A comprehensive transcriptomic view of renal function in the malaria vector, *Anopheles gambiae*

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Abstract

Renal function is essential to maintain homeostasis. This is particularly significant for insects that undergo complete metamorphosis; larval mosquitoes must survive a freshwater habitat whereas adults are terrestrial, and mature females must maintain ion and fluid homeostasis after blood feeding. To investigate the physiological adaptations required for successful development to adulthood, we studied the Malpighian tubule transcriptome of *Anopheles gambiae* using Affymetrix arrays. We assessed transcription under several conditions; as third instar larvae, as adult males fed on sugar, as adult females fed on sugar, and adult females after a blood meal. In addition to providing the most detailed transcriptomic data to date on the *Anopheles* Malpighian tubules, the data provide unique information on the renal adaptations required for the switch from freshwater to terrestrial habitats, on gender differences, and on the contrast between nectar-feeding and haematophagy. We found clear differences associated with ontogenetic change in lifestyle, gender and diet, particularly in the neuropeptide receptors that control fluid secretion, and the water and ion transporters that impact volume and composition. These data were also combined with transcriptomics from the *Drosophila melanogaster* tubule, allowing meta-analysis of the genes which underpin tubule function across Diptera. To further investigate renal conservation across species we selected four *Drosophila melanogaster* genes with orthologues highly enriched in the *Anopheles* tubules, and generated RNAi knockdown flies. Three of these genes proved essential, showing conservation of critical functions across 150 million years of phylogenetic separation. This extensive data-set is available as an online resource, MozTubules.org, and could potentially be mined for novel insecticide targets that can impact this critical organ in this pest species.

Keywords: *Anopheles gambiae*, Malpighian tubules, transcriptomics, haematophagy, *Drosophila melanogaster*

Introduction

Ion and water homeostasis is essential in insects

The extraordinary success of insects can be ascribed in part to their ability to maintain a stable internal environment in a range of extreme habitats. Ion and water homeostasis is essential at every stage of the insect life-cycle, and the Malpighian tubules, in conjunction with the hindgut, are the primary organ of fluid and salt balance (Beyenbach et al., 2010). The tubules produce a dilute urine from which the hindgut can reabsorb water, ions, and organic solutes, while excreting toxic or undesirable compounds.

Recently, the combination of classical physiology and post-genomic technologies in the genetic model organism, *Drosophila melanogaster*, has greatly accelerated our understanding of insect renal function (Beyenbach et al., 2010). Transcriptomic analysis has identified a range of abundantly expressed transporters that have been shown experimentally to underpin tubule function (Chintapalli et al., 2012; Wang et al., 2004); and by comparing epithelial transcriptomes by meta-analysis of the online FlyAtlas.org resource (Chintapalli et al., 2007b; Chintapalli et al., 2013), it has been shown that the key fluid secreting epithelia of *Drosophila* (salivary glands, midgut, tubules, hindgut) all shared a transcriptomic fingerprint, or 'epitheliome' (Chintapalli et al., 2013). Although unsupported transcriptomic studies should be interpreted cautiously, downstream experimental testing has validated the models so obtained (Allan et al., 2005; Cabrero et al., 2014; Wang et al., 2004). However, laboratory *Drosophila* lead rather uniform and protected lives in controlled environments at high humidity on a uniform, predictable diet. Most advanced (holometabolous) insects exploit entirely different environments during their growing (larval) and mating (adult) stages, and so their renal system might require extensive remodelling in the pupa.

This is particularly evident in the malaria vector mosquito, *An. gambiae*. Larvae feed and grow in freshwater, whereas adults are terrestrial; thus the excretory system is faced by diametrically opposing demands (excess water, then the risk of desiccation) as the insect grows. Furthermore, there is marked sexual dimorphism in the adult lifestyle; whereas both males and females feed on sugary nectar, only the females take a complete blood meal. During post-blood meal diuresis, when the mosquito has imbibed 2-3 times her unfed mass (Lehane, 2005), urine is voided unaltered by the hindgut, and so in addition to being the primary organ of osmotic and ion regulation the tubules are essential for body volume reduction. Prioritisation of tubule physiological function – to shift from an aquatic to terrestrial environment, and from processing small volumes of nectar to ion rich blood – is crucial for development and reproduction, and likely requires transcriptomic changes in the diuretic peptide receptors, water and ion transporters expressed.

The mosquito thus allows us to study, within the same organism, the different transcriptomic profiles needed to perform renal function across ontogeny, changes in lifestyle and habitat, and different diets. Accordingly, we generated the most comprehensive transcriptomic data-set to date, showing that a common core transport signature continues from larval to adult life, providing a backdrop to more specialised modifications of function. Our resulting datasets are in the public domain (at MozTubules.org) to permit further community analysis.

Materials & Methods

Anopheles rearing

The *An. gambiae* Keele strain (Hurd et al., 2005) was used for Affymetrix sample preparation, and was maintained in the Institute of Infection, Immunity and Inflammation, University of Glasgow. Mosquitoes were reared in an insectary room maintained at 26°C and 80% humidity with a 12 h day: night cycle. Adult mosquitoes were given access to 5% glucose in 0.05% para-aminobenzoic acid water, and larvae fed with ground TetraMin tropical fish food (Tetra, USA). Adult mosquitoes were offered a human blood-meal through an artificial membrane once per week. Blood was obtained from the Glasgow and West of Scotland Blood Transfusion Service, and ethical approval obtained from the Scottish National Blood Transfusion Service. Pupae were collected on a daily basis, and placed in mosquito cages to hatch and age to 3-5 days for experimental use.

Affymetrix sample preparation

Malpighian tubules from approximately 400 uninfected *An. gambiae* larvae, sugar-fed adult males, sugar-fed adult females, and females 3 hours after a blood feed were dissected (Table 1). Insects were briefly chilled at 4 °C, and dissected in a sterile 1:1 mixture of *Anopheles* saline (in mmol l^{-1}: NaCl, 117.5; $MgCl_2$, 8.5; $CaCl_2$, 2; $NaHCO_3$, 10.2; NaH_2PO_4, 4.6; HEPES, 8.6; glucose, 20; adjusted to pH7) and Schneider's medium (Invitrogen, UK), before being transferred to RLT buffer containing 1% β-mercaptoethanol (Qiagen, UK). Four replicates were prepared for each sample, and four whole-mosquito samples, each containing 30 adult *Anopheles* maintained on sugar-water, were also prepared. Tubules were pooled in each sample to permit total RNA extraction using RNeasy columns (Qiagen, UK), in accordance with the manufacturer's protocol. Hybridisation to the GeneChip was performed by the University of Glasgow Polyomics Facility. Each cRNA sample was prepared independently using the Affymetrix standard protocol, and the samples were hybridised to the Affymetrix GeneChip *Plasmodium/Anopheles* Genome Array for analysis of the transcriptome.

Anopheles data analysis

The *Plasmodium/Anopheles* Genome array contains probe sets to approximately 14,900 *An. gambiae* transcripts, in addition to 4,300 *Plasmodium falciparum* transcripts, which were irrelevant to this study as the mosquitoes were not infected with the malarial parasite. The array was designed using Ensembl (Build 2, 2003), GenBank and dbEST. The Affymetrix GeneChips were analysed using the Affymetrix MAS5 software, in which the average expression was set to 100. Annotation of the probe sets was obtained from NetAffx (Affymetrix, USA). Subsequent microarray analysis was performed using GeneSpring 6 (Agilent Technologies, USA), or with Bioconductor under R, using default settings for robust multichip average (RMA) with normalization against all arrays used. Gene up-regulation or down-regulation was assessed using Student's *t*-tests between two biological groups, and did not require a minimum average fold-change.

Peptide synthesis

Drosophila DH_{31} and DH_{44} (both with and without a N-terminal cysteine) were synthesized by Cambridge Peptides (UK). The modified peptide was subsequently coupled to either Bodipy543-TMR-C_5-maleimide (BioRad, US) or to Alexa488-C_5-maleimide (Invitrogen, US), to make fluorescent DH_{31} (DH_{31}-F; Alexa488-C_5-

maleimide-CTVDFGLARGYSGTQEAKHRMGLAAANFAGGPamide) and DH_{44} (DH_{44}-F; Bodipy543-TMR-C_5-maleimide-CNKPSLSIVNPLDVLRQRLLLEIARRQMKENSRQVELNRAIL-KNVamide) respectively, with the final working concentration adjusted according to peptide purity.

Receptor binding assay

The relative DH31-R1 and DH44-R1 expression levels in both larval and adult Malpighian tubules (MTs) were explored using an *ex vivo* receptor binding assay, which allows direct visualization of ligand-receptor interaction, and thus relates fluorescent intensity to receptor levels (Halberg et al., 2015). Briefly, MTs were carefully dissected from cold-anesthetized animals, under *Anopheles* saline (Halberg et al., 2015), and the tubules from both larvae and adults mounted on poly-L-Lysine covered 35 mm glass bottom dishes. The untreated tubules were then used to adjust baseline filter and exposure settings to minimize autofluorescence during subsequent image acquisition. Next, the tissues were incubated in *Anopheles* saline supplemented with the labelled neuropeptide (10^{-7} M) and DAPI (1 µg/ml), and following a light wash in saline, images were recorded (optical section 20 µm) on an inverted confocal microscope (Zeiss LSM 510 Meta) using the previously determined filter and exposure settings. From these images, intensity profiles were generated using the existing Zeiss LSM 510 Meta confocal software, and an average intensity profile in x-y position of (n = 21-23) tubules from both larvae and adults was generated for each peptide.

