THE MOLECULAR CHARACTERIZATION OF A

DIURETIC HORMONE RECEPTOR (GPRDIH1) FROM FEMALES OF THE

YELLOW FEVER MOSQUITO, *Aedes aegypti* (L.)

A Dissertation

by

CHRISTOPHER LLOYD JAGGE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2009

Major Subject: Entomology

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Approved by:

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ABSTRACT

The Molecular Characterization of a

Diuretic Hormone Receptor (GPRdih1) from Females of the

Yellow Fever Mosquito, *Aedes aegypti* (L.). (December 2009)

Christopher Lloyd Jagge, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Patricia V. Pietrantonio

In the yellow fever mosquito, *Aedes aegypti* (L.), hemolymph-circulating diuretic hormones act upon the renal organs (Malpighian tubules) to regulate primary urine composition and secretion rate; however, the molecular endocrine mechanisms underlying rapid water elimination upon adult eclosion and blood feeding are not fully understood. Bioinformatic analysis of the current *Aedes aegypti* genome assembly reveals only a single predicted corticotropin releasing factor (CRF)-like diuretic hormone 44 (DH_{44}) gene, but two DH_{44} receptor genes. The tissue expression profiles of the DH_{44} receptor(s), and specifically the identity of the DH_{44} receptor(s) in the Malpighian tubule, are undetermined in any mosquito species.

This dissertation shows that Vectorbase gene ID AAEL008292 encodes a DH_{44} receptor (*Aaeg*GPRdih1) transcribed in Malpighian tubules. Sequence analysis and transcript localization indicate that *Aaeg*GPRdih1 is the co-ortholog of the *Drosophila melanogaster* DH_{44} receptor (CG12370-PA). The presence of conserved amino acid residues between *Aaeg*GPRdih1 and vertebrate CRF receptors suggests this mosquito

receptor modulates multiple G protein-dependent intracellular signaling pathways. Quantitative PCR analysis of a time course of Malpighian tubule cDNA reveals *Aaeg*GPRdih1 abundance increases paralleling periods of observed urination. This suggests that target tissue receptor biology is linked to the known periods of release of diuretic hormones from the nervous system, pointing to a common up-stream regulatory mechanism. Higher relative abundance of *Aaeg*GPRdih1 transcript in female Malpighian tubules 24 hours after blood feeding suggests a role for *Aaeg*GPRdih1 in the excretion of nitrogen waste. RNA-mediated silencing to establish the significance of *Aaeg*GPRdih1 to mosquito Malpighian tubule physiology was inconclusive.

ACKNOWLEDGMENTS

Without the help and assistance of many individuals, the work in this dissertation would not have been possible. It is not possible to name without exclusion all those whose advice, assistance, and technical expertise have been greatly appreciated and absolutely essential to my work, but I would like to thank most especially Dr. Patricia Pietrantonio, my committee chair and advisor, and before that employer, who recruited me to the program, helped me secure a competitive assistantship from the department, and provided magnificent facilities and precious resources. Never once was funding a hindrance to research, and from her I learned that "cheap is expensive!" Patricia's creativity and vision were the source of ideas from which I drew, and her expertise and prodding were the foundation on which I built. Thank you Patricia.

Additionally, the graduate faculty and support staff of the Department of Entomology did much to support, encourage, and enhance my work. Thank you.

My lovely wife Jenny and my brother William provided me each in their own way the emotional strength I needed to endure the most difficult challenge of my life.

My parents deserve special thanks for training up a free thinking and independent individual. I don't know how they did it.

And lastly to the humble mosquito for yielding me some of her secrets.

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CHAPTER I

INTRODUCTION[*]

Yellow Fever Mosquito *Aedes aegypti* Biology and Impact as a Pathogen Vector

The yellow fever mosquito, *Aedes aegypti* (Linnaeus), is a holometabolous Nematoceran fly in the order Diptera (Family: Culicidae). Native to Africa, *Aedes aegypti* is an introduced species in other tropical and subtropical areas of the world. *Aedes aegypti* females oviposit on wet surfaces, or at the junction of a solid surface and a pool of water. Subsequent submersion of the eggs stimulates hatching. First instar larvae emerge from small, 0.6 mm eggs (Buxton & Hopkins, 1927) and undergo three larval molts. Fourth instar larvae (7-8 mm) molt to a pupal stage which undergoes ecdysis to the adult. Other than the terrestrial egg and adult, all stages are aquatic. Adult mosquitoes, both male and female, will feed on sugar-water sources such as flower nectar; however, 24-40 hours after emergence (Bishop & Gilchrist, 1946) females seek a large, protein-rich blood meal, which stimulates oogenesis. This blood meal volume may exceed the female's hemolymph volume by ten times (Briegel, 1990; Beyenbach, 2003b). The obligatory haematophagia of female mosquitoes directly relates to their capacity to vector human pathogens including the flaviviruses which cause yellow fever, dengue fever and dengue hemorrhagic fever (Gubler, 2002; Gubler,

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This dissertation follows the style and format of *Insect Molecular Biology*.

[*] Portions of this chapter reprinted with permission from "Diuretic hormone 44 receptor in Malpighian tubules of the mosquito *Aedes aegypti*: evidence for transcriptional regulation paralleling urination." by C.L. Jagge and P.V. Pietrantonio, 2008. *Insect Molecular Biology,* 17:413-426 , © 2008 Blackwell Publishing Ltd.1

2004; Gubler, 2006). A yellow fever vaccine has been developed, but distribution and administration within remote or politically unstable endemic areas is difficult and expensive (Wolfson *et al.*, 2009). There is no commercial vaccine for the dengue virus, contracted annually by 50-100 million people worldwide (Gubler, 2002). With the ease of international and intercontinental travel, the impact and occurrence of these diseases is not limited to developing countries (Wilder-Smith & Gubler, 2008).

The most effective means of disease control and prevention has been to directly control the mosquito vector (Luz *et al.*, 2009); however, resistance to several classes of pesticides has been observed in these mosquitoes, including those most widely used for vector population control: synthetic pyrethroids, organophosphates, and organochlorines (Canyon & Hii, 1999; Sunaiyana *et al.*, 2006; Montella *et al.*, 2007; Rodriguez *et al.*, 2007). Insecticide resistance by vector populations threatens the success of health campaigns targeting these diseases (Zaim & Guillet, 2002). The long-term goal for blood-feeding vector borne disease control must include the development of new pesticides with novel and selective modes of action.

Mosquito Urination

Mosquito females demonstrate two observable periods of heightened fluid evacuation (urination): (1) Gillet (1983) observed a high rate of urination commencing immediately upon adult emergence from the pupal exuviate; the rate rapidly dropped to basal levels within minutes but is followed by a gradual, steady increase, peaking 11-17 hours after eclosion. This post-eclosion peak of urination then declines to basal levels

on the second day after emergence. During this first day, the newly emerged adult voids water and nitrogen waste resulting from the catabolism of larval muscle tissues and retained during the pupal stage (Romoser *et al.*, 2000; von Dungern & Briegel, 2001a). (2) The female's urination rate increases after consuming a protein-, ion-, and water-rich blood meal (Stobbart, 1977; Williams *et al.*, 1983). Human blood plasma is hypo-osmotic to mosquito hemolymph (295 mOsmol/kg compared to 354 mOsmol/kg), and contains a greater concentration of sodium (Williams *et al.*, 1983) so the excess water and sodium are voided. The postprandial urination proceeds rapidly, with approximately 40% of the blood-meal volume (water) eliminated one hour after feeding (Williams *et al.*, 1983). Additionally, engorgement increases the difficulty in flying, so a rapid urination reduces the chance of predation (Roitberg *et al.*, 2003; Nelson & Jackson, 2006). The ion composition of the postprandial urine is not constant. Urine is highly enriched in sodium ions within the minutes following the blood meal, but after 30 minutes the potassium content of the urine begins to rise, and after 2 hours, the potassium concentration peaks (Williams *et al.*, 1983; Beyenbach, 2003b; Coast *et al.*, 2005).

Disruption of the physiological process of urination has been proposed as a method of insect control (Keeley *et al.*, 1992; Gäde, 2004; Pietrantonio *et al.*, 2001; Pietrantonio *et al.*, 2005) because of the precise and coordinated control of this rapid diuresis and excretion.

Malpighian Tubule Function and Anatomy

Insect renal organs were first described in the seventeenth century by the Italian scientist Marcello Malpighi. These eponymous "Malpighian" tubules structurally originate at the junction of the posterior midgut and anterior hindgut (Figure 1.1); however, the number varies among insect species. For example, the mosquitoes (Diptera: Culicidae) have five Malpighian tubules (Beyenbach *et al.*, 1993), fruit flies (Diptera: Drosophilidae) have four (Wessing & Eichelberg, 1978), the fire ant *Solenopsis saevissima* (Hymenoptera: Formicidae) has six (Arab & Caetano, 2002), the American cockroach *Periplaneta americana* (Blattaria: Blattidae) has over 150 (Wall *et al.*, 1975), and the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae) has over 200 (Savage, 1956).

For each Malpighian tubule, a single cell epithelium forms a "tube-like structure" with an internal lumen: the distal end of each organ is closed (blind-ended) and surrounded by the insect hemolymph, while the proximal end empties into the pylorus of the hindgut (Jones & Brandt, 1981) (Figure 1.1). The Malpighian tubules and hindgut work in tandem to regulate the water and solute composition of the hemolymph. Similar to renal organs in other animals, the *Aedes aegypti* Malpighian tubules continuously function, producing primary urine with a combined rate of approximately 4.6 µl per day, twelve times the hemolymph volume.
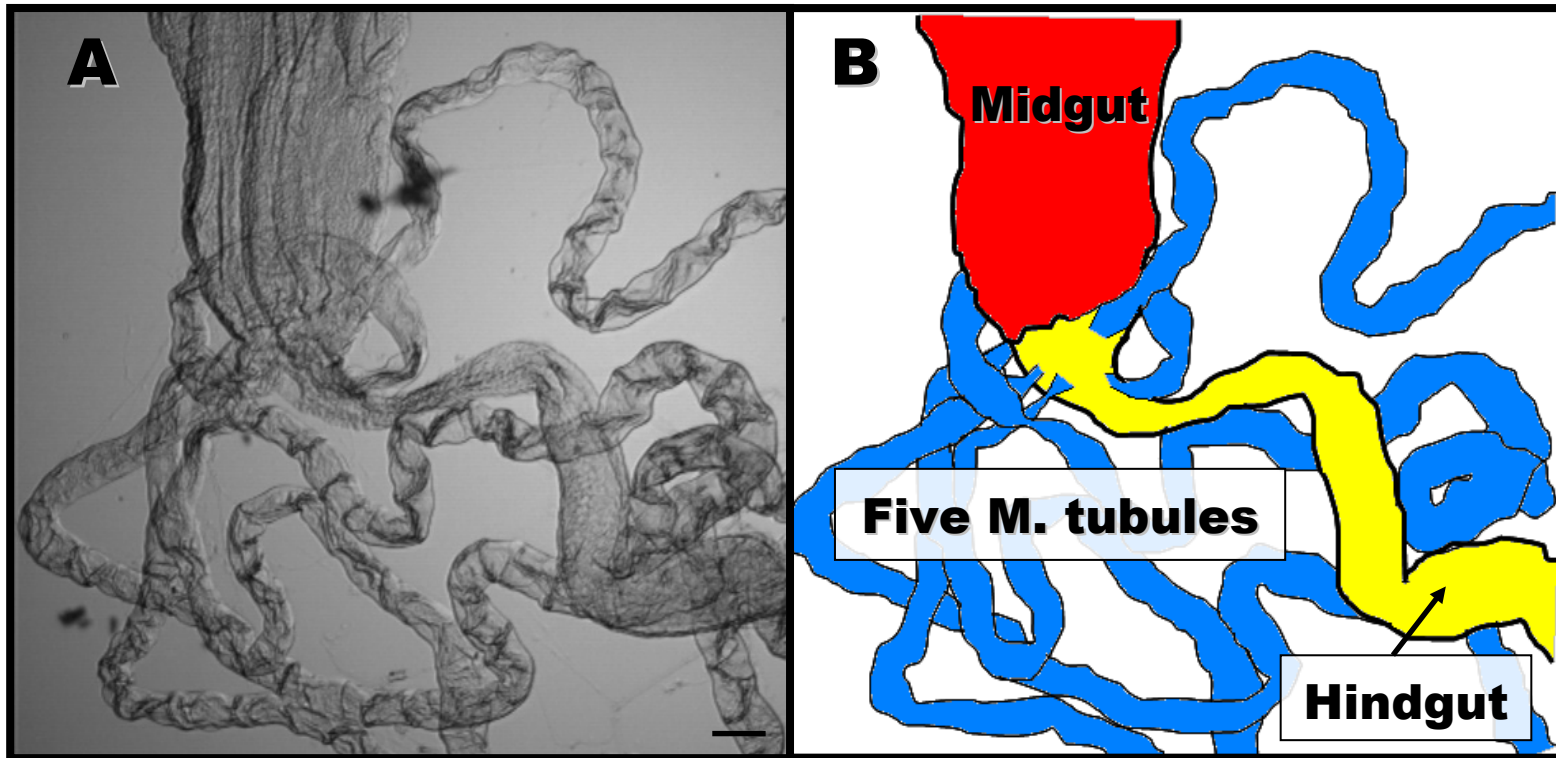
Figure 1.1   Light micrograph (A) and colored schematic (B) highlighting the general morphology of the posterior alimentary canal and excretory system of unfed females of *Aedes aegypti*. The proximal end of each of the five functionally identical Malpighian (M.) tubules (blue) (Beyenbach *et al.*, 1993) joins the alimentary canal at the junction of the midgut (red) and hindgut (yellow). The M. tubules are also extensively connected to and interact with the respiratory system of the mosquito (not shown) by a network of very fine trachea (Pietrantonio *et al.*, 2000; Pietrantonio *et al.*, 2001). Solid black line in panel (A) is 50 μm bar.

