $\Delta\Delta$Ct was calculated using a 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 (Regensberg, Germany). The sequence encoding the peptide: HTRKLVNAINGKKDIRSELDFPAQKNKKNENAANGNANLKVNYERMNGGANKYADNLNLGGNGLLPPSMVNDIAKETETIKIAVEPDREPEVNSALLPVEGATDIDHSPERYPTSEFH was cloned on pET16b vector as an *Nde*I/*Bam*HI fragment, under an Isopropyl β-D-1-thiogalactopyranosid (IPTG) 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 nickel nitrilotriacetic acid ($Ni\text{-}NTA^{2+}$) affinity chromatography. The purified peptide was used as an antigen to develop antibodies in rabbits.

Immunofluorescence and confocal microscopy

Three–five-day-old sugar-fed female mosquitoes from the N'Gousso strain were fixed in a cold solution of 4% formaldehyde (methanol free, Thermo Scientific, Waltham, MA) in phosphate-buffered saline (PBS) for 4 h, cryo-protected in 30% sucrose/PBS at 4 °C for 12 h, immobilized in Optimal Cutting Temperature O.C.T. (Tissue-Tek, SAKURA, The Netherlands) and stored at −80 °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 PBS, supplemented with 2 mM $MgSO_4$ and 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (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% bovine serum albumin, 0.1%

TRITON X-100 (Sigma) in PBS]. Then the tissues were stained with rabbit primary antibody in 1/500 dilution in the blocking serum, followed by goat antirabbit [Alexa 488, Molecular Probes (Eugene, OR, USA), 1/1000], which gave the green colour. Finally, nucleic acid was stained using TO-PRO-3 Iodide (Molecular Probes 1/1000). Images were obtained on a Leica TCS-NT Confocal laser scanning microscope with LAS AF Lite (Leica-TCS MP5, Mannheim, German) software.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Amino acid sequences of *Anopheles gambiae* ATP-binding cassette 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* ATP-binding cassette gene family including chromosomal location, predicted length of translated protein and expression data in different mosquito tissues (Baker *et al.*, 2011; Pitts *et al.*, 2011; Gabrieli *et al.*, 2014; Ingham *et al.*, 2014). The *Aedes aegypti* orthologues [from Lu *et al.* (2016)] are also included along with RNAsequencing (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 Fig. 5.

Table S4. Primer sequences used in quantitative PCR (qPCR) of *Anopheles gambiae* ATP-binding cassette subfamily G.

Figure S1. Phylogenetic tree of ATP-binding cassette subfamily A (ABCA), ABCB and ABCC 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 neighbour-joining method. The percentages 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 ATP-binding cassette subfamily D (ABCD), ABCE and ABCF in mosquitoes. See legend to Fig. S1 for further details.

Figure S3. Phylogenetic tree of the ATP-binding cassette subfamily G (ABCG) subfamily in mosquitoes. See legend to Fig. S1 for further details. Note that exons from CPIJ002198 and CPIJ002199 were merged to form a full-length ABCG protein (labelled CPIJ020198_9*).