***Drosophila* rearing and stocks**

Drosophila were reared on standard diet (Ashburner, 1989) in vials, at 21°C with a 12:12 h photoperiod and at 45-55% room humidity. Where required, they were anesthetized by brief exposure to carbon dioxide. Crosses were maintained at 21°C. Stocks used were: $white^{honey}$ (for out-crossing of stocks), *Actin*-Gal4 (ubiquitous Gal4 expression) and c42-Gal4 (Malpighian tubule principal cell-specific Gal4 expression).

Generation of RNAi alleles

RNAi alleles for *CG15406, Picot, ZnT35C* and *CG8028* were generated using the pWiz vector (Lee and Carthew, 2003). Regions of approximately 600 bp were selected and cloned into the pWiz vector to form an inverted repeat. Inserts were verified by sequencing, and sent for commercial germ-line transformation by BestGene (USA). Multiple inserts for each construct were balanced to determine the chromosomal localisation, and knockdown validated by quantitative RT-PCR.

Quantitative RT-PCR

Dissected *Anopheles* or *Drosophila* tubules were stored in RLT buffer, and RNA extracted using an RNAeasy Mini Kit (Qiagen, UK). PCR was performed in a One-Step real-time PCR machine (Applied Biosystems, UK) using Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems, UK). Expression was quantified using the $\Delta\Delta C_t$ method, using *RPL32* as a reference gene.

Emergence Counts of *Drosophila* Crosses

Gal4-driven UAS-RNAi crosses were set up in vials in quadruplicate, and reared in standard conditions at 21°C. Each UAS-RNAi was maintained over a balancer chromosome, to allow calculation of the number of Gal4>UAS-RNAi flies expected from the cross. Progeny were collected and sorted each day, and the number of flies of each genotype was counted daily until all flies had emerged. The number of observed flies of each genotype was compared to the expected number of flies for each genotype, and $\chi 2$ analysis performed. Each

Gal4 driver and UAS parent was also crossed to *white*^{honey} and maintained at 21° C, to ensure there was no survival phenotype associated with the Gal4 or UAS transgene alone.

Statistics

Significance of differences was assessed with unpaired Student's *t*-test or χ^2 test (two tailed) as appropriate. All testing used GraphPad Prism Version 5 (GraphPad, USA) or OriginPro 8.5 (OriginLab, USA) software. Throughout, the critical level is taken as $P<0.05$.

Results and Discussion

The transcriptome of the *Anopheles* Malpighian tubules was assessed in larvae and adults, and additionally compared to a whole-mosquito sample (Table 1). This comparative approach allows analysis of the physiological functions prioritised at each life-stage, and identification of genes regulated by each transition. Statistical comparisons, using Student's *t*-test to assess significance, show clear changes in the transcriptomes associated with environment, gender and diet (Fig. 1). The most striking divergence is between the larval and adult tubules, where the expression of approximately 3300 genes varies between the life-stages; 1429 transcripts are up-regulated in the larval tubules in comparison to the adult, and 1887 up-regulated in the adult tubules in comparison to the larval. Similarly, 1500 genes are differentially expressed in the male and female adult tubule, and 3 h after a blood-feed >1200 genes are significantly up-regulated or down-regulated. These data demonstrate that at each life-stage the renal transcriptome is tailored specifically to meet the physiological needs and challenges of the insect.

An epithelial expression signature common to dipteran tubules?

The *Drosophila* tubule transcriptome identifies a set of key transport genes that are expressed at very high levels compared with the organismal average, and this is believed to reflect the core transport capability of the tubule (Wang et al., 2004). For example, exactly one gene for each of the 13 V-ATPase subunits is highly expressed, and mutants of these genes show a conserved tubule phenotype (Allan et al., 2005). Further meta-analysis of the FlyAtlas.org expression resource (Chintapalli et al., 2007b) showed that this core transcriptional signature is well-conserved across the major transporting epithelia (salivary gland, midgut, tubules and hindgut), suggesting that insect ion transport may be relatively tightly defined (Chintapalli et al., 2013). How well is this motif conserved between species? That is, can a similar signature be identified in *Anopheles* tubules; and if so, is it shared between larvae and adults, despite their radically different osmoregulatory needs?

As can be seen in Table 2, across around 150 Million years of divergent evolution, the motif of basic core transporters associated with insect epithelial function is conserved remarkably well. So, although there are many differences in lifestyle between fly and mosquito, we can assert that the underlying machinery is completely conserved. This is graphically illustrated in Fig. 2.

Of course, this *in silico* analysis benefits from experimental validation, and the powerful and elegant transgenic approaches of *Drosophila* (notably cell-type specific RNAi knockdown) are not easily obtained in mosquitos. However, there is ample physiological evidence that the processes, if not the individual genes, are important in mosquito tubule function. V-ATPase has been cloned and localized immunocytochemically in mosquito tubule (Patrick et al., 2006; Patrick and Gill, 2002; Zhuang et al., 1999), so this dataset allows the subunits involved to be identified. The data further show that the larval and adult tubules use the same

subset of V-ATPase genes to encode the plasma membrane V-ATPase (Table 2). Similarly, the Na^+, K^+ ATPase (Patrick and Gill, 2002), the aquaporin DRIP (Liu et al., 2011) and the apical exchanger (Rheault et al., 2007; Xiang et al., 2011; Xiang et al., 2012b) have all been described in mosquito tubule; these array data provide a more comprehensive survey of which genes in a particular family show highly enriched expression in the tubule.

Given an underpinning similarity in the core machinery, what differences in gene expression can be ascribed to unique specializations associated with changes in mosquito age, habitat and diet?

Cell signalling in Larval versus adult tubules

Anopheles larvae mature from egg to pupae in a freshwater environment, where they must balance continual water uptake and excretion against preservation of vital ions such as Na^+ and K^+. In contrast, adults live in a terrestrial environment and must carefully conserve water gained from nectar-feeding to avoid desiccation. As expected, the transcriptome of the Malpighian tubules alters significantly between these two opposing states, particularly for a subset of diuretic peptide receptors, ion transporters and water channels (Table 3). Receptors for the diuretic peptides Anoga-DH_{31} and Anoga-DH_{44} have been identified in the *Anopheles* tubules; GPRDIH1 is the putative receptor for Anoga-DH_{44} (Jagge and Pietrantonio, 2008), and GPRCAL1 for Anoga-DH_{31} (Coast et al., 2005). Our data show that in the larval tubules *GPRDIH1* is strongly expressed, with very low *GPRCAL1* expression, and in adults the opposite - *GPRCAL1* is strongly expressed, with lower *GPRDIH1* (Table 3).

This suggests a clear switch from *Anoga-DH_{44}/GPRDIH1* signalling in larvae to *Anoga-DH_{31}/GPRCAL1* in the adult. This switch also results in alteration of the ionic composition of the secreted fluid – Anoga-DH_{31} is natriuretic, whereas Anoga-DH44 has a non-specific effect on Na^+ and K^+ transport (Coast et al., 2005; Kwon et al., 2012). By contrast, expression of the kinin receptor is stable between larva and adult in both *Anopheles* and *Drosophila*. Data from the gene expression database FlyAtlas shows some similarities with *Drosophila*, as *DH31* receptor (*DH31-R1*) is massively enriched in the adult tubules compared to the larval tubules, and *DH44-R2* isn't enriched in either life-stage (Chintapalli et al., 2007a).

To validate these data, we used a newly developed technique to directly visualise neuropeptide receptors in the tubule, using fluors with high quantum yield (Halberg et al., 2015). Both receptors are expressed exclusively in the principal cells of both larvae and adult; however, there is a clear shift from DH44 to DH31 in the adult, consistent with the array data.

Similarly, expression of the aquaporin family of water channels alters significantly during the transition from larva to adult. Four of the seven predicted *Anopheles* aquaporins (Drake et al., 2010) are expressed in the tubules, and each is massively up-regulated in adult when compared to larva (Fig. 4*A*, Supplementary Table 1). This implies that the adult tubules, alongside the hindgut, perform a highly active role in water recycling, which is essential for elimination of toxins. Likewise, almost all of the subunits, which comprise the tubule H^+ V-ATPase, are significantly up-regulated in adult tubules in comparison to larvae (Fig. 4*B*, Supplementary Table 1). The H^+ V-ATPase is responsible for energising the tubule by creating a proton gradient, which can direct secondary transport of other ions such as Na^+ and K^+, and so an increase in expression suggests a more active epithelium. *AgNHA2*, which is expressed in the apical membrane of the stellate cells (Xiang et al., 2012a) is up-regulated two-fold, as is *AgNHA1*, which is co-localised in the principal cell apical membrane with the H^+ V-ATPase (Fig. 4*C* and 4*D*). Xiang *et al* (2012) hypothesise that the principal cell H^+ V-ATPase

generates an outside positive voltage, which causes AgNHA1 to drive Na^+ into the lumen in exchange for H^+. The Na^+ is then driven into the stellate cell by AgNHA2, where it can be returned to the haemolymph by a basal Na^+ K^+ -ATPase, and retained as an essential resource. As water flux appears to be increased in the adult tubules, enrichment of the NHAs may serve as a mechanism to retain sodium ions. An additional putative Na^+/H^+ exchanger, *AgNHE3* (Fig. 4*E*) is also massively up-regulated, and although it is yet uncharacterised the *Aedes* orthologue can be basally or apically located in the principal cells (Pullikuth et al., 2006). The putative $Na^+/K^+/Cl^-$ symporter *AGAP010249* and inwardly-rectifying potassium channel *AGAP007818* are also enriched (Fig. 4*F* and 4*G*), and could potentially have roles in potassium retention. Although the importance of each of these genes must be tested *in vivo*, their up-regulation as the mosquito changes from an aquatic to terrestrial environment implies a significant role in water and ion conservation in *Anopheles*.