Water and/or ions may then be reabsorbed into the hemolymph either in the proximal Malpighian tubule or hindgut (Beyenbach, 2003b; Coast, 2007). Malpighian tubules also transport xenobiotics and metabolic waste into the alimentary canal for excretion, most notably excess nitrogen in the form of uric acid and urea (von Dungern & Briegel, 2001a) resulting from protein catabolism.

In *Aedes aegypti* females, each of the five, ~3 mm long, functionally homogenous Malpighian tubules (Beyenbach *et al.*, 1993) is composed of approximately 54 cells (Cabrero *et al.*, 2004) of two different cell types. The larger, bulbous, principal cells comprise ~80% of the total cell number of each Malpighian tubule, and are arranged alternately along the length of the tubule, forming most of the length, circumference, and mass of each (Christophers, 1960; Clements, 1992; Weng *et al.*, 2008) (Figure 1.2). Seemingly identical principal cells may have ion transport capacity variations which are difficult to determine (Cabrero *et al.*, 2004; Patrick *et al.*, 2006; Pullikuth *et al.*, 2006).

The smaller, more slender and elongated stellate cells, comprise ~20 % of the total cell number of each Malpighian tubule, with each having cellular projections (hence "star" shaped) that place them in direct physical contact with the adjacent 3 or 4 principal cells (Patrick *et al.*, 2006). Stellate cells are only present in the distal two-thirds of each organ (Patrick *et al.*, 2006). Each cell of a Malpighian tubule is connected to adjacent cells via gap junctions though the exact localization and distribution remain to be resolved (Weng *et al.*, 2008). The hemolymph-facing basal lamina (Clements, 1992) of each Malpighian tubule is extensively infolded into the surface of each
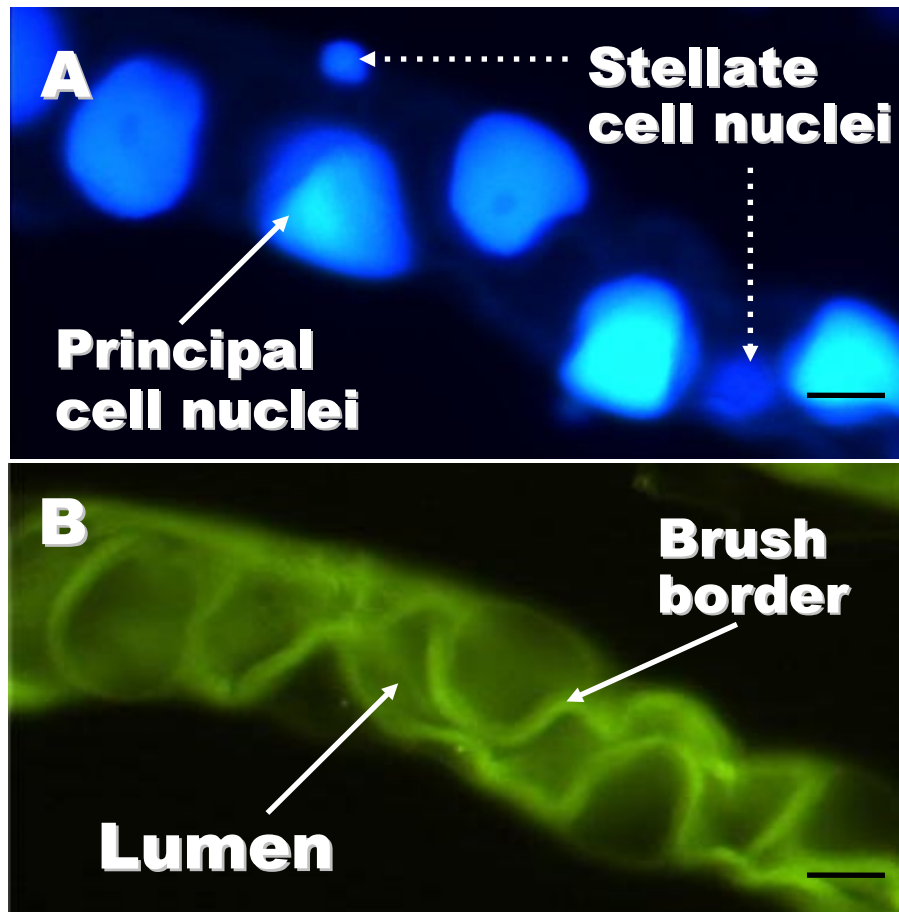
Figure 1.2   Whole mount fluorescence micrographs of a portion of the distal Malpighian tubule from a female of *Aedes aegypti*. (A) Nucleic acid staining using DAPI (4',6-diamidino-2-phenylindole) shows the nucleus of the two cell types comprising the Malpighian (M.) tubule: the larger, more numerous principal cells (solid arrows) and the smaller and fewer stellate cells (dashed arrows). (B) The M. tubules have a natural, green auto-fluorescence when excited by UV light and observed with a FITC filter. Notice in this longitudinal image, the principal cell apical membrane brush border and the M. tubule lumen (white arrows) can be clearly discerned. Black line in both panels (lower right corner) is 25 μm.

principal cell. The apical plasma membrane of each principal cell is also densely folded with numerous microvilli forming a brush-border, greatly increasing the surface area of the lumen-facing side of each principal cell. Each microvillus in the brush border contains a mitochondrion (Beyenbach, 2003b). The basal and apical membranes of each stellate cell are also extensively folded; however, the microvilli comprising the brush border of the stellate cell's apical membrane lack mitochondria (Clements, 1992).

Malpighian Tubule Transport

Diuresis, the production of primary urine production by Malpighian tubules, occurs as a result of the transport of ions (Na^+/K^+/Cl^-) across the basal and apical membranes and the establishment of a transepithelial voltage and osmotic gradients. Water from the hemolymph then follows the ion-driven osmotic gradient into the Malpighian tubule lumen (Pannabecker, 1995; O'Donnell & Spring, 2000; Dow & Davies, 2001; O'Donnell *et al.*, 2003; Dow & Davies, 2003; Beyenbach, 2003b; Rheault & O'Donnell, 2004; Coast, 2007). The Malpighian tubules also contain an extensive array of transporters for the active transport of organic solutes (Wang *et al.*, 2004; Chintapalli *et al.*, 2007). The composition of the urine voided by the mosquito may be quite different than the composition of the primary urine since selective ion and/or water recycling occurs in the hindgut (Beyenbach, 2003b; Coast, 2007). In *Aedes aegypti*, the Malpighian tubules secrete a fluid approximately isosmotic to the hemolymph; however, the volume and solute composition of the primary urine varies depending on hormonal

regulation and on the diet of the animal (nectar or blood) (Williams *et al.*, 1983; Clements, 1992; Coast *et al.*, 2005).

The transport activity of *Aedes aegypti* Malpighian tubule is energized by a vacuolar ATPase (V-ATPase), a multi-subunit protein pump which actively transports protons from the cytosol, across the apical membrane and into the Malpighian tubule lumen (Weng *et al.*, 2003; Wieczorek *et al.*, 2009). The V-ATPase is only present in principal cells (Patrick *et al.*, 2006). Using the inhibitor bafilomycin, V-ATPase functionality and fluid transport in the Malpighian tubule can be completely inhibited, indicating the V-ATPase is solely responsible for the polarization of the apical membrane (Beyenbach *et al.*, 2000; Weng *et al.*, 2003). The V-ATPase is ATP dependent, hydrolyzing ATP to ADP as an energy source, which results in the expulsion to the lumen of two protons for each molecule of ATP (Beyenbach & Wieczorek, 2006). This high energy demand explains the observation that each microvillus of the brush border contains an ATP-generating mitochondrion (Beyenbach, 2003b). Apical membrane cation (Na^+/K^+) antiporters then exchange lumen protons for cellular cations, moving cations into the lumen while maintaining a stable lumen pH of 7.19±0.09 (Petzel *et al.*, 1999). A recently identified cation antiporter (AeNHE8) from *Aedes aegypti* has been localized by immunohistochemistry to the apical membrane and is capable of exchanging sodium and potassium ions for protons (Kang'ethe *et al.*, 2007). AeNHE8 has also been localized to subapical compartments (Piermarini *et al.*, 2009). The contribution of AeNHE8 to ion transport is currently undetermined. Two other distinct cation-proton antiporters (AgNHA1, AgNHA2) localized to the Malpighian tubule

apical membrane have been identified from *Anopheles gambiae* larvae (Rheault *et al.*, 2007) and have orthologs in *Drosophila melanogaster* (Day *et al.*, 2008), but it is currently unknown if these are expressed in Malpighian tubules of adult mosquitoes.

The V-ATPase holoenzyme is composed of at least 14 different protein subunits (Beyenbach & Wieczorek, 2006) which can be grouped into two dissociable protein complexes. The V_1 complex, which in the holoenzyme is responsible for the hydrolysis of ATP to ADP, is soluble and cytosolic. The V_0 complex, which functions as the proton channel, is membrane bound. Only the V_1-V_0 holoenzyme is able to energize the Malpighian tubule (Beyenbach & Wieczorek, 2006). Antibodies generated against the B subunit of the V_1 complex localized the complex to the cytoplasm and apical membrane of principal cells in *Aedes aegypti* (Weng *et al.*, 2003; Patrick *et al.*, 2006). In *Aedes aegypti* Malpighian tubules, blocking protein kinase A (PKA) eliminated the normal acidification of the cytosol observed in cAMP-stimulated Malpighian tubules, likely by preventing V-ATPase holoenzyme assembly (Petzel *et al.*, 1999). In the salivary gland of the dipteran *Calliphora vicina*, cAMP activates PKA phosphorylation of subunit C of the V_1 complex inducing the translocation of the V_1 complex from the cytoplasm to the apical membrane (Voss *et al.*, 2007; Rein *et al.*, 2008). Subunit C participates in the interaction between the V_0 and V_1 complexes (Inoue & Forgac, 2005). This apparent regulatory mechanism of V-ATPase holoenzyme assembly is likely also active in the Malpighian tubule principal cells: an increase in cAMP would result in an increase in V-ATPase holoenzyme assembly, generating an increase in ion and fluid movement into the lumen.

Cations have several routes to cross the Malpighian tubule basal membrane. Pharmacological studies using channel and transporter blockers established the presence of cation channels and a cation-chloride ($Na^+/K^+/2Cl^-$) co-transporter in the basal membrane of *Aedes aegypti* Malpighian tubules (Petzel *et al.*, 1987; Hegarty *et al.*, 1991; Scott *et al.*, 2004) (Figures 1.3 and 1.4). The movement of ions via this co-transporter is coupled to the concentration of sodium ions as a result of the favorable electrochemical gradient established by the action of the apical membrane V-ATPase. A cation-chloride co-transporter has been identified in midguts of *Aedes aegypti* females (Filippov *et al.*, 2003); however, the precise molecular identity of the basal co-transporter has not been established in mosquitoes. A Na^+-dependent co-transporter from *Maduca sexta* is abundant in Malpighian tubules of that lepidopteran (Reagan, 1995b; Gillen *et al.*, 2006). A cation-proton exchanger (AeNHE3) localized in the basal membrane of proximal and distal principal cells has been identified in *Aedes aegypti* (Pullikuth *et al.*, 2006). AeNHE3 belongs to the Cation Proton Antiport (CPA) superfamily of metal/proton exchangers (Brett *et al.*, 2005; Rheault *et al.*, 2007), and moves extracellular cations into the cell in exchange for intracellular protons towards the hemolymph (Pullikuth *et al.*, 2006). Additionally, a Na^+/K^+–ATPase is present in the basal membrane of all stellate cells and proximal principal cells in *Aedes aegypti*, providing a means to actively exchange extracellular potassium for intracellular sodium ions, thus moving sodium from the cell into the hemolymph (Patrick *et al.*, 2006). Since immunohistochemical studies suggest functional and transport capacity variations exist along the length of the Malpighian tubules (Cabrero *et al.*, 2004; Patrick *et al.*, 2006;

Pullikuth *et al.*, 2006), additional co-localization studies are needed to determine the ion transport potential of any specific principal cell. For example, localization of a the cation-proton exchanger AeNHE3 in the basal membrane of principal cells in distal (blind end) Malpighian tubule (Pullikuth *et al.*, 2006), and absence of a Na^+/K^+–ATPase in the same cells (Patrick *et al.*, 2006) suggest those cells are specialized for the general transport of cations into the lumen (Figure 1.3). Principal cells in the proximal tubule having both the cation-chloride co-transporter and the Na^+/K^+–ATPase in the basal membrane seem to be specialized for potassium excretion, with sodium recycling to the hemolymph (Pullikuth *et al.*, 2006; Patrick *et al.*, 2006) (Figure 1.4).

Chloride ions are predicted to move into the lumen passively along the electrochemical gradient. In *Drosophila melanogaster*, chloride ions enter the Malpighian tubule lumen transcellularly through the stellate cells (O'Donnell *et al.*, 1998) and a similar mechanism is suggested in *Anopheles gambiae* (Radford *et al.*, 2004). Though chloride channels have been identified in the apical membrane of stellate cells in *Aedes aegypti* (O'Connor & Beyenbach, 2001), the current literature supports that after a blood meal, chloride ions enter the lumen along a paracellular route in *Aedes aegypti* (Beyenbach *et al.*, 2009).
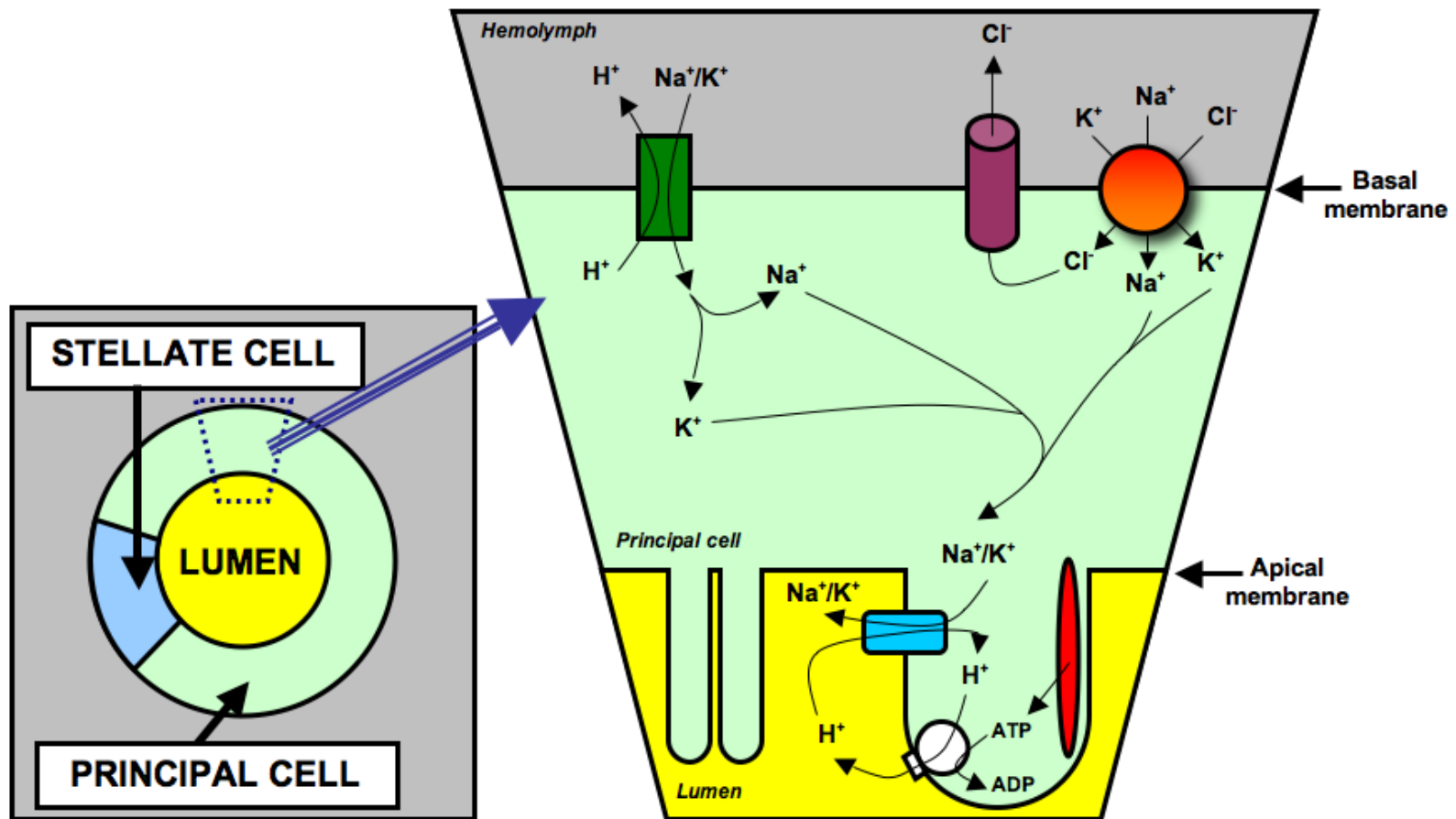
Figure 1.3 Schematic of the primary transport systems responsible for the movement of ions across the principal cells of the distal Malpighian tubules of *Aedes aegypti*. Inset shows a cross section of a stylized <u>distal</u> (towards the blind end) Malpighian (M.) tubule, and the enlarged principal cell region is shown by the dotted line. Ions move from the hemolymph (top; grey) across the basal membrane and into the principal cell cytosol (middle; light green) by the action of a cation/proton exchanger (AeNHE3; dark green) (Pullikuth *et al.*, 2006) and a cation-chloride co-transporter (orange) (Scott *et al.*, 2004). A chloride channel (purple) is hypothesized to return chloride ions to the hemolymph (Beyenbach, 2003b). A vacuolar ATPase (V-ATPase; white) energizes the apical membrane brush border by transporting protons from the principal cell cytosol to the M. tubule lumen (bottom; yellow) (Weng *et al.*, 2003). The protons are then exchanged for cations by an unidentified cation-proton exchanger (blue) (Kang'ethe *et al.*, 2007; Rheault *et al.*, 2007). Individual mitochondrion (red) in each microvillus of the brush border provide the ATP for the V-ATPase (Beyenbach, 2003b).

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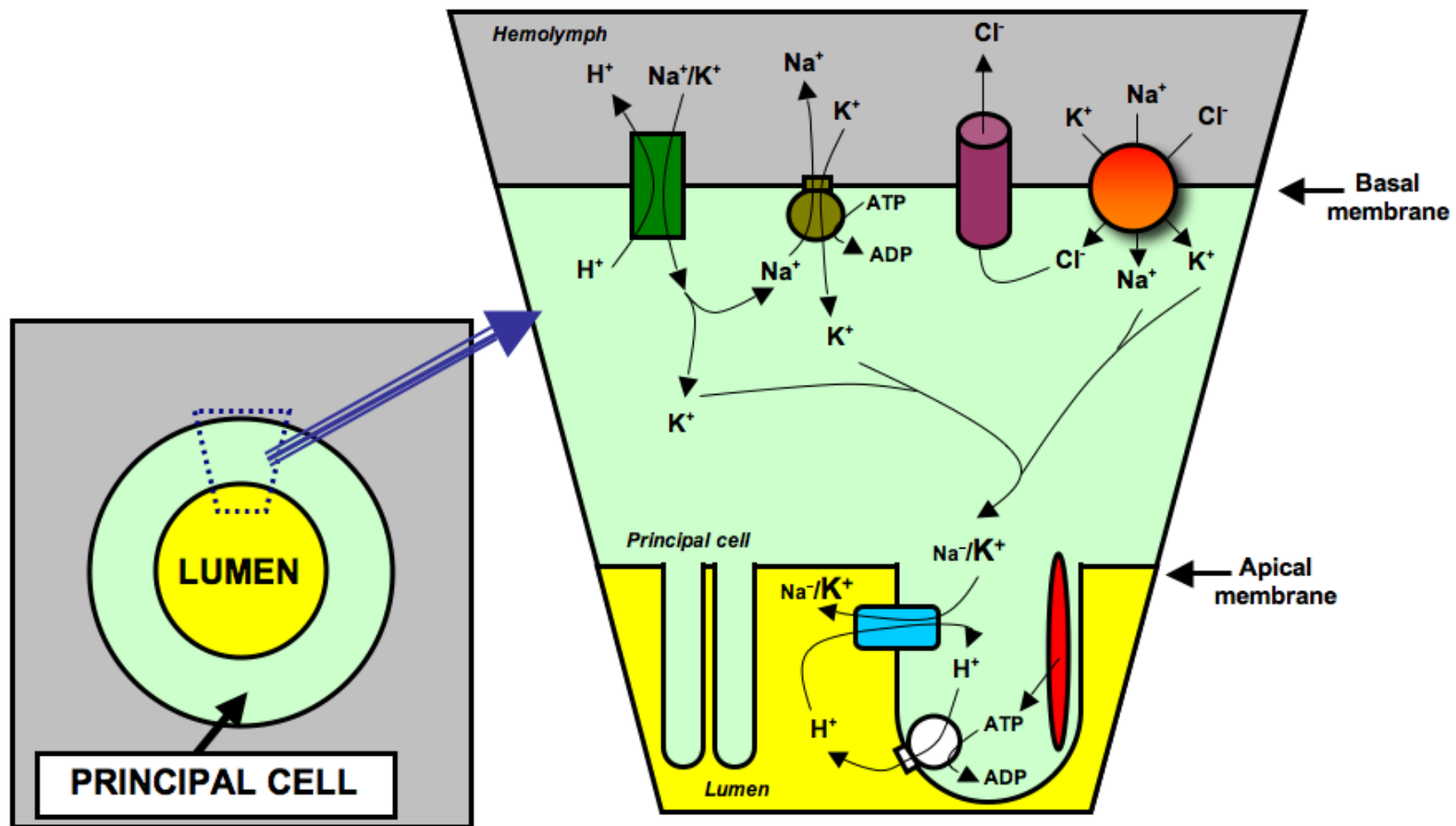
Figure 1.4   Schematic of the primary transport systems responsible for the movement of ions across the principal cells of the proximal Malpighian tubules of *Aedes aegypti*. Inset shows a cross section of a stylized Malpighian tubule, and the enlarged region in shown by the dotted line. In addition to the channels and transporters present in principal cells of the distal tubule, proximal tubule principal cells also contain a basal membrane Na^+/K^+–ATPase (brown) (Patrick *et al.*, 2006) which acts to return sodium ions to the hemolymph. However, in the presence of cAMP the activity of the Na^+/K^+–ATPase is reduced (McMullen & Storey, 2008), thus providing a mechanism for the same principal cell to alter the concentration of cations secreted into the Malpighian tubule lumen.