Male versus female tubules

Male and female adult *Anopheles* maintained on the same sugar-water diet have vastly different tubule transcriptomes. Female *Anopheles* maintained on a sugar diet appear to 'anticipate' a future blood-feed, with numerous ion and water transporters maintaining a transcription level midway between the male tubule, and the state required for post-blood-meal diuresis. The expression of *Aquaporin-8* and *AgNHA2* (Fig. 5*A* and 5*B*) exemplify this trend - both have lower expression in female than male tubules, and the transcript level decreases further upon a blood-meal. This may allow the tubule to function on a nectar-diet, but quickly adapt for post-prandial diuresis. The reduction in transcription of *AgNHA2* after a blood-meal is consistent with the hypothesis that the function of *AgNHA2* is to preserve haemolymph sodium content (Xiang et al., 2012a), and so expression is decreased during natriuresis. Similarly, there are significant differences in the expression of the diuretic peptide receptors – male tubules transcribe twice as much *GPRLKK* (kinin receptor) and *GPRDIH1* (*Anoga-DH44* receptor) as their female counterparts (Fig. 5*C* and 5*D*). If the primary role of Anoga-DH31 is to flush sodium after a blood meal it is perhaps not surprising that male mosquitoes have increased expression of alternative diuretic peptide receptors, as they are unlikely to require such extensive natriuresis.

Sugar versus blood-feeding

The Malpighian tubule transcriptome is not static; it continually reacts to shifts in mosquito development and feeding to maintain organism ion and water balance. In the hours following a blood-meal the transcriptome alters regularly, as noted by Esquivel *et al*., who investigated the tubules of *Aedes albopictus* 3h, 12h and 24h after a blood-feed (Esquivel et al., 2014). From their analysis it is evident that the tubule initially prioritises post-prandial diuresis, progressing towards detoxification, amino-acid metabolism and proteolytic activity 12 – 24 hours post-feed. Our microarray dataset assesses the *Anopheles* transcriptome 3 h after a blood-feed, highlighting the initial wave of gene transcription. A variety of transporters are enriched after blood-feeding, such as *AgPrestinB* and *AGAP004633,* which have roles in sulphate/oxalate transport (Hirata et al., 2012) and the folate transporter *AGAP004562* (Fig. 6*A,* 6*B* and 6*C*). All three of these genes have likely roles in the detoxification of digestive blood products absorbed into the haemolymph from the midgut. *AGAP004652* is only expressed in the tubule upon blood-feeding, highlighting the plasticity of the tissue, and the urgency with which they are able to adapt physiologically. There is also significant up-regulation of four genes with putative roles in amino acid transport, *AGAP010865, AGAP000022, AGAP001627,* and *AGAP005653* (Fig. 6*D*, 6*E*, 6*F* and 6*G*), which may be in response to red-blood cell

digestion, or the amino-acid metabolism and proteolytic activity noted by Esquivel *et al.* We also see a doubling of *Aquaporin 1* transcription, suggesting it may be involved in the initial purge of fluid from the mosquito, although *aquaporin-8* is not enriched (Fig. 6*H* and 5*A*). This is consistent with *Aedes aegypti*, where three of the six aquaporins identified are up-regulated 3 h after a blood-meal (Drake et al., 2010). Of the genes most highly enriched in the tubules upon blood-feeding , only one is 'named' - *Prestin* - and the vast majority appear to be involved in enzymatic and metabolic processes (Table 4).

It is reasonable to expect that, in specializing to handle a blood meal, some transcripts would be downregulated. However, this was not the case; although 58 transcripts were upregulated 5-fold or higher in blood-fed female tubules, only one transcript (significantly of *AGAP006736*, a homologue of *Drosophila* sugar-responsive transcription factor *sugarbabe*), was upregulated more than 5x in sugar-fed, compared to blood-fed, females. Therefore, the effect of a blood meal is to express a new set of genes in addition to the baseline expression level seen in sugar-fed mosquitoes.

Testing the functional significance of co-enrichment between transcriptomes of *Anopheles* and *Drosophila*

Although the fundamental pathways essential for secretion appear common to both *Anopheles* and *Drosophila*, the conservation of other renal processes has not been rigorously tested. We would expect mechanisms of secretion to be essential for survival, but what about less obvious transport functions of the tubule? We mined the *Anopheles* microarray data in combination with FlyAtlas (Chintapalli et al., 2007a), to identify transporters that are significantly up-regulated in the *Anopheles* tubule, that have conserved orthologues that also show up-regulated expression in the *Drosophila* tubules. We selected four genes for functional analysis: the putative sugar transporters *AGAP007752* and *CG15406*, the inorganic phosphate transporters *AGAP011251* and *Picot*, the zinc transporters *AGAP009005* and *Znt35c*, and the monocarboxylate transporters *AGAP002587* and *CG8028*. Fig. 7 contains quantitative-PCR (qPCR) data for expression of each gene in whole adult insect (combined male and female), larval tubules, male tubules, female tubules and female tubules 3 h and 24 h after a blood-feed (*Anopheles* only). *AGAP007752* and *AGAP011251* are highly enriched in the adult tubules, *AGAP009005* is enriched at all life-stages but highest in the male, and *AGAP002587* is female-specific, and massively up-regulated after a blood-meal. Ubiquitous knockdown of their *Drosophila* orthologues using RNAi shows that *CG15406, Picot* and *ZnT35C* are all essential for development to adulthood (Fig. 8). Knockdown of *CG8028* has no effect on fly survival, even with a 90% decrease in transcript levels. Knockdown of *CG15406* and *Picot* using a tubule principal cell-specific driver (c42-Gal4) results in reduced survival to adulthood, suggesting a vital function of these genes in the tubules. Of our four gene pairs, the only *Drosophila* orthologue that is not essential for survival is *CG8028* - partner of *AGAP002587*, which shows the highest level of enrichment in the *Anopheles* tubules after a blood-meal. On this premise, it would appear that genes that show conservation of both sequence and enriched expression in the Malpighian tubules of *Anopheles* and *Drosophila* are likely to be essential for survival.

The Moztubule resource

Although these array data are available online at NCBI GEO, they are more conveniently studied at: moztubule.org. This resource provides a rapid comparison for gene expression levels across the divergent ontogenetic and physiological conditions of this dataset. This is analogous to a subset of our FlyAtlas.org expression dataset for *Drosophila* (Chintapalli et al., 2007b; Dow, 2006).

Comparison with other datasets

The deposition of microarray data in the public domain allows comparison between datasets. As well as the *Drosophila* data in FlyAtlas.org, there are other transcriptomic results from *Anopheles*, though none are precisely comparable. A previous Affymetrix study provided compartmentalized expression profiles along larval midgut, allowing spatial patterns of digestion and absorption to be inferred (Neira Oviedo et al., 2008). However, the data cannot be directly compared, as the Malpighian tubules and hindgut were combined in this study. This is significant because this effectively combines the tissues that excrete the primary urine, and reabsorb desirable products from the excreta. Also, the reference sample used to normalize gene expression in this study was whole larvae - whereas our study used whole adults – resulting in two incompatible comparisons. A second study looked at changes in whole mosquito 24 h after a blood meal (Marinotti et al., 2005). This study looked at longer term changes than our 3 h timepoint, and used whole mosquitoes, an approach which we have shown under-represents the contribution of individual tissues (Chintapalli et al., 2007b). Similarly, the UC Irvine Atlas (Marinotti et al., 2006; Sieglaff et al., 2009) is a whole-animal time series after a blood meal. The valuable mozatlas resource (Baker et al., 2011) contains tubule datasets, but contrasts male sugar-fed with female blood-fed adults. As a rough check, the genes identified as blood-meal enriched in Table 4 were searched in mozatlas, and 16/23 were shown to be enriched in female compared with male tubules (data not shown). There is thus a reasonable correspondence between these independently generated datasets.

Conclusions and significance

The *Plasmodium* parasites which cause malaria are spread exclusively by mosquitoes of the genus *Anopheles*, and can be ingested or transmitted when the female imbibes human blood (Trampuz et al., 2003). *An. gambiae* is perhaps the most efficient vector of human *Plasmodium*, due to its long life-span and preference for biting humans. To develop to a life stage where it can transmit malaria, the mosquito must undergo a series of physiological adaptations, and each transition requires substantial alteration of the mosquito renal excretory system - the Malpighian tubules - and as such offers the opportunity to target a process crucial for insect development. Nearly all known insecticides target the CNS (Nauen and Bretschneider, 2002); however, preliminary studies targeting the Malpighian tubules of *Aedes aegpyti* using a small molecule inhibitor of inward-rectifying potassium channels (Raphemot et al., 2013), show that the insect excretory organs are a promising alternative target. The present study provides the most in-depth analysis of the *Anopheles* Malpighian tubule to date, by tracking thousands of the transcriptome modifications required for development to an adult capable of transmitting disease. The data demonstrate how a simple epithelium can switch function with both precision and speed, as the osmotic and ionic demands associated with eclosure and haematophagy demand alteration in both control and physiology of transport. In addition, the adult comparisons demonstrate that even on the same sugar diet, there is a distinct ‘male’ and ‘female’ tubule, tailored for both the present and future requirements of the mosquito. These life-stage, gender and life-style specializations may even allow targeted insecticide development; for example the design of compounds, which specifically target the larval tubules and can be applied in stagnant water pools. Perhaps surprisingly, comparative transcriptomics between *Anopheles* and *Drosophila* – across 150 M years of divergent evolution - identifies a core renal “epitheliome” of transporters conserved between the two species. The basic mechanisms of renal secretion can therefore adapt not only during the life-cycle of an insect, but also to support multiple species with opposing life-styles. This data-set is made

available as an online resource, MozTubules.org, to allow the mosquito community to access data on their own genes-of-interest.