Hormonal Regulation of Malpighian Tubule Function

Malpighian tubules in adult mosquitoes are not innervated (Jones & Brandt, 1981) and thus not under the direct control of the nervous system. Their function is modulated and regulated by the action of hemolymph-circulating hormones, including those which regulate primary urine composition and secretion rate (for reviews see Gäde, 2004; Schooley *et al.*, 2005). These hormones include: (1) Corticotropin releasing factor (CRF)-like Diuretic Hormone 44 (DH_{44}) which signals via the second messenger cAMP to regulate general cation (Na^+/K^+) transport (Cabrero *et al.*, 2002; Coast *et al.*, 2005; Lovejoy & Jahan, 2006); (2) Calcitonin-like Diuretic Hormone 31 (DH_{31}) signals via the intracellular cAMP cascade, and is specific for stimulating Na^+ transport (natriuresis) (Petzel *et al.*, 1985; Coast, 2001; Coast *et al.*, 2005); (3) Mosquito kinins regulate anion (Cl^-) transport through the release of intracellular calcium (Ca^{++}) (Hayes *et al.*, 1989; Veenstra *et al.*, 1997; Terhzaz *et al.*, 1999; Radford *et al.*, 2002; Radford *et al.*, 2004; Yu & Beyenbach, 2002; Yu & Beyenbach, 2004; Pietrantonio *et al.*, 2005); (4) Periviscerokinins (also called CAP_{2b} or CAPA peptides) increase cation transport by increasing cyclic-GMP via Ca^{++} and nitric oxide pathways (Davies *et al.*, 1995; Riehle *et al.*, 2002) (Figure 1.5). These hormones activate intracellular signaling cascades by interacting with unique <u>G</u>uanine nucleotide-binding <u>P</u>rotein -<u>C</u>oupled <u>R</u>eceptors (GPCRs) in the Malpighian tubule basolateral membrane.

Cyclic-AMP, the second messenger synthesized in response to DH_{44} and DH_{31} receptor signaling, activates the basal membrane cation-chloride co-transporter and likely contributes to regulating the assembly of the apical membrane vacuolar-ATPase

holoenzyme (Hegarty *et al.*, 1991; Beyenbach, 2003b; Coast *et al.*, 2005; Beyenbach & Wieczorek, 2006; Voss *et al.*, 2007; Rein *et al.*, 2008). Though the exact mechanisms by which different hormones (DH_{44} and DH_{31}) produce variable physiological results from the same intracellular second messenger are unknown, the recent discovery in another dipteran that activity of the Na^+/K^+–ATPase is inhibited by cAMP-dependent protein kinase A phosphorylation (McMullen & Storey, 2008) suggests that cAMP can both activate and inhibit various ion transport proteins within the same cell. *Aedes* kinins, through the Ca^{++} signaling cascade, are hypothesized to open the septate junctions connecting adjacent principal cells or principal-stellate cells (Beyenbach, 2003a; Yu & Beyenbach, 2004), and may also recruit water transport proteins (aquaporins) to the basal and apical membranes of stellate cells (Spring *et al.*, 2009).

In *Drosophila melanogaster*, periviscerokinins activate specific cGMP-dependent protein kinases in both the basal and apical membranes (MacPherson *et al.*, 2004), and modulate apical membrane mitochondrial function increasing ATP production (Terhzaz *et al.*, 2006). ATP production activates the vacuolar ATPase and increases cation and fluid transport. Periviscerokinins likely have a similar function in mosquitoes.
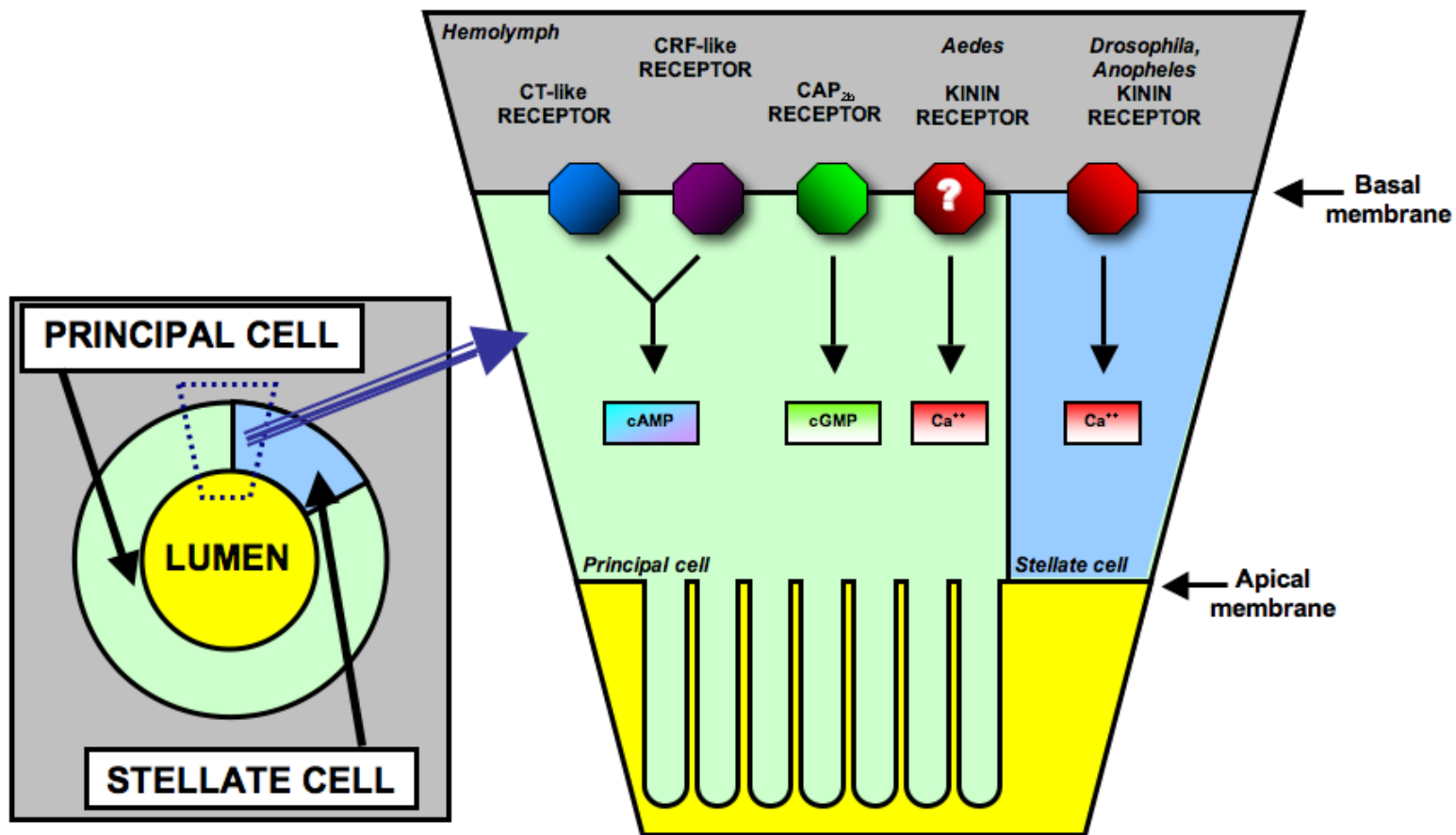
Figure 1.5   Representation of the primary hormones and their cognate receptors which drive ion transport in the Malpighian tubules of *Aedes aegypti* and the intracellular second messengers by which they act.   The corticotropin releasing factor (CRF)- and calcitonin (CT)-like peptides elevate cAMP (Coast, 2001; Cabrero *et al.*, 2002; Beyenbach, 2003b; Coast *et al.*, 2005) while the periviscerokinins (CAP2b) elevate cGMP (Davies *et al.*, 1995; Riehle *et al.*, 2002); both increase cation transport across the Malpighian tubules.   Mosquito kinins elevate intracellular calcium and subsequently chloride transport (Radford *et al.*, 2002; Radford *et al.*, 2004; Yu & Beyenbach, 2002; Yu & Beyenbach, 2004).   In *Anopheles gambiae* and *Drosophila melanogaster*, the kinin receptor has been localized to the stellate cell (Radford *et al.*, 2002; Radford *et al.*, 2004), though Beyenbach claims the *Aedes* kinin receptor is present in the principal cells (Yu & Beyenbach, 2004; Beyenbach *et al.*, 2009).

The Corticotropin Releasing Factory Peptide Family

In vertebrates, the hypothalamus and adrenal glands respond to certain stimuli by secreting peptides, among them, members of the corticotropin releasing factor (CRF) family. These peptides have a multitude of "gut-brain axis" effects, such as appetite and feeding modulation, gastric transit and emptying, and heat-induced swelling, as well as adrenal steroidogenesis stimulation and hormone release (for reviews see Tsatsanis *et al.*, 2007; Stengel & Taché, 2009). The vertebrate CRF peptide family is well characterized and includes four paralogous lineages: CRF, Urocortin (Urocortin, Urotensin, Sauvagine), Urocortin 2, and Urocortin 3 (Chang & Hsu, 2004). CRF, also sometimes designated CRH (corticotropin releasing hormone), has been identified from boney fish, amphibians, and mammals, and is likely present in all terrestrial vertebrates. Urocortins were first identified in fish (Urotensin I) and amphibians (Sauvagine), but are known now also from mammals (Lovejoy & Balment, 1999; Lovejoy & Jahan, 2006). Human urocortin 2 and 3 were discovered more recently (Lewis *et al.*, 2001; Reyes *et al.*, 2001). An amino acid sequence alignment for the four CRF-related peptides identified from humans showing their residue similarity is presented in Table 1.1.

Table 1.1   Sequence alignment for the four peptides identified from humans belonging to the corticotropin releasing factor (CRF) family.   Bold, shaded letters indicate identical or conservative replacement of amino acid residues among peptides.

Representative	Aligned CRF Family Peptide Sequences from Human	Reference
CRF	SEE**PP**I**SLDL**T**FHLL**RE**VLE**M**ARA**EQL**A**QQ**A**HS**N**RK**L**ME**I**I**	(Shibahara *et al.*, 1983)
Urocortin	DN**PSLSIDL**T**FHLL**RT**LLELAR**TQSQ**RERA**EQ**NRI**I**FDS**V	(Donaldson *et al.*, 1996)
Urocortin 2	IV**LSLDVP**IG**LL**Q**ILLE**Q**ARA**RAA**REQA**TT**NAR**I**LAR**V	(Reyes *et al.*, 2001)
Urocortin 3	F**TLSLDVP**TNIMNLLFNIAKAKN**LRAQA**AAN**AHL**M**AQ**I	(Lewis *et al.*, 2001)

The first isolation of a CRF-like peptide in insects was that of a "diuretic hormone" (Manse-DH; Manse-DH$_{41}$) from the lepidopteran, *Manduca sexta*.   The posterior brain, including the corpora cardiaca and corpora allata, was dissected from the heads of ten thousand pharate adults (~420 g); nine steps of protein extraction and purification yielded approximately 1.5 nmol of peptide which was used for sequencing (Kataoka *et al.*, 1989).   Manse-DH is also produced in abdominal ganglia (Kim *et al.*, 2006c).   The mature Manse-DH is a 41-residue amidated peptide with 40% sequence identity to sauvagine, one of the first CRF-family peptides identified from the skin of the frog *Phyllomedusa sauvagei* (Kataoka *et al.*, 1989).   Manse-DH was shown to significantly increase fluid excretion in intact *M. sexta* larvae (Kataoka *et al.*, 1989) and to produce a dose-dependent decrease in food consumption and water loss when injected into the larvae of another lepidopteran, *Heliothis virescens* (Keeley *et al.*, 1992).

After the discovery of Manse-DH, similar peptides were identified from roaches (Kay *et al.*, 1992), orthopterans (Lehmberg *et al.*, 1991; Kay *et al.*, 1991), and the flies *Musca domestica* and *Stomoxys calcitrans* (Clottens *et al.*, 1994).   Current database queries reveal CRF-like diuretic hormones have been identified or predicted from at

least 16 insect species representing seven different orders of insects (Table 1.2), as well as from non-insect arthropods, such as the blacklegged tick *Ixodes scapularis* (Christie, 2008). All insect CRF-like peptides directly isolated or predicted to date have a C-terminal amidation except those from beetles (Coleoptera) which have a terminal carboxylate (Furuya *et al.*, 1998). Comparison of the vertebrate CRF-Urocortin peptide consensus sequence (Lovejoy & Jahan, 2006) with the consensus sequence for the insect DH$_{44}$ peptides (Table 1.3) indicates sequence conservation between the vertebrate and invertebrate peptides even after an estimated 500 million years of divergence (Lovejoy & Balment, 1999).

Noting sequence similarities between Manse-DH and *Acheta domesticus* diuretic hormone (Table 1.2), Reagan and colleagues (Reagan *et al.*, 1993) used circular dichroism to investigate potential conformation similarities. They showed Manse-DH could exist as a random coil or an α-helical coil, and hypothesized that the peptide is stabilized into a helical conformation upon binding to the cognate receptor. Further structural studies showed that a peptide fragment containing the "V-L-R" motif (absolutely conserved among all insect CRF-like peptides to date; Table 1.2) had a high probability of forming an α-helix (Nittoli *et al.*, 1999). Vertebrate CRF-family peptides have a similar conserved "L-L-R" residue motif (Table 1.3, underlined) and a similar predicted secondary structure, being generally disordered in solution but forming an α-helical structure when bound to the cognate receptor (Hoare, 2005; Grace *et al.*, 2007), indicating that both the primary sequence and secondary structure have been conserved in this peptide family across invertebrate and vertebrate taxa.

Table 1.2 Sequence alignment of identified CRF-like peptides in insect orders. Bold, shaded letters indicate identical amino acid residues among all sequences. The consensus sequence is also shown.   Alignment preparation used the ClustalW2 tool at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw2/).   Dashes (-) indicate an alignment gap inserted by the ClustalW2 tool.

Order: Species	Aligned Insect Diuretic Hormone (DH$_{44}$) sequences	Reference
ORTHOPTERA		
Acheta domesticus	TGAQ**SLSI**VAPLD**VLR**QRLMN**E**LN**R**RRMRELQGSRIQQ**N**RQL**L**TSI	(Kay *et al.*, 1991)
Locusta migratoria	MGMGP**SLSI**VNPMD**VLR**QRLLL**E**IA**R**RRLRDAE-EQIKA**N**KDF**L**QQI	(Lehmberg *et al.*, 1991)
BLATTARIA		
Diploptera punctata	TGTGP**SLSI**VNPLD**VLR**QRLLL**E**IA**R**RRMRQTQ-NMIQA**N**RDF**L**ESI	(Furuya *et al.*, 2000b)
Periplaneta americana	TGSGP**SLSI**VNPLD**VLR**QRLLL**E**IA**R**RRMRQSQ-DQIQA**N**REI**L**QTI	(Kay *et al.*, 1992)
ISOPTERA		
Zootermopsis nevadensis	TGAVP**SLSI**VNPLD**VLR**QRLLL**E**IA**R**RRMRQSQ-DQIQA**N**REM**L**QTI	(Baldwin *et al.*, 2001)
HYMENOPTERA		
Apis mellifera	IG**SLSI**VNSMD**VLR**QRVLL**E**LA**R**RKALQDQ-AQIDA**N**RRL**L**ETI	(Huising & Flik, 2005)
COLEOPTERA		
Tribolium castaneum	AGALGESGA**SLSI**VNSLD**VLR**NRLLL**E**IA**R**KKAKEG----ANR**N**RQI**L**LSL	(Li *et al.*, 2008)
Tenebrio monitor	AGALGESGA**SLSI**VNSLD**VLR**NRLLL**E**IA**R**KKAKEG----ANR**N**RQI**L**LSL	(Furuya *et al.*, 1998)
DIPTERA		
Anopheles gambiae	TKP**SLSI**VNPLD**VLR**QRIIL**E**MA**R**RQMRENT-RQVEL**N**KALL**R**EI	(Coast *et al.*, 2005)
Culex salinarius	TKP**SLSI**VNPLD**VLR**QRIIL**E**MA**R**RQMRENT-RQVER**N**KAILREI	(Clark *et al.*, 1998)
Drosophila melanogaster	NKP**SLSI**VNPLD**VLR**QRLLL**E**IA**R**RQMKENS-RQVEL**N**RAILKNV	(Cabrero *et al.*, 2002)
Musca domestica	NKP**SLSI**VNPLD**VLR**QRLLL**E**IA**R**RQMKENT-RQVEL**N**RAILKNV	(Clottens *et al.*, 1994)
Stomoxys calcitrans	NKP**SLSI**VNPLD**VLR**QRLLL**E**IA**R**RQMKENT-RQVEL**N**RAILKNV	(Clottens *et al.*, 1994)
LEPIDOPTERA		
Bombyx mori	KMP**SLSI**NNPME**VLR**QRLLL**E**VA**R**KQMREANQRQAVA**N**RLF**L**QNV	(Tanaka, unpublished)
Manduca sexta	RMP**SLSI**DLPMS**VLR**QKLSL**E**KE**R**K----VHALRAAA**N**RNF**L**NDI	(Kataoka *et al.*, 1989)
Hyles lineata	RMP**SLSI**DLPMS**VLR**QKLSL**E**KE**R**K----VQALRAAA**N**RNF**L**NDI	(Furuya *et al.*, 2000a)
CONSENSUS	..P**SLSI**VNPLD__**VLR**__QRLLL**E**.A**R**R........Q...**N**R..**L**..I	

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Early structural analysis using truncated fragments of the first identified insect CRF-like peptide (Manse-DH) shows that the peptide possesses separate binding and activation domains; the C-terminus is required for ligand binding while the N-terminus is required for receptor activation (Reagan *et al.*, 1993; Reagan, 1995a). Vertebrate CRF and several other structurally similar peptides have a similar differentiation between the N- and C-terminal domains (Hoare, 2005), including the parathyroid hormone (Jüppner, 1994), glucagon-like peptide-1 (Lopez de Maturana *et al.*, 2003), and CRF (Perrin *et al.*, 2003). Considering that both N- and C-terminal regions of these ligands seem to be required for full receptor activation, a 'Two-Domain' model has been proposed to explain the ligand-receptor interaction of these peptides (Hoare, 2005). In this model the C-terminal portion of the peptide first interacts with the N-terminal extracellular domain of the receptor. In addition to stabilizing the α-helical structure of the ligand, this binding acts as an affinity trap increasing the local ligand concentration. Subsequent interaction of the N-terminus of the stabilized ligand with extracellular regions of the receptor causes conformational changes in the receptor's transmembrane domains, thus inducing intracellular signaling (Hoare & Usdin, 2001; Al-Sabah & Donnelly, 2003; Hoare, 2005).

Table 1.3   Alignment of the vertebrate CRF/Urocortin consensus sequence and the insect CRF-like diuretic hormone consensus sequence. Bold, shaded letters indicate identical or conservative replacement of amino acid residues between sequences. Alignment preparation used the ClustalW2 tool at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw2/). Dashes (-) indicate an alignment gap inserted by the ClustalW2 tool.

Representative	Aligned Consensus Peptide Sequences	Reference
CRF/Urocortin	..E**PP**I**SL**DLTFH**LLR**E--M**LEM**A**R**.E--N...**Q**A.S**NR**K.MD.**I**	(Lovejoy & Jahan, 2006)
Insect DH44	...**PSLSI**VNPLD**VLR**QRLL**LE**.A**R**R.......**Q**...**NR**..L..**I**	Table 1.2

There is only one diuretic hormone 44 -encoding gene predicted from each of the genomes of *Drosophila melanogaster*, *Anopheles gambiae,* and *Aedes aegypti* (Hewes & Taghert, 2001; Riehle *et al.*, 2002; Nene *et al.*, 2007). Cabrero and colleagues (2002) synthesized *Drosophila melanogaster* DH_{44} (Drome-DH_{44}) and using a modified Malpighian tubule secretion assay (Ramsay, 1954; Dow *et al.*, 1994), demonstrated the Drome-DH_{44} synthesized peptide ($\geq$100 nM) doubled the fluid secretion rate of isolated Malpighian tubules. Drome-DH_{44} also increased the production of cyclic-AMP 150%, with the increase localized to the principal cells only. Using a whole organism bioassay, Cady and Hagedorn (1999) demonstrated that CRF-like DH_{44} from the salt water mosquito, *Culex salinarius*, when injected directly into *Aedes aegypti* females, increased urination. Coast and colleagues (2005) synthesized the *Anopheles gambiae* CRF-like DH_{44} (Anoga-DH_{44}) after improving the original Anoga-DH_{44} sequence prediction from the *Anopheles* genome (Riehle *et al.*, 2002). Application of Anoga-DH_{44} to isolated Malpighian tubules from both *Anopheles gambiae* and *Aedes aegypti* increased fluid secretion rates three-fold (Coast *et al.*, 2005) in a manner that also elevated cAMP.