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Figure legends:

Fig. 1: Transcripts significantly up-regulated in the *Anopheles* Malpighian tubules during each life-stage. Significance tested using Student's *t*-test on pairwise comparisons, $P<0.05$, N=4.

Fig. 2. The *Anopheles* tubule epitheliome conforms to the *Drosophila*-derived consensus model. A model epithelial cell, with *Drosophila* transporter genes highly enriched across the four major fluid-secreting epithelia (salivary glands, midgut, tubules and hindgut), as described elsewhere. (Chintapalli et al., 2013). Where a gene is highly enriched in the *Anopheles* larval and adult tubule (Table 1), the Drosophila gene name is coloured green.

Fig. 3. Ontogenetic switching of neuropeptide control of renal function. The calcitonin/DH_{31} receptor gene GPRCAL1 is highly expressed in the adult tubules, whereas the CRF/DH_{44} receptor GPRDIH1 is larval-enriched. Receptor binding assays reiterate the relative difference in receptor expression levels in the tubules of larval and adult *Anopheles* **(a, c).** DH_{31} and DH_{44} receptors are expressed exclusively in the principal cells of both larval and adult tubules**.** The DH_{31}-F and DH_{44}-F fluorescent intensities **(b, d)**, and thus receptor levels, are significantly higher in adult and larval tubules, respectively (*; $P<0.05$, unpaired, two-sample *t*-test). SC, stellate cell; PC, principal cell.

Fig. 4. *Aquaporin-8* (*A*), *Vha55* (*B*), *AgNHA2* (*C*), *AgNHA1* (*D*), *AgNHE3* (*E*), *AGAP010249* (*F*) and *AGAP007818* (*G*) are all significantly enriched in the adult Malpighian tubules when compared to the larval tubules. (Student's *t* test, *N*=4; significant changes in expression are marked with an asterisk).

Fig. 5. *Aquaporin-8* (*A*), *AgNHA2* (*B*), *GPRLKK* (*C*) and *GPRDIH1* (*D*) have significantly lower expression in female adult tubules than male. (Student's *t* test, *N*=4; significant changes in expression are marked with an asterisk).

Fig. 6. *Prestin* (*A*), *AGAP004633* (*B*), *AGAP004562* (*C*), *AGAP010865* (*D*), *AGAP000022* (*E*), *AGAP001627* (*F*), *AGAP005652* (*G*) and *Aquaporin 1* are all significantly enriched in the female Malpighian tubules 3 h after a blood-meal. (Student's *t* test, *N*=4; significant changes in expression are marked with an asterisk).

Fig. 7. Q-PCR expression of four orthologous gene pairs. (*A*) and (*B*) putative sugar transporters *AGAP007752* and *CG15406*; (*C*) and (*D*) inorganic phosphate co-transporters *AGAP011251* and *Picot;* (*E*) and (*F*) zinc transporters *AGAP009005* and *ZnT35C*; (*G*) and (*H*) monocarboxylate transporters *AGAP002587* and *CG8028*. Significance determined by Student's *t*-test, N=4.

Fig. 8. *CG15406, Picot* and *ZnT35C* are essential for survival. When driven with *Actin*-Gal4, *CG15406*-RNAi*, Picot*-RNAi and *ZnT35C*-RNAi flies do not survive to eclosure (black bars). When driven by the tubule-specific driver c42-Gal4, *CG15406* and *Picot* flies have reduced viability to eclosure (grey bars). *CG8028*-RNAi has no effect on viability. Emergence of each genotype was calculated by comparing the number of Gal4>UAS-RNAi flies which eclosed for each cross, to the expected number of eclosures (Control). Where bars or errors are not visible, they are too small to plot. Significant differences (χ^2 test, *N*=4) are marked with an asterisk.

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Table 1. *Anopheles* samples compared by microarray analysis.

Sample	Gender	Life-Stage & Tissue	Diet
Whole	Male & female	Whole adult	Sugar-water
Larval	Male & female	L3 larval tubules	Fish food
Adult	Male & female	Adult tubules	Sugar-water
Male	Male only	Adult tubules	Sugar-water
Female (sugar)	Female only	Adult tubules	Sugar-water
Female (blood)	Female only	Adult tubules	Sampled 3 h after a blood meal

Table 2. Comparison of *Anopheles* and *Drosophila* Malpighian tubule datasets.

	Anopheles				*Drosophila*				
Function		Larval MT	Adult MT	Whole		Larval MT	Adult MT	Whole	Orthologue Relationship
V-ATPase V_0 subunits	*VATA* (A)	219	445	52	*Vha68-2* (A)	4898	6242	1905	1 –to-many
	AGAP002884 (B)	351	766	90	*Vha55* (B)	2817	4016	1071	1-to-1
	AGAP005845 (C)	570	871	275	*Vha44* (C)	2524	2131	619	1-to-1
	AGAP010298 (D)	1089	2443	619	*Vha36-1* (D)	2417	3336	859	1-to-many
	AGAP002401 (E)	69	121	23	*Vha26* (E)	3979	6448	1976	1-to-1
	AGAP002473 (F)	330	582	185	*Vha14-1* (F)	3995	3210	989	1-to-1
	AGAP001823 (G)	554	960	128	*Vha13* (G)	4227	4963	2247	1-to-1
	AGAP009486 (H)	934	1586	218	*VhaSFD* (H)	3002	3711	917	1-to-1
V-ATPase V_1 subunits	*AGAP001587* (a)	402	463	159	*Vha100-1* (a)	383	204	242	Many-to-many
	AGAP001588 (a)	4553	5900	502	*Vha100-2* (a)	1758	3657	662	Many-to-many
	AGAP003711 (a)	355	239	112	*Vha100-5* (a)	636	419	180	Many-to-many
	AGAP028154 (c)	729	1065	128	*Vha16-1* (c)	3409	3433	1308	1-to-1
	AGAP000721 (d)	163	285	94	*VhaAC39-1* (d)	1964	2650	747	1-to-1
	AGAP003588 (e)	891	1384	304	*VhaM9.7-1* (e)	416	459	178	1-to-many
					VhaM9.7-2 (e)	3032	3706	1158	1-to-many
	AGAP009334 (c")	91	155	42	*VhaPPA1-1* (c")	4187	5532	1130	1-to-many
	AGAP003879	2477	3402	514	*VhaAC45*	3690	4606	1210	1-to-1
CPA exchangers	*AGAP009446*	13	15	10	*Nhe1*	442	521	120	1-to-1
	AGAP009036	41	226	47	*Nhe2*	51	6	9	1-to-1
	AGAP008718	10	10	11	*Nhe3*	86	28	81	1-to-1
	AGAP002093	15	29	85	*Nha1*	38	155	99	1-to-1
	AGAP002324	274	697	52	*Nha2*	376	874	45	1-to-1
Na^+, K^+ - ATPase subunits	*AGAP002858*	1193	1200	360	*ATPalpha*	996	5427	683	1-to-1
	AGAP007790	768	1360	1021	*Nervana1*	2272	4556	1102	1-to-1
	AGAP007791	49	48	47	*Nervana2*	14	14	193	1-to-1
	AGAP009595	33	106	420	*Nervana3*	2	11	389	1-to-1
Water channels	*AGAP008842*	170	834	55	*Drip*	1024	589	116	1-to-1
	AGAP008843	175	580	566	*CG7777*	1785	1188	1025	1-to-1
	AGAP010325	463	2903	384	*CG4019*	1339	674	455	1-to-many
	AGAP010326	139	1887	89	*CG17644*	3	3	26	1-to-many
Chloride channels	*AGAP000943*	695	865	537	*Clic*	153	146	246	1-to-1
	GPRMGL4	29	30	18	*ClC-a*	660	1162	106	1-to-1
	AGAP007499	76	106	42	*ClC-b*	250	130	136	1-to-1
	AGAP005777	78	50	30	*ClC-c*	875	1335	317	1-to-1
	AGAP011745	10	10	11	*Best1*	300	551	163	1-to-1
Potassium channels	*AGAP001280*	245	395	64	*Ir*	844	1099	201	1-to-1
	AGAP001283	59	29	39	*Irk2*	13	805	157	1-to-many
	AGAP001284	40	86	16					
	AGAP007818	699	1949	41	*Irk3*	2898	4932	115	1-to-many
	AGAP006347	11	11	14	*KCNQ*	180	245	37	1-to-1
Additional Ion exchangers	*AGAP009736*	58	36	15	*NDAE1*	383	111	22	1-to-1
	AGAP010389	75	70	45	*Prestin*	318	339	92	1-to-1
	AGAP011426	1214	3026	46	*NaPi-T*	1490	2613	43	1-to-1
	AGAP010249	242	440	161	*CG10413*	609	741	370	1-to-1
	AGAP012251	3811	3830	1539	*Picot*	2982	3621	470	1-to-many
	AGAP009005	1189	5182	276	*ZnT35C*	1960	4645	210	1-to-many
	AGAP007752	367	3910	289	*CG15406*	626	2008	34	many-to-many
	AGAP002587	10	1022	25	*CG8028*	4193	4941	165	1-to-1
	AGAP006249	124	87	37	*CG12773*	650	216	107	1-to-1
	AGAP001557	9	9	9	*Ncc69*	16	7	70	1-to-1
Carbonic anhydrase	*AGAP010052*	379	36	115	*CAH1*	1082	978	478	1-to-1
	AGAP002359	22	1915	118					

Key transport genes in the *Drosophila* tubules and their *Anopheles* orthologues show strikingly similar patterns of expression. *Drosophila* genes identified as core components of the insect transport epitheliome are listed on the right, with colour intensity indicating the level of enriched expression compared with the whole fly average. On the left, expression of the equivalent *Anopheles* orthologues (as published in Ensembl Metazoa) in larval and adult tubules are shown with analogous enrichments. Data displayed is the average signal strength for each gene, as determined by this study and Chintapalli *et al.,* 2007b.