Insect Diuretic Hormone Receptors Are Family B GPCRs

Classical "guanine nucleotide-binding protein"-coupled receptors (GPCRs) are membrane-spanning proteins having an extracellular amino- (N-) terminal domain, seven transmembrane-spanning domains, three extracellular loops, three intracellular loops, and an intracellular carboxyl- (C-) terminal domain (Kobilka, 2007). Interaction of a ligand with the extracellular regions of its cognate receptor alters the conformation of the intracellular domains of the receptor, subsequently affecting the intracellular signaling cascade. GPCRs comprise one of the largest protein families in the genomes of animal taxa, for example, comprising over 1% of the genes in the genomes of the mosquitoes *Anopheles gambiae* (Hill *et al.*, 2002) and *Aedes aegypti* (Nene *et al.*, 2007). The classic signaling pathways for GPCRs are initiated by receptor interactions with the heterotrimeric guanine nucleotide-binding proteins (G-proteins) (for a comprehensive review see Oldham & Hamm, 2008) (Figure 1.6), though members of the G protein receptor kinase (GRK), and β-arrestin protein families also interact directly with the intracellular loops of GPCRs (Reiter & Lefkowitz, 2006). Additionally, protein-protein interactions between GPCRs and GPCR interacting proteins (GIPs) are responsible for the localized assembly of multi-protein signaling units called "receptosomes" (Bockaert *et al.*, 2004). The importance of G protein-independent signaling cascades regulated by GPCRs is also being realized (Sun *et al.*, 2007).
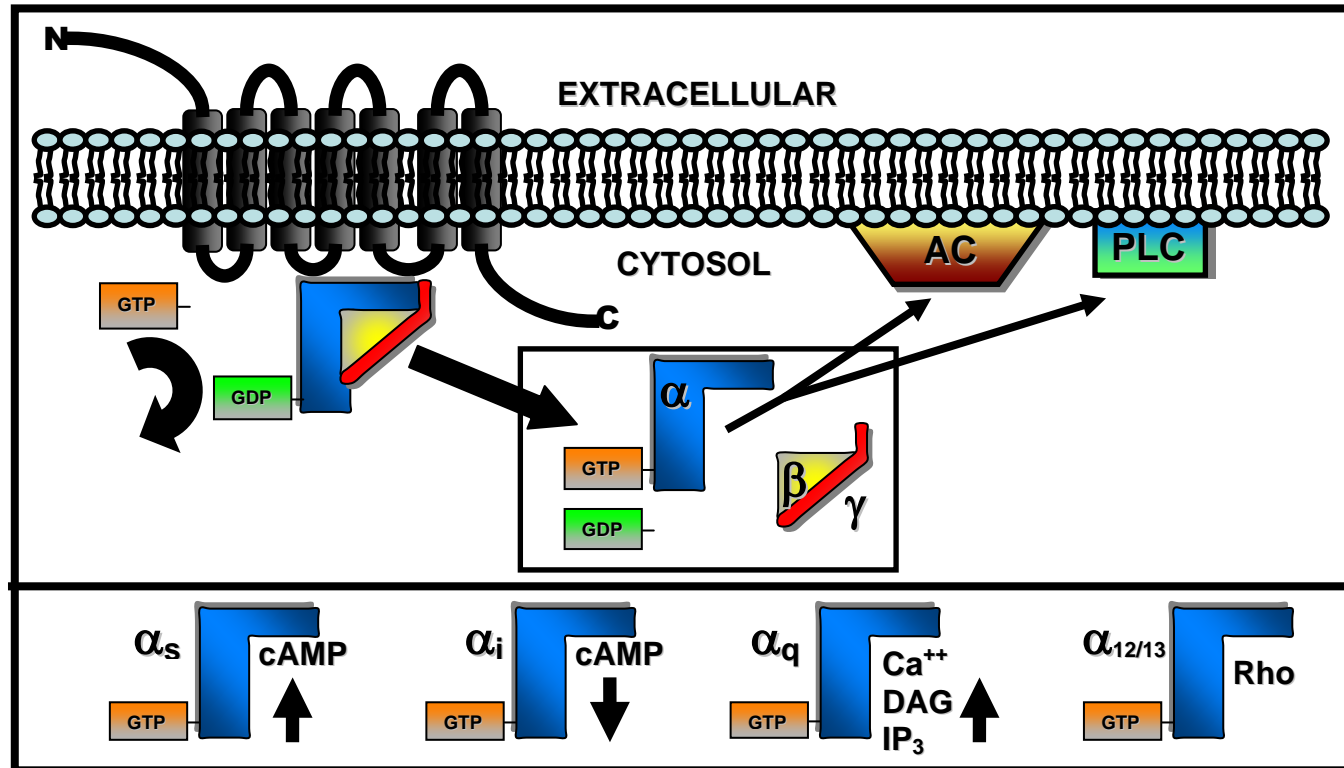
Figure 1.6 Receptor-mediated G protein signaling diversity and respective second messengers. Heterotrimeric G proteins are composed of three different subunits: GDP-bound α- (blue), β- (yellow) and γ- (red). Several isoforms of all three subunits exist. Upon extracellular ligand binding (not shown) to a membrane-bound receptor (black), a conformational change permits interaction of the G protein with the activated receptor and catalyses a GTP for GDP exchange on the α-subunit. The heterotrimer disassociates into two subunits (α and $\beta\gamma$; inset) which can interact with downstream effector proteins, such as adenylyl cyclase (AC) or phospholipase C (PKC), thus transducing an extracellular event into an intracellular signal. G protein signaling can result in the modulation of several different second messengers. Specificity is dependent on the isoform identity of the heterotrimer subunits. Isoforms of the α-subunit are classified on their ability to stimulate (α_s) or depress (α_i) cAMP synthesis, increase intracellular calcium, diacylglycerol, and inositol triphosphate (α_q) or activate the Rho signaling pathway ($\alpha_{12/13}$) (McCudden *et al.*, 2005; Oldham & Hamm, 2008). The ligand-receptor mediated signal is terminated by hydrolysis of GTP to GDP, allowing the reassembly of the heterotrimer. The $\beta\gamma$-complex also has downstream effectors (not shown), such as N-type calcium channels (Smrcka, 2008).

GPCRs have been classified into several subfamilies based on conserved structural features, and though members of the several subfamilies lack broad sequence similarities, all maintain the classic seven transmembrane-domain structure and are hypothesized to originate from a common ancestor (Graul & Sadée, 2001; Fredriksson *et al.*, 2003). Fredriksson *et al.* (2003) identified five distinct clades within the GPCR superfamily in a phylogenetic analysis of GPCRs identified in the human genome. His proposed GRAFS classification system names each clade for a significant GPCR within each specific clade: <u>G</u>lutamate, <u>R</u>hodopsin, <u>A</u>dhesion, <u>F</u>rizzled, and <u>S</u>ecretin. This naming system has been extended to all vertebrates. An alternate naming scheme uses roman letter nomenclature (Family A, B, C, etc…) in an attempt to relate all GPCRs from both vertebrates and invertebrates (Kolakowski, 1994). Unifying these nomenclatures, Secretin-type receptors fall into Family B.

Family B GPCRs have been found in all animal species investigated thus far, but not in bacteria, fungi or plants. A single GPCR identified from thale cress, *Arabidopsis thaliana*, has some similarity to secretin-type receptors (Josefsson & Rask, 1997; Fredriksson & Schiöth, 2005) but its relationship within the greater GPCR superfamily is unclear; however, if this relationship is established, it would suggest a Family B-type receptor as the most ancient of GPCRs (Fredriksson & Schiöth, 2005). All Family B GPCRs have a long N-terminal domain (~100-160 amino acids) containing six positionally conserved cysteine residues resulting in three disulfide bonds (Perrin *et al.*, 2001; Grace *et al.*, 2004; Martin *et al.*, 2005). Nuclear magnetic resonance (NMR) structural analysis of the N-terminal domain of a CRF receptor from mouse indicates the

long protein domain folds on itself, creating a ligand binding pocket, with the disulfide bonds providing stability (Grace *et al.*, 2004). Ligand binding provides further stability to this domain (Grace *et al.*, 2004; Grace *et al.*, 2007).

All investigated vertebrate Secretin-type GPCRs couple to the G protein $G_{\alpha s}$, simulating production of the second messenger cyclic-AMP (cAMP) (Harmar, 2001). Some Secretin-type receptors, including members of the CRF receptor family, additionally have been shown to couple to the G protein type $G_{\alpha q}$ -mediated signaling pathway which leads to the production of inositol triphosphate (IP_3), diacylglycerol (DAG) and an increase in intracellular calcium (Huang *et al.*, 1996; Wietfeld *et al.*, 2004; Dickson *et al.*, 2006; Siu *et al.*, 2006) (Figure 1.6). Signaling through G protein-independent pathways, such as β-arrestin mediated signaling, is known for Rhodopsin- and Secretin-type GPCRs (Azzi *et al.*, 2003; Johnson *et al.*, 2003; Bockaert *et al.*, 2004; Johnson *et al.*, 2005; Lefkowitz & Shenoy, 2005; Siu *et al.*, 2006), but the extent and physiological effects of these alternative pathways has not been widely investigated.

After further phylogenetic analyses Harmar (2001) refined Family B GPCRs into three subclasses (designated B1, B2, B3). The GPCRs for the insect DH_{44} peptides belong to subclass B1; they were the first invertebrate members of the Secretin-type Family B receptor subfamily identified (Reagan, 1994). The first insect CRF-like receptor was discovered in the tobacco hornworm, *Manduca sexta* (Reagan, 1994). In his pioneering work, Reagan used expression cloning and radioactively-labeled *M. sexta* CRF-like diuretic hormone to identify the receptor from a Malpighian tubule cDNA library of 1.1 million recombinant plasmids. Reagan similarly used degenerate primers

and a polymerase chain-reaction (PCR) based screening method to identify the CRF-like diuretic hormone receptor from the Malpighian tubules of the house cricket, *Acheta domesticus* (Reagan, 1996). CRF-like hormone receptors have been experimentally identified from Malpighian tubule cDNA prepared from silk moth, *Bombyx mori* (Lepidoptera; Ha *et al.*, 2000) and from Malpighian tubule/gut cDNA of the planthopper, *Nilaparvata lugens* (Hemiptera; Price *et al.*, 2004). Additionally antibodies against the planthopper CRF-like receptor identified the receptor in the basolateral membrane of the Malpighian tubules (Price *et al.*, 2004).

In each fully-sequenced insect genome thus far, two paralog CRF-like DH_{44} receptor genes are predicted, including the honey bee, *Apis mellifera* (Hymenoptera; Hauser *et al.*, 2006a), the red flour beetle, *Tribolium castaneum* (Coleoptera; Hauser *et al.*, 2008), and three species of Diptera: the fruit fly *Drosophila melanogaster* (Brody & Cravchik, 2000), the malaria mosquito *Anopheles gambiae* (Hill *et al.*, 2002), and recently, the yellow fever mosquito, *Aedes aegypti* (Nene *et al.*, 2007).

In adult *Drosophila melanogaster*, the head and nervous system are enriched in transcript for the receptor encoded by gene CG8422, while in the nervous system, alimentary canal, and Malpighian tubules are enriched for the transcript for the paralog receptor gene CG12370 (Chintapalli *et al.*, 2007). The location of the CG8422 receptor in nervous tissue was confirmed by histochemistry and the same experiments failed to detect any receptor in Malpighian tubules (Johnson *et al.*, 2004). The presence of two differentially expressed receptors indicates a complicated mechanism for DH_{44} function

exists, with the coordination of a number physiological processes and tissues orchestrated by this hormone.

RNA Interference as a Physiological Tool

Introduction of double-stranded RNA (dsRNA) into insects, whether by viral infection (Uhlirova *et al.*, 2003; Sanchez-Vargas *et al.*, 2004; Campbell *et al.*, 2008), direct injection (Blandin *et al.*, 2002; Jose & Hunter, 2007), feeding (Araujo *et al.*, 2006; Walshe *et al.*, 2009), or transgenic manipulation (Torrie *et al.*, 2004; Day *et al.*, 2006) has been observed to decrease the transcript abundance, and hence translation of endogenous genes with homology to the exogenous dsRNA (Cerutti, 2003). RNA-dependent silencing in eukaryotes appears to be an ancient gene-regulatory mechanism (Meister & Tuschl, 2004; Cerutti & Casas-Mollano, 2006) in addition to an innate form of "molecular immunity" and defense against both dsRNA viral infection (Keene *et al.*, 2004; Lu *et al.*, 2004; Stram & Kuzntzova, 2006; Wang *et al.*, 2006; Campbell *et al.*, 2008; Cirimotich *et al.*, 2009; Sanchez-Vargas *et al.*, 2009) and genome invasion by transposons and retroviruses (Bagasra & Prilliman, 2004; Cerutti & Casas-Mollano, 2006; Obbard *et al.*, 2009). This silencing phenomena termed RNA interference (RNAi) was originally described in the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998), but similar homology-dependent gene silencing mechanisms have now been described in plants, fungi, invertebrates and mammals (Cerutti, 2003). Further phylogenetic analysis indicates the basic RNAi machinery is conserved in five of the six supergroups of eukaryotes (Cerutti & Casas-Mollano, 2006). In insects, RNAi has been experimentally

observed in at least eight different insect orders including Diptera (Elbashir *et al.*, 2001a; Jose & Hunter, 2007); however, the tissue-specific efficacy of RNAi remains to be determined. Among insects the cellular response to exogenous dsRNA was first described in the fruit fly *Drosophila melanogaster* (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001b; Kim *et al.*, 2006b), and in mosquitoes the RNAi response has been exploited to develop transgenic strains of dengue-resistance *Aedes aegypti* (Olson *et al.*, 2002; Travanty *et al.*, 2004; Franz *et al.*, 2006; Sanchez-Vargas *et al.*, 2009).