Table 3. Receptor expression levels in tubules

		Anopheles				*Drosophila*			
Function		Larval MT	Adult MT	Whole		Larval MT	Adult MT	Whole	Relationship
DH31-r	AGAP009770	21	82	13	CG2843	35	992	28	1-to-1
DH44-r	AGAP005464	59	34	13	CG8422	3	4	7	1-to-many
Kinin-r	AGAP010851	74	96	14	CG10626	348	348	78	1-to-1
Tyramine-r	AGAP004034	8	7	8	CG7431	37	4	3	1-to-1

Table 4. Genes more than 10x overexpressed in female blood-fed compared with female sugar-fed tubules.

Gene identifier	Description/annotation	Blood-feed enriched
AGAP010118	N-acetyltransferase	60.41
AGAP004581	Major heat shock 70 kDa protein	51.90
AGAP004793	transaminase	47.70
AGAP004793	Major facilitator superfamily (MFS)	46.99
AGAP012247	phosphoserine phosphatase	44.74
AGAP008849	D-isomer specific 2-hydroxyacid dehydrogenase	30.55
AGAP004350	Major Facilitator Superfamily	28.68
AGAP006594	Major Facilitator Superfamily	18.98
Prestin	Sulphate transporter	18.42
AGAP004633	Sulphate transporter	16.49
AGAP008900	MFS probably sugar transporter	15.40
AGAP003205	MFS probably sugar transporter	13.46
AGAP005948	kynurenine 3-monooxygenase	13.01
AGAP006958	Heat shock protein 83	11.90
AGAP000907	AMP-dependent synthetase/ligase	11.67
AGAP004212	Calreticulin (calcium binding)	11.05
AGAP000732	OATP/MFS-like sugar transporter	10.72
AGAP010725	Sulphate transporter	10.54
AGAP013063	fibroblast growth factor receptor binding	10.17

Supplementary Tables:

	Gene name	**Larval Average ± S.E.M.**	**Adult Average ± S.E.M.**	***P*-value**	**Adult enrichment**
Aquaporins:					
AGAP008842	-	1040 ± 107	6378 ± 262	0	6.1
AGAP008843	*aquaporin-8*	374 ± 53	5008 ± 246	0	13.4
AGAP010325	*aquaporin AQPAn.G*	448 ± 58	2043 ± 200	0.003	4.6
AGAP010326	*aquaporin 1*	308 ± 14	922 ± 63	0.002	3.0
AGAP008766	-	44 ± 7	23 ± 4	-	-

AGAP008767	*Aquaporin-12A*	18 ± 9	14 ± 4	-	-
AGAP010878	-	3 ± 1	2 ± 0	-	-
H^+ V-ATPase subunits					
AGAP003153	V_1 subunit A	658 ± 24	1279 ± 69	0.001	1.9
AGAP002884	V_1 subunit B	873 ± 54	1573 ± 40	0	1.8
AGAP009486	V_1 subunit SFD	1950 ± 103	3295 ± 254	0.008	1.7
AGAP010298	V_1 subunit D	2546 ± 82	4913 ± 226	0.001	1.9
AGAP002401	V_1 subunit E	258 ± 20	346 ± 45	0.148	-
AGAP002473	V_1 subunit F	805 ± 21	1555 ± 204	0.034	1.9
AGAP001823	V_1 subunit G	1511 ± 49	2532 ± 269	0.03	1.7
AGAP001588	V_0 subunit a	11588 ± 297	14132 ± 612	0.017	1.2
AGAP028154	V_0 subunit c	2347 ± 276	3245 ± 385	0.112	-
AGAP009334	V_0 subunit c"	208 ± 17	381 ± 20	0.001	1.8
AGAP003588	V_0 subunit e,h	2573 ± 160	3671 ± 154	0.003	1.4
AGAP000721	V_0 subunit d	47 ± 5	108 ± 16	0.028	2.3

Supplementary table 1: Expression of *Anopheles* aquaporins and H^+ V-ATPase subunits as determined by microarray. Where no *P*-value is present, the gene was not detected in both the larval and adult data-sets. Where no enrichment value is present, the *P*-value showed no significant difference between the larval and adult samples.

Figures.

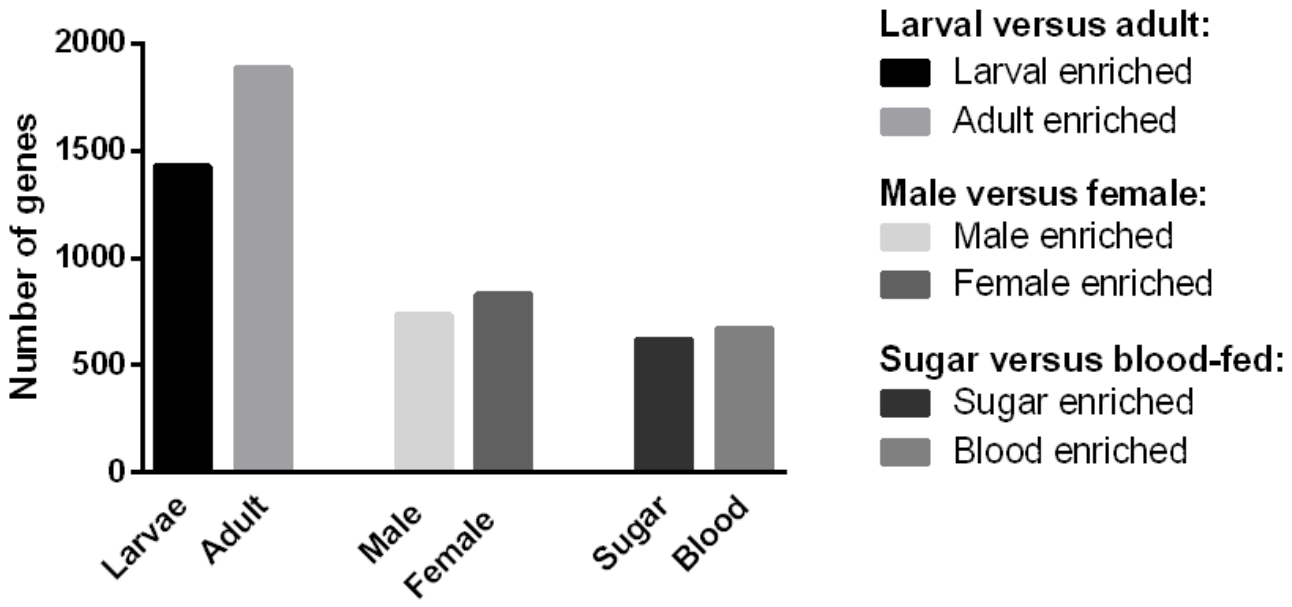

Fig. 1.

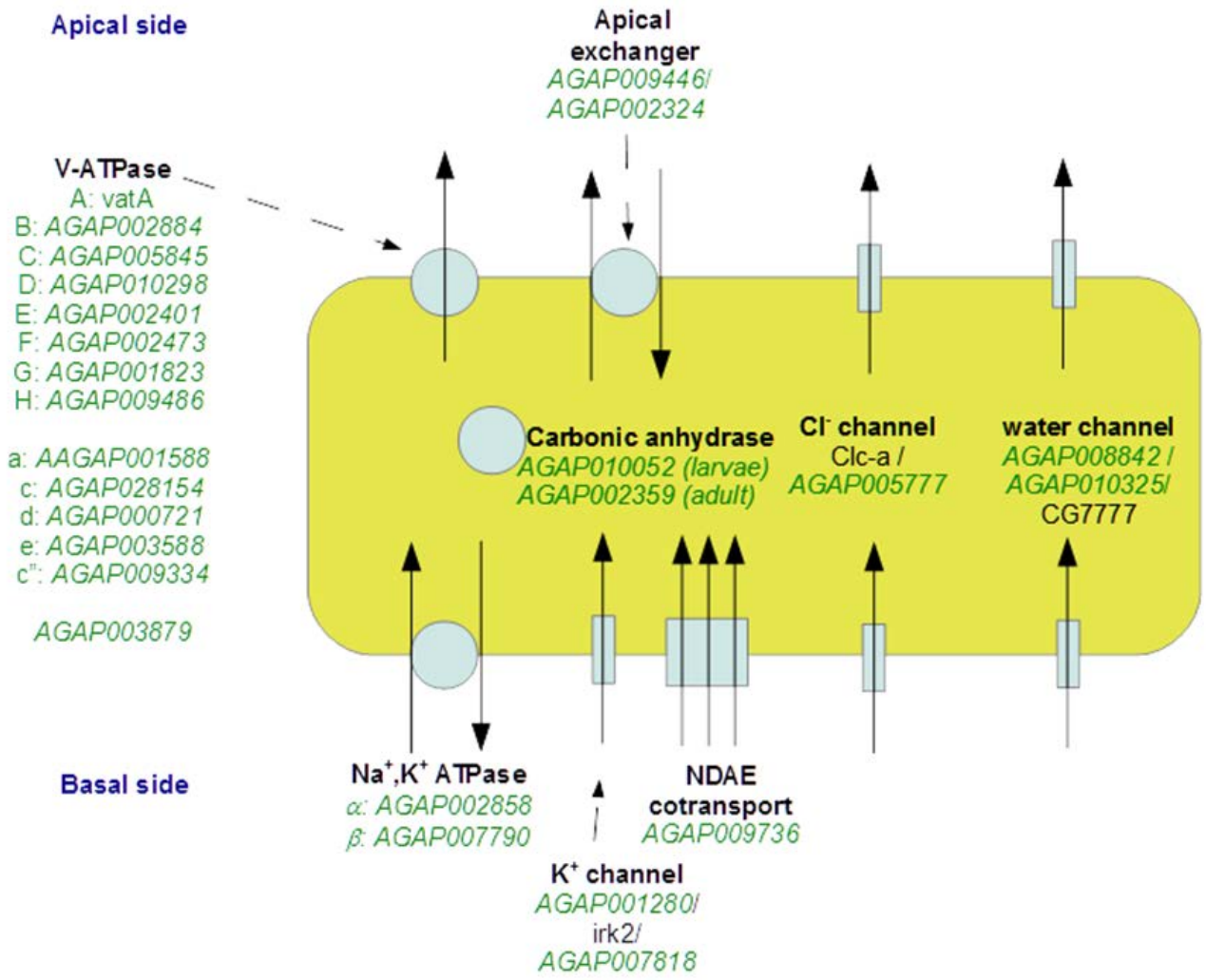
Apical side
Apical exchanger
AGAP009446/
AGAP002324
V-ATPase
A: vatA
B: AGAP002884
C: AGAP005845
D: AGAP010298
E: AGAP002401
F: AGAP002473
G: AGAP001823
H: AGAP009486
a: AAGAP001588
c: AGAP028154
d: AGAP000721
e: AGAP003588
c'': AGAP009334
AGAP003879
Carbonic anhydrase
AGAP010052 (larvae)
AGAP002359 (adult)
Cl- channel
Clc-a /
AGAP005777
water channel
AGAP008842 /
AGAP010325/
CG7777
Basal side
Na+,K+ ATPase
α: AGAP002858
β: AGAP007790
NDAE
cotransport
AGAP009736
K+ channel
AGAP001280/
irk2/
AGAP007818

Fig . 2.

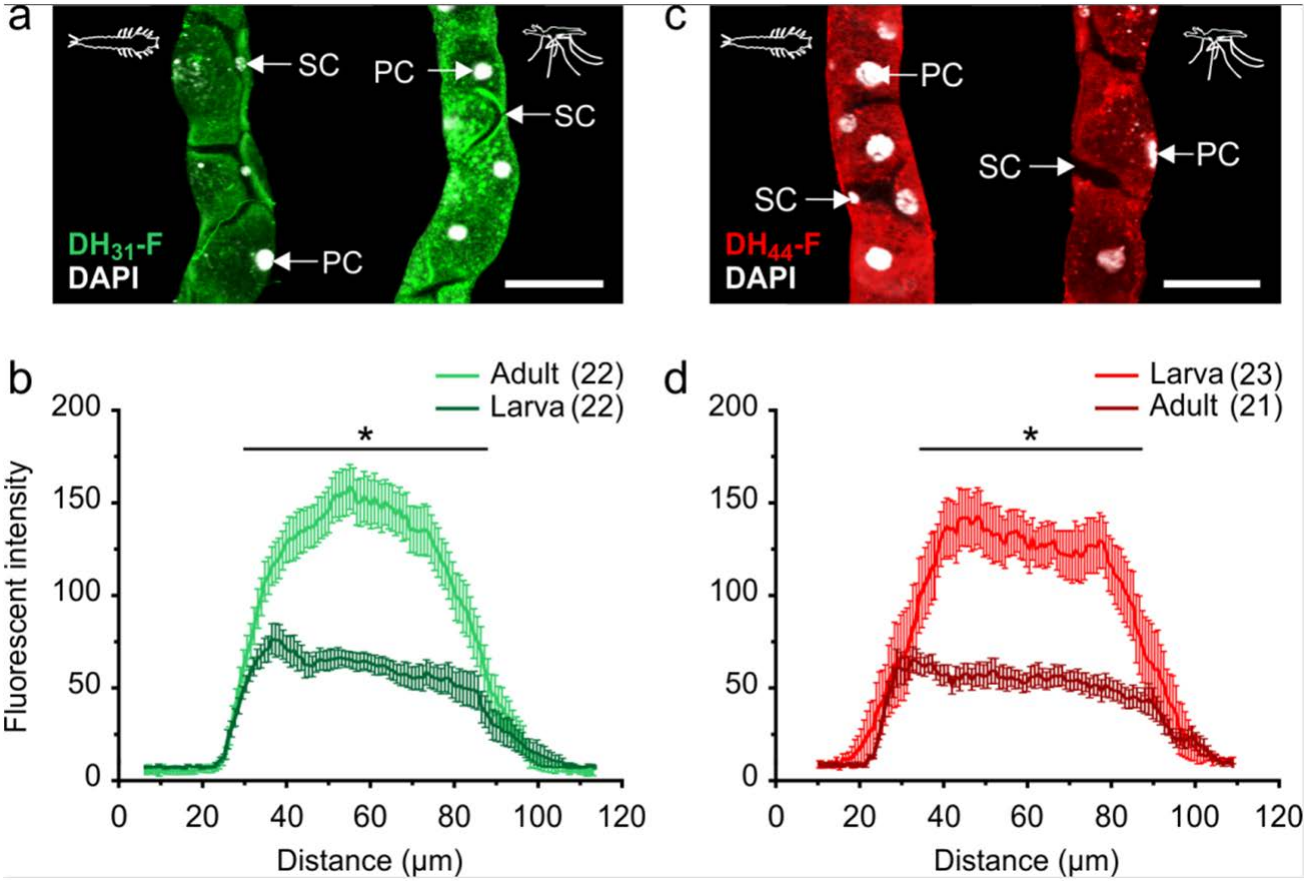
a
SC
PC
SC
PC
DH31-F
DAPI
c
PC
SC
SC
PC
DH44-F
DAPI
b
Adult (22)
Larva (22)
*
Fluorescent intensity
200
150
100
50
0
0
20
40
60
80
100
120
Distance (μm)
d
Larva (23)
Adult (21)
*
200
150
100
50
0
0
20
40
60
80
100
120
Distance (μm)

Fig. 3.