RNAi proceeds mechanistically in two steps (Cerutti, 2003): In the <u>initiation step</u>, long dsRNAs are processed by the protein Dicer (Bernstein *et al.*, 2001; Kim *et al.*, 2006b) into 21-22 nucleotide dsRNA duplexes, each possessing a 5' phosphate and a two nucleotide 3' overhang (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001b; Meister & Tuschl, 2004). In *D. melanogaster* Dicer is a multi-domain protein having a dsRNA binding domain, an RNA helicase domain, a PAZ domain and RNaseIII endonuclease domains (Meister & Tuschl, 2004). In the <u>effector step</u>, the short dsRNA duplex products of Dicer, termed small interfering RNAs (siRNA), are incorporated into a RNA-induced silencing complex (RISC) composed of a multi-domain Argonaute protein (Carmell *et al.*, 2002). The two nucleotide overhang of the siRNA is required for introduction into RISC (Elbashir *et al.*, 2001a). RISC then recognizes and cleaves endogenous mRNA with homology to the RISC-incorporated siRNA (Tomari & Zamore, 2005; Kim *et al.*, 2006a). Cleaved mRNA is then a target for degradation (Hammond *et al.*, 2000). Widespread systemic RNAi has been observed in several organisms, most notably *C. elegans* (Smardon *et al.*, 2000; Sijen *et al.*, 2001). Dipterans

lack several of the genes responsible for a robust, systemic RNAi effect including genes for a dsRNA channel protein and an RNA-dependent RNA polymerase, yet retain a cell-autonomous RNAi (Feinberg & Hunter, 2003; Cerutti & Casas-Mollano, 2006; Jose & Hunter, 2007; Tomoyasu *et al.*, 2008) based on dsRNA uptake via receptor-mediated endocytosis (Saleh *et al.*, 2006). Therefore, RNAi can be used to study gene function in a cell- or tissue-specific manner depending on the permeability of the target tissue to dsRNA uptake (Boisson *et al.*, 2006). The use of RNAi to study *Drosophila melanogaster* physiology is already widespread, and ongoing projects are attempting to knockout every gene in the *Drosophila* genome (Flockhart *et al.*, 2006). RNAi used to generate gene knockouts is especially useful in non-model organisms and those organisms without fully sequenced genomes or for genes with unknown functions, and may uncover novel functions for previously characterized genes.

Purpose of This Study

The *Aedes aegypti* female mosquito obtains a protein-rich blood-meal to acquire the nutrients required for egg production, thus linking feeding behavior, digestion, excretion and reproductive success. In response to the rapid increase in gut volume from acquiring this blood-meal, metabolic equilibrium is actively and precisely maintained by the release of specific hormones and factors (Gäde, 2004; Beyenbach, 2003b). These regulate the movement of excess water, ions, and metabolic waste through the alimentary canal (crop, midgut, hindgut) and renal organs (Malpighian tubules), resulting in production and excretion both of liquid and solid waste. Investigation of the

hormones and receptors which regulate this process of excretion will identify the critical signaling components and potential targets for physiological disruption.

In order to better understand the biological significance of the CRF-like hormone receptors in *Aedes aegypti* and to determine the identity of the CRF-like hormone receptor(s) in Malpighian tubule, I pursued the following three objectives:

(1) Clone and identify the biologically significant CRF-like diuretic hormone 44 receptor from Malpighian tubules of females of *Aedes aegypti*;

(2) Establish the expression profile of this receptor transcript from Malpighian tubules from females of different ages;

(3) Attempt to investigate the role of this receptor by using RNA silencing to knockout or knockdown gene function.

CHAPTER II

IDENTIFICATION OF THE DIURETIC HORMONE 44 RECEPTOR IN

MALPIGHIAN TUBULES OF THE YELLOW FEVER MOSQUITO *Aedes aegypti*

(DIPTERA: CULICIDAE): EVIDENCE FOR TRANSCRIPTIONAL REGULATION

PARALLELING URINATION [*]

Introduction

The female of the mosquito *Aedes aegypti* (L.) is the vector of the arboviruses causative of yellow fever and dengue fever. The transmission of these human pathogens directly relates to the obligatory haematophagia of the female for reproductive success. Although similarities in Malpighian tubule signaling are evident between Drosophilidae (fruit flies) and Culicidae (mosquitoes), the aquatic habitat of mosquito larvae and pupae (Donini *et al.*, 2006), and the haematophagia of the adult female impose unique physiological constraints for rapid elimination of water. Fluid excretion is elevated immediately upon adult emergence. The excretion rate then rapidly drops within minutes, followed by a gradual increase peaking approximately 11–17 h later (post-eclosion diuresis) before decreasing steadily to a basal level on the second day post-emergence (Gillett, 1983). The rate of fluid excretion also spikes immediately after consuming a sodium-rich blood meal (postprandial diuresis) (Stobbart, 1977; Williams

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et al., 1983), the volume of which may exceed the female's hemolymph volume by 10 times (Briegel, 1990; Beyenbach, 2003b). These two periods of increased excretory water loss show physiological differences, both in duration and urine composition, indicating different mechanisms of hormonal control (Williams *et al.*, 1983; Coast *et al.*, 2005).

In dipteran Malpighian tubules, DH_{44} and DH_{31} signal via the second messenger cAMP, increasing transepithelial cation (Na^+ and/or K^+) transport and hence, water movement into the Malpighian tubule lumen (Coast, 2001; Coast *et al.*, 2005; Cabrero *et al.*, 2002). DH_{31} specifically stimulates transepithelial Na^+ transport (Petzel *et al.*, 1985; Coast *et al.*, 2005), while DH_{44} elicits the non-specific transport of cations (Na^+ and K^+) for the production of primary urine (Cabrero *et al.*, 2002; Beyenbach, 2003b; Coast *et al.*, 2005). Insect kinins increase intracellular calcium (Radford *et al.*, 2002; Radford *et al.*, 2004; Yu & Beyenbach, 2002; Yu & Beyenbach, 2004) to increase anion (Cl^-) movement toward the Malpighian tubule lumen (Coast *et al*., 2002; Gäde, 2004; Predel & Wegener, 2006). Additionally, DH_{44} and insect kinins directly trigger the central nervous system behavioral sequence for pre-ecdysis (Kim *et al.*, 2006c; Kim *et al.*, 2006d), suggesting that a greater repertoire of regulatory functions for these 'diuretic hormones' remains to be discovered. These hormones activate intracellular signaling cascades by interacting with unique 'G protein'-coupled receptors (GPCRs) in the basolateral membrane. For both mosquitoes and fruit flies, the Malpighian tubule kinin receptor has been characterized (Radford *et al.*, 2002; Radford *et al.*, 2004; Pietrantonio *et al.*, 2005). The known insect diuretic hormone receptors, first cloned and described

from *Manduca sexta* (Reagan, 1994) and *Acheta domestica* (Reagan, 1996), now also include those from the silk moth *Bombyx mori* (Ha *et al.*, 2000) and the planthopper *Nilaparvata lugens* (Price *et al.*, 2004). Genomic sequences with high identity to diuretic hormone receptors are predicted from the jewel wasp, *Nasonia vitripennis*, the honey bee, *Apis mellifera* (Hauser *et al.*, 2006a) and the red flour beetle, *Tribolium castaneum* (Hauser *et al.*, 2008). Prediction of the genomic repertoire of GPCRs from the fruit fly, *Drosophila melanogaster* (Brody & Cravchik, 2000; Hewes & Taghert, 2001; Hauser *et al.*, 2006b) and the malaria mosquito, *Anopheles gambiae* (Hill *et al.*, 2002), identified putative DH_{44} and DH_{31} receptor genes. *Drosophila* receptors for DH_{44} (Johnson *et al.*, 2004) and DH_{31} (Johnson *et al.*, 2005) have been cloned. In the yellow fever mosquito *Aedes aegypti*, neither DH_{44} nor DH_{31} receptor cDNAs have been cloned; however, in the first released annotation of the *Aedes aegypti* genome (www.vectorbase.org), incomplete sequences (AAEL008292, AAEL005894, AAEL008287) with high similarity to *Drosophila melanogaster* DH_{44} receptor transcripts (CG8422 and CG12370) are present (Nene *et al.*, 2007).

Confirming activity of predicted GPCRs potentially involved in diuresis and characterizing their temporal and spatial expression in target tissues is essential for a complete understanding of the regulatory mechanisms of water balance, especially for insects such as the female mosquito in which blood-feeding behavior is fundamental to reproductive success. In this manuscript we report the cloning of a Malpighian tubule cDNA (EU273351) encoding a putative DH_{44} receptor (*Aaeg*GPRdih1) from *Aedes aegypti*. Additionally, a partial cDNA clone (EU273352) of a paralog receptor

(*Aaeg*GPRdih2) was isolated from female head cDNA and detected at very low levels in Malpighian tubule cDNA. Methods *in silico* were used to predict the sequences for the corresponding co-ortholog receptors from the non-annotated genome of the Southern house mosquito, *Culex (pipiens) quinquefasciatus* Say. Our results indicate that the *Aaeg*GPRdih1 transcript is the most abundant DH_{44} receptor transcript in *Aedes aegypti* female Malpighian tubules and quantitative PCR analysis (QPCR) revealed that the transcript's abundance fluctuates during periods that correlate with the physiological demand for urination, mirroring urine excretion rate patterns discussed above. Ours is the first study to show temporal variation of diuretic hormone receptor transcript abundance in a target tissue from an arthropod, specifically a blood-feeding insect.

Results

The goals of our study were to determine which DH_{44} receptor participates in diuresis in female *Aedes aegypti* Malpighian tubules and to estimate relative transcript abundance during distinct physiological stages. Malpighian tubule cDNA was used as template for DNA amplification using degenerate and specific primers designed based on insect DH_{44} receptor sequences (Table 2.1). The 2715-bp cDNA sequence (EU273351) (Figure 2.1) contained an open reading frame (ORF) encoding a 443 amino acid residue protein, hereafter referred to as *Aaeg*GPRdih1, with a predicted molecular mass of 49.98 kDa.

Table 2.1   DNA primers used for *Aaeg*GPRdih1 and GPRdih2 cloning and transcriptional analysis. Sequence position numbers for *Aaeg*GPRdih1 correspond to base pair positions in Figure 2.1. *Aaeg*GPRdih2 sequence positions correspond to base pair positions in Appendix Figure A-1.