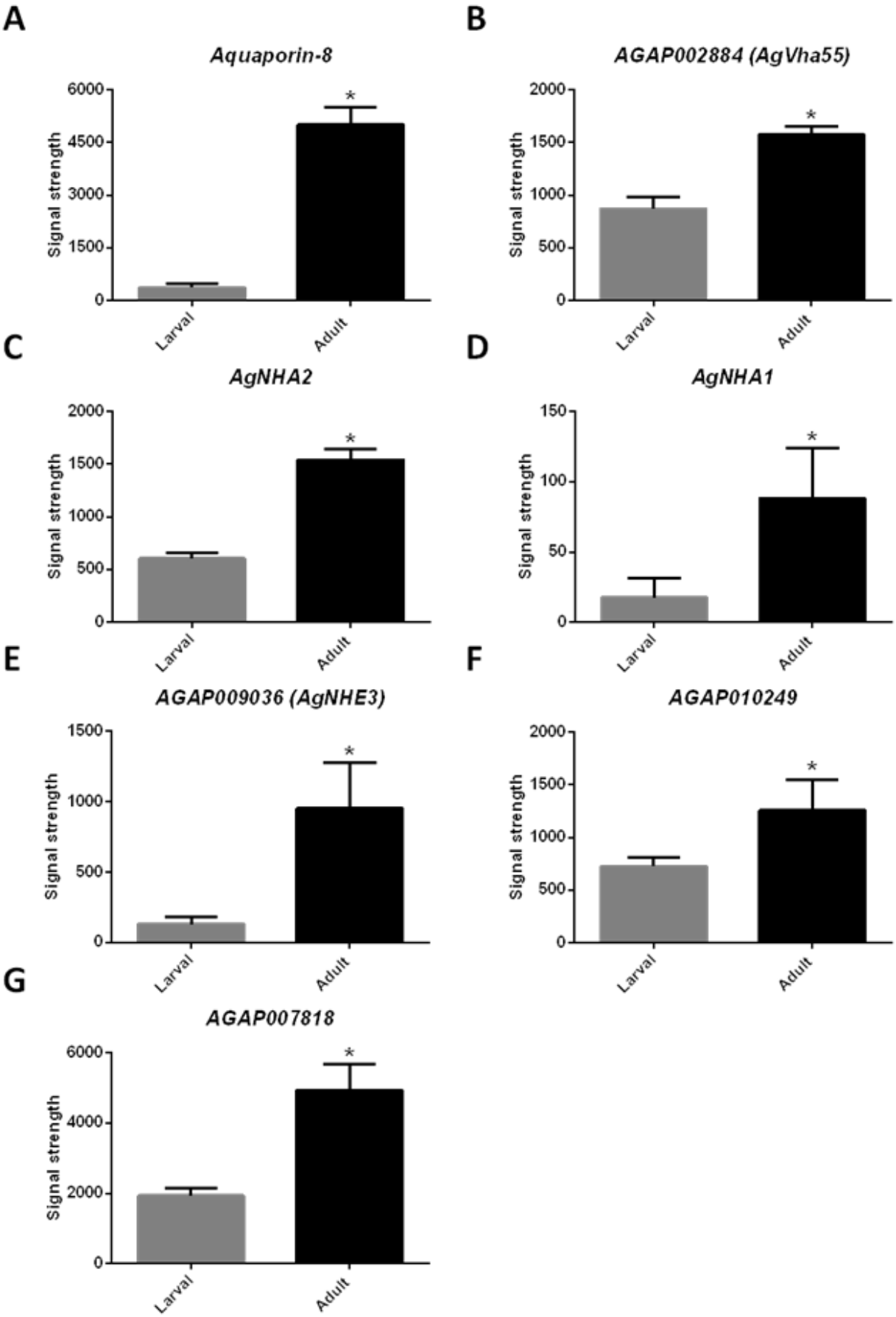
A
Aquaporin-8
Signal strength
6000
4500
3000
1500
0
*
Larval
Adult
B
AGAP002884 (AgVha55)
Signal strength
2000
1500
1000
500
0
*
Larval
Adult
C
AgNHA2
Signal strength
2000
1500
1000
500
0
*
Larval
Adult
D
AgNHA1
Signal strength
150
100
50
0
*
Larval
Adult
E
AGAP009036 (AgNHE3)
Signal strength
1500
1000
500
0
*
Larval
Adult
F
AGAP010249
Signal strength
2000
1500
1000
500
0
*
Larval
Adult
G
AGAP007818
Signal strength
6000
4000
2000
0
*
Larval
Adult

Fig. 4.

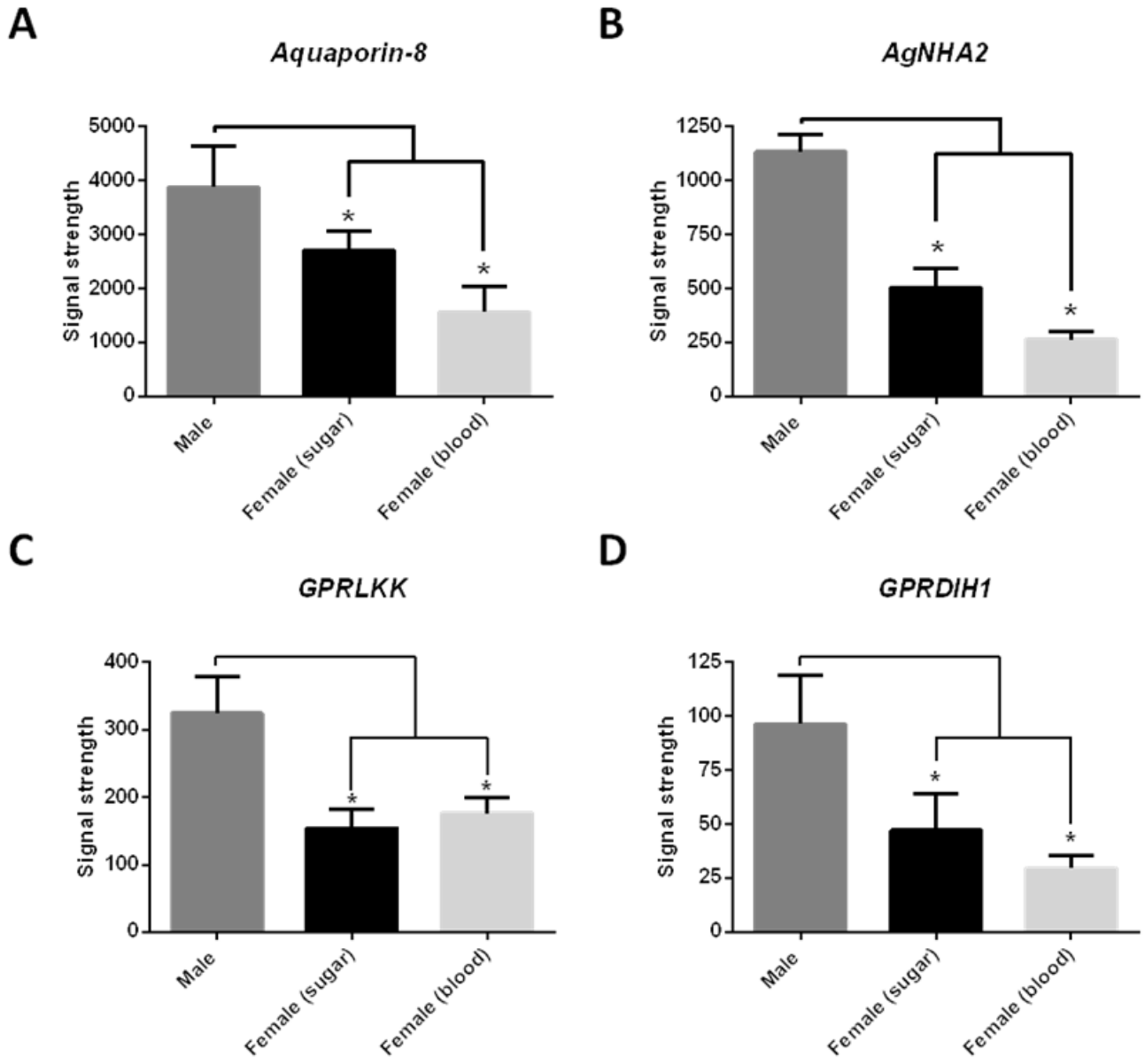
A
Aquaporin-8
Signal strength
5000
4000
3000
2000
1000
0
Male
Female (sugar)
Female (blood)
B
AgNHA2
Signal strength
1250
1000
750
500
250
0
Male
Female (sugar)
Female (blood)
C
GPRLKK
Signal strength
400
300
200
100
0
Male
Female (sugar)
Female (blood)
D
GPRDIH1
Signal strength
125
100
75
50
25
0
Male
Female (sugar)
Female (blood)

Fig. 5.

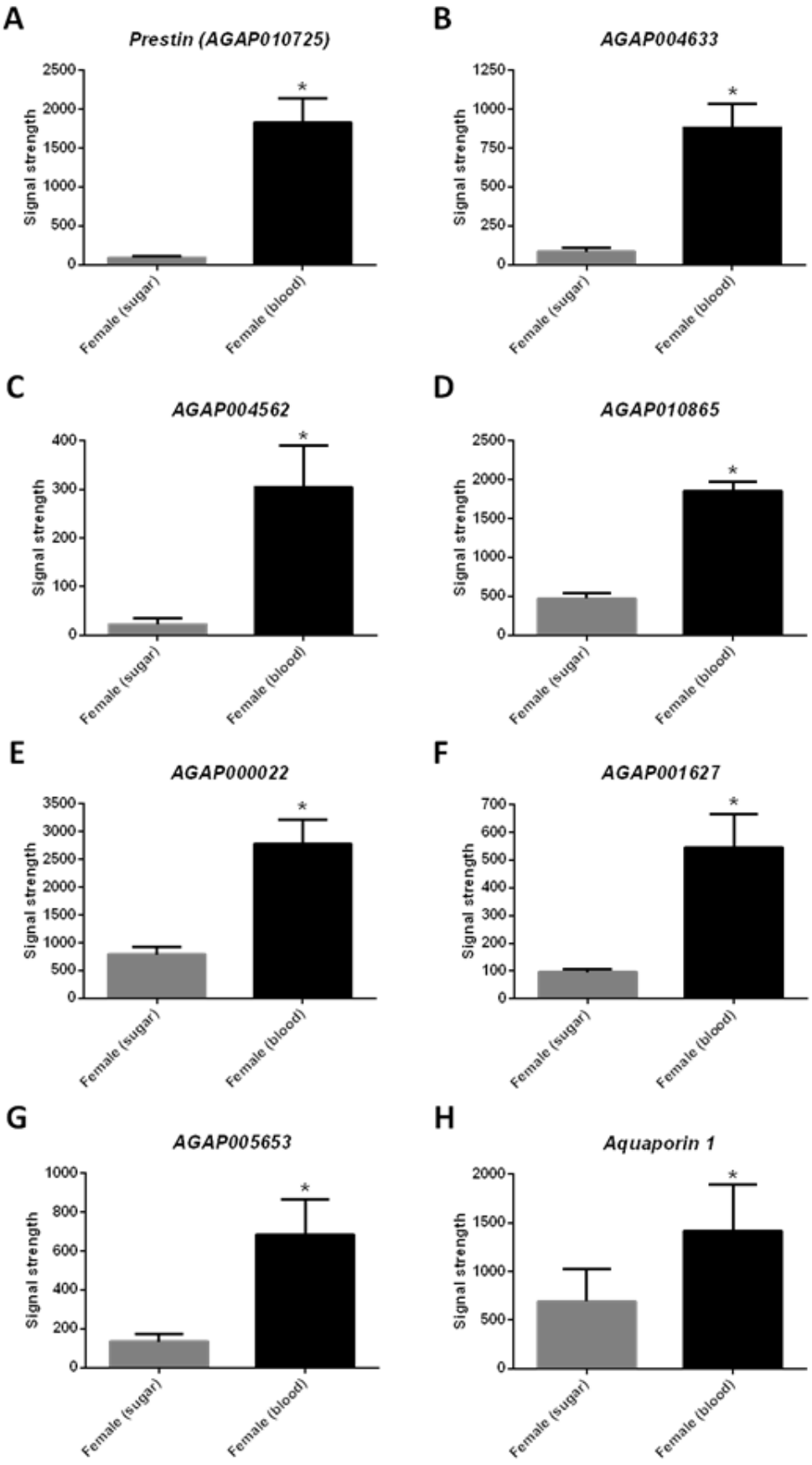
A
Prestin (AGAP010725)
Signal strength
2500
2000
1500
1000
500
0
*
Female (sugar)
Female (blood)
B
AGAP004633
Signal strength
1250
1000
750
500
250
0
*
Female (sugar)
Female (blood)
C
AGAP004562
Signal strength
400
300
200
100
0
*
Female (sugar)
Female (blood)
D
AGAP010865
Signal strength
2500
2000
1500
1000
500
0
*
Female (sugar)
Female (blood)
E
AGAP000022
Signal strength
3500
3000
2500
2000
1500
1000
500
0
*
Female (sugar)
Female (blood)
F
AGAP001627
Signal strength
700
600
500
400
300
200
100
0
*
Female (sugar)
Female (blood)
G
AGAP005653
Signal strength
1000
800
600
400
200
0
*
Female (sugar)
Female (blood)
H
Aquaporin 1
Signal strength
2000
1500
1000
500
0
*
Female (sugar)
Female (blood)