Name	Sequence Position	Sequence	Orientation
GPRdih1 and GPRdih2 cloning			
P133	505 bp – 537 bp	5'CACGYCAACYTGTTCYTCACSTACATCATGTCG3'	Sense
P134	922 bp – 950 bp	5'CTGCTGCGYATCATGTGGGTKCTRATCAC3'	Sense
P144	1213 bp – 1239 bp	5'GGAAATGATGCCCAGCCGAATGTTGCG3	Anti-sense
P143	1184 bp – 1210 bp	5'CATCTCGCCAACGGTAGTAGTGTAACC3'	Anti-sense
P197F	205 bp – 234 bp	5'GAGTTCAAGGGCGTCGCTTATGATGCACGC3'	Sense
P197R	205 bp – 234 bp	5'GCGTGCATCATAAGCGACGCCCTTGAACTC3'	Anti-sense
P206	1437 bp – 1467 bp	5'GTGACGATGATGAATTTGCACTTTAGACCGAG3'	Anti-sense
Tissue expression analysis of AaegGPRdih1 and -GPRdih2 transcripts			
dih1-start	-19 bp – 11 bp	5'CCAAGTGTCCTCAACAAGGATGAACGACTC3'	Sense
dih1-stop	1318 bp – 1351 bp	5'CGGGTTGATGCAATCAAACTTAAATCAACGGCAC3	Anti-sense
dih2-stop	1437 bp – 1467 bp	5'GTGACGATGATGAATTTGCACTTTAGACCGAG3'	Anti-sense
5HT$_7$-start	-15 bp – 12 bp	5'GCACCCTCTTTATGTATGGATCCAACG3'	Sense
5HT$_7$-stop	1377 bp – 1403 bp	5'GCCTAGACTCATAGGAAGCTCTCCCGC3'	Anti-sense
Amplicon design for quantitative PCR			
P180	927 bp – 949 bp	5'GCGAATCATGTGGGTTCTCA3'	Sense
P181	976 bp – 995 bp	5'TTCCGGTATTGCCTCGTTTC3'	Anti-sense
P203	1167 bp – 1188 bp	5'AGGTCAAGTCCGACAACGAAGT3'	Sense
P204	1217 bp – 1233 bp	5'GCGAGGCGAGCATTCCT3'	Anti-sense

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                                    GTAGCTAGATGACCGTCGATGAATCTGCATACGGAGCCGAATTAGTCTCCCGGA    -180
GTCGAGTATGAATGAAAAGTGCAAAACTTTCCGGTTACGAAAGGTTGTAGAGAAAAGAAGTGTTTACAAATCTCGATGAGGCACACTAAA    -90
TTAAAACAAGCGACCGGGAAGGAGTGCGAAGCAAGTGATAACGATAAGAAGTGCTCCGATTGGTGTAGGACCCAAGTGTCCTCAACAAGG    -1
ATGAACGACTCATCATCAGCGTCAGTGGAGTCGTTGTTAGCCCTGGCGGGTGCCGGTGGAGATTTCGCCGGTTCCGGTAGTGGCGATGGC    90
 M  N  D  S  S  S  A  S  V  E  S  L  L  A  L  A  G  A  G  G  D  F  A  G  S  G  S  G  D  G      30

TTTTCCACCAATGATTTTGAACGAAACTTTGCGGGATCTGTGCCACCAACAGAACGACACCCTGTTTCTGGAGCTGTCCTGTCCTCCATTG    180
 F  P  P  M  I  L  N  E  T  L  R  D  L  C  H  Q  Q  N  D  T  L  F  L  E  L  S  C  P  P  L      60

TTCGATTCGATCTCGTGCTGGCCAAGGACACCGCCGGCAACAACGGCAGTTTTGCCGTGTTTCTCCGAGTTCAAGGGAGTGCAGTACGAT    270
 F  D  S  I  S  C  W  P  R  T  P  P  A  T  T  A  V  L  P  C  F  S  E  F  K  G  V  Q  Y  D      90

GCGACACACGAACGCCACCCGCTATTGCAACATCGACGGAACGTGGAACAGCTTCGCGAACTACGATGCCTGCAAGCACCTGGAACTGCCA    360
 A  T  Q  N  A  T  R  Y  C  N  I  D  G  T  W  N  S  F  A  N  Y  D  A  C  K  H  L  E  L  P     120

TCGTCCGATCCAACGCTCGAATCGTTCATCGAATTACCGATAGTGATCTATTTTGTAGGATATACCATAAGCTTGCTGGCACTTTTTTGC    450
 S  S  D  P  T  L  E  S  F  I  E  L  P  I  V  I  Y  F  V  G  Y  T  I  S  L  L  A  L  F  C     150

GCCGTCACCGTTCTAGTTTACTTTAAGGAGCTACGATGCCTCAGGAATACTATTCACGTCAATCTCTTCGTCACCTACATCATGTCGTCC    540
 A  V  T  V  L  V  Y  F  K  E  L  R  C  L  R  N  T  I  H  V  N  L  F  V  T  Y  I  M  S  S     180

AGCTTGTGGATCATCATACTCTCTCAACAGTTGGCTGCCAAGCAGGGTCTGGTGGATTGCATCTTCCTGGTGACGCTATTCCACTACTTT    630
 S  L  W  I  I  I  L  S  Q  Q  L  A  A  K  Q  G  L  V  D  C  I  F  L  V  T  L  F  H  Y  F     210

AGTACGACCAACTTCTTCTGGATGCTGGTGGAAGGTCTCTATCTCTACATGCTGGTGGTTCAGACGTTCTCCGGCGATTATCTACGCTTT    720
 S  T  T  N  F  F  W  M  L  V  E  G  L  Y  L  Y  M  L  V  V  Q  T  F  S  G  D  Y  L  R  F     240

TGGAAGTATTCCATCATCGGCTGGGGAGGTCCTTTAATCTTTGTCGGAGCGTGGGCCATTGCCAAGTCCTTTTATCCGTATGACCTGATG    810
 W  K  Y  S  I  I  G  W  G  G  P  L  I  F  V  G  A  W  A  I  A  K  S  F  Y  P  Y  D  L  M     270

CCGGGGCATCCGAACAAGCTGGAAATCGAATGCTCGTGGATGCGGGAATCTCACATCGACTGGATTATTCAGGGCCCATCCTGTGCTGTG    900
 P  G  H  P  N  K  L  E  I  E  C  S  W  M  R  E  S  H  I  D  W  I  I  Q  G  P  S  C  A  V     300

CTGGTAATCAATCTCATCTTCCTGCTGCGAATCATGTGGGTTCTCATCACCAAGCTCCGATCGGCCAATACGGTCGAAACGAGGCAATAC    990
 L  V  I  N  L  I  F  L  L  R  I  M  W  V  L  I  T  K  L  R  S  A  N  T  V  E  T  R  Q  Y     330

CGGAAGGCATCCAAGGCACTGCTGGTACTTATTCCGCTGCTGGGCATCACCTATCTGGTGGTCATCTACGGGCCTCACGAGGGCGTGGGC    1080
 R  K  A  S  K  A  L  L  V  L  I  P  L  L  G  I  T  Y  L  V  V  I  Y  G  P  H  E  G  V  G     360

AGTCAGATCTTTGCGGTCACCAGAGCAGTCCTGTTGAGCACACAGGGCTTGGTAGTGTCGTTACTCTACTGCTTCCTCAACTACGAGGTG    1170
 S  Q  I  F  A  V  T  R  A  V  L  L  S  T  Q  G  L  V  V  S  L  L  Y  C  F  L  N  Y  E  V     390

CGAGGAACTCTACGGTTACACTACTACCGTTGGCGAGATGAGCGCAACATTCGGCTGGGCATCATTTCCAAACATCGAAGACCGACCATC    1260
 R  G  T  L  R  L  H  Y  Y  R  W  R  D  E  R  N  I  R  L  G  I  I  S  K  H  R  R  P  T  I     420

AGTGGAACTGAAAGTATACGTATTACCACTACGACAACCACAACAACAACCACATCGGTGCCGTTGATTTAAGTTTGATTGCATCAACCC    1350
 S  G  T  E  *S* I  R  I  T  T  T  T  T  T  T  T  T  T  T  T  S  V  P  L  I  .                443

GAACGGACTGATGACCGAGCAGAGGCGAGCAGAATGGAAATTGAGACGGAAAAATATAAAAATGTCGACACAAACAACCACCCACTGCTG    1440
ATTCCAGTTTTTTTTTTGTTACAACCGAGGCAACGTTCGAACGAGTTTTCCTCAGTTTTTTTTTTTCTTAAACGATCTCCACGTGATGACA    1530
AATTCAATGACGATGGTGTTGATCCGTCGTTGGATGTTGTGACTAATTAAGTGTCACCCCTGGAGTATAGTGTCGTGATAATGAAAAGCTT    1620
GGCACCACCCTCCTCAAGGCACCACCACCACCACAACCACAACCATCACAATAACCTTGGCGGCAGCAACTGAGCTTGCCGTACATAAAA    1710
TTCAGCTTCCGGTCAAATTTATACTATGGACCTTCGGACAAGTTTGGAATCAACGCTGTCCAGGGAGTTGTAAACGAAACAACTTTGCGG    1800
AGAGAATTGTTTGTTAGACGACACGGCAACTGCTACAATTAAATTGAATTTCAAAACAAACATCATCGTCTCACGAATGGAAACTTTTAA    1890
TTCAATACTCCGACGACGACGACAATGATCTTTTGTGTGCAGGACAATGGCGAGAGTTGCATTGGGACGGTGTGAAGTATTTACCGAAG    1980
TAGAAGTCAATACAATTTGCAACAGACAGACAGACAGGCAGTCAGTAGGACGACATAGAATCTCATCTCGAGCCAAGTGGGACATGTTTG    2070
CACTTCATTTGGCGGTTGAATTCAGTTGGCGAAGGATTTCGCTCGGAAGGTTGAGATTAAAACGAAATTTGCGGACGGGCTGCGGTGGGA    2160
GATGTAAATATTTACGAATTCGATTGAAATTTCTAGGCGAGTCGCTTGTGTGTTTGGATCGGATTTCACTGTCAGCATGTGTGGAGTTGG    2250
ATGTATGATAAGGAAAGTCAGAGTATGGCAGGCAGCTTCAACGTAACTGATGTACAATGTATTTAGGTTAGGTTTATGTAGTTTAATTAT    2340
CCAAACGGGTTTAAAGAGAGACCAAACCAAAGCTTACACATGTTAGTATAGATTAAATTGTCATTTTTGTATATATTTGAAACATTGAAA    2430
CATGCAGATATAAGATTAAAAGAACATTGATGTAAAAAAAAAAAAAAAAAAAA                                       2481
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Figure 2.1 *Aaeg*GPRdih1 cDNA from Malpighian tubules of females of *Aedes aegypti*. (GenBank accession number EU273351; 2715 bp: 234 5'UTR, 1329 bp ORF, 918 3'UTR) Predicted seven transmembrane regions are underlined (◆━━━◆). Conserved N-terminal amino acids in this receptor family are indicated: six cysteine residues (♦: C^{44}, C^{57}, C^{66}, C^{80}, C^{99}, C^{114}), two tryptophan residues (▲: W^{67}, W^{105}), and residues responsible for salt bridge formation (■: D^{62}, R^{97}). Four predicted Asn-glycosylation sites are underlined. Residues responsible for suspected interaction with either G_s (T^{317}, K^{318} and E^{389}) or G_q (V^{314}, L^{315} and K^{318}) subtypes of the G_α subunit of the G-protein complex are double-underlined (Huang *et al.*, 1996; Couvineau *et al.*, 2003). S^{425} (indicated by *S*) is also conserved among insect diuretic hormone receptors and may be phosphorylated by PKC, a signal for internalization and desensitization. Ten consecutive threonine residues (T^{429}-T^{438}) offer additional potential sites for phosphorylation.

This sequence was confirmed using *Aedes aegypti* genomic sequences (AAGE02014128, AAGE02014129, AAGE02014130). Domain searching (protein family database) identified the protein as a Family B, secretin-type GPCR with seven transmembrane (TM) regions as predicted by transmembrane hidden Markov model (TMHMM) (Figure 2.1). The predicted receptor has residues in the first extracellular domain consistent with Family B GPCRs, including six conserved cysteine residues (C^{44}, C^{57}, C^{66}, C^{80}, C^{99}, C^{114}), two tryptophan residues (W^{67}, W^{105}) and residues likely to be responsible for the formation of a salt bridge (D^{62}, R^{97}) (Hoare, 2005). The receptor's N-terminus is not predicted to contain a signal peptide (data not shown). The first extracellular domain contains four predicted Asn-glycosylation sites (N-X-S/T) at residues N^{2}, N^{37}, N^{48} and N^{94}. The 'NetPhosK' tool (www.cbs.dtu.dk/services/) predicted potential protein-kinase A phosphorylation sites at threonine residues T^{393}, T^{419}, T^{429} and T^{430}, and possible protein-kinase C (PKC) phosphorylation sites at threonine and serine residues T^{393}, S^