Fig. 6.

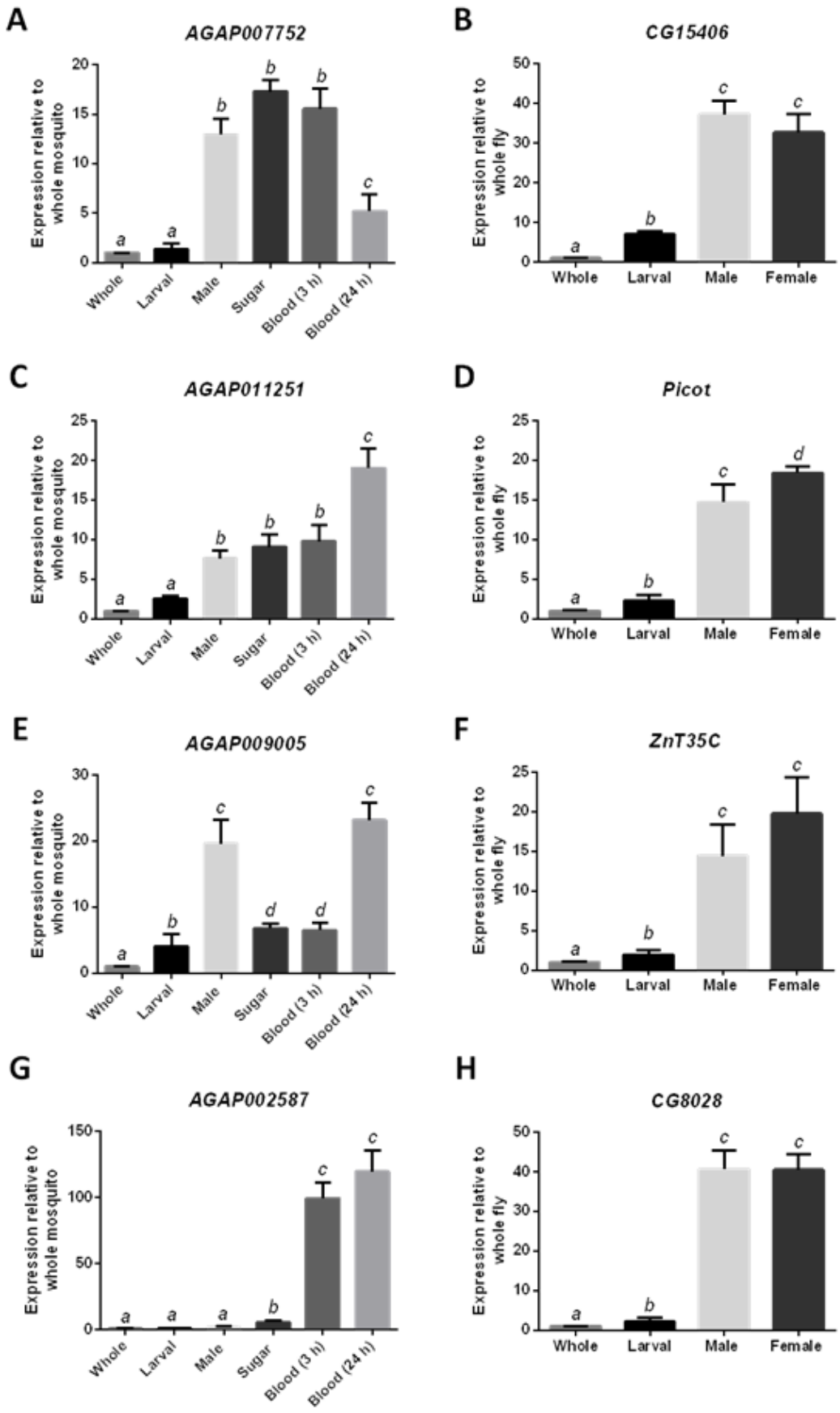
A
AGAP007752
Expression relative to whole mosquito
Whole
Larval
Male
Sugar
Blood (3 h)
Blood (24 h)
B
CG15406
Expression relative to whole fly
Whole
Larval
Male
Female
C
AGAP011251
Expression relative to whole mosquito
D
Picot
Expression relative to whole fly
E
AGAP009005
Expression relative to whole mosquito
F
ZnT35C
Expression relative to whole fly
G
AGAP002587
Expression relative to whole mosquito
H
CG8028
Expression relative to whole fly

Fig. 7.

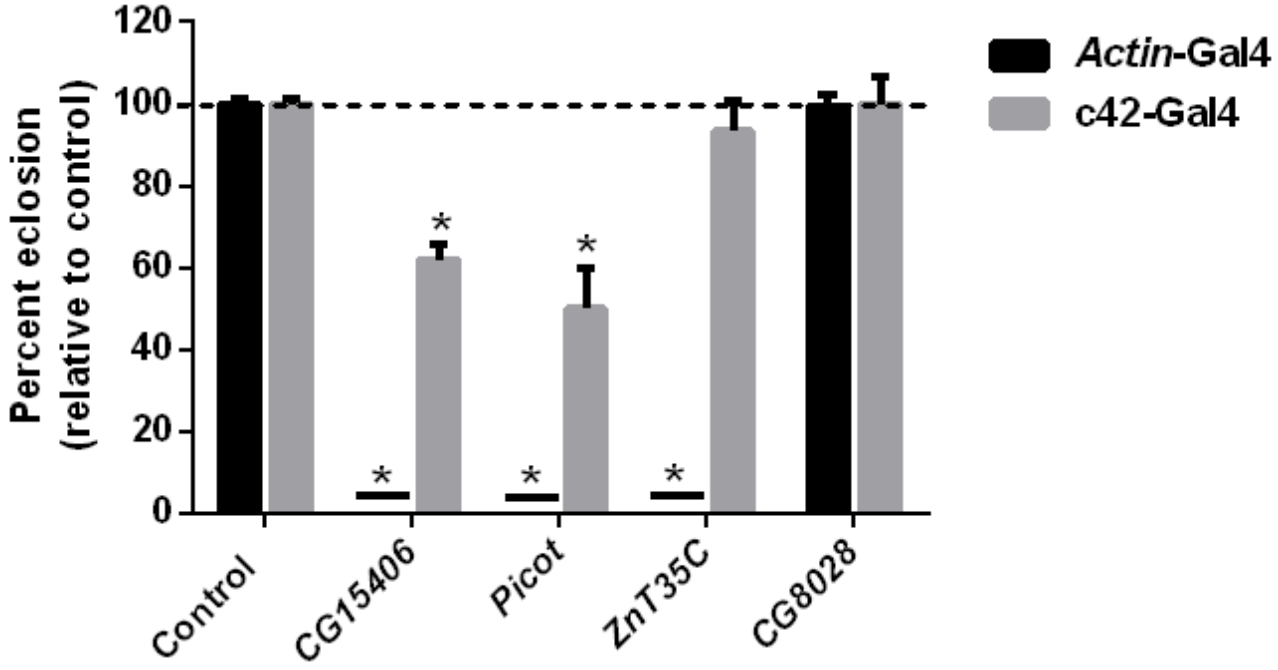
Percent eclosion
(relative to control)
120
100
80
60
40
20
0
*
*
*
*
*
Control
CG15406
Picot
ZnT35C
CG8028
Actin-Gal4
c42-Gal4

Fig. 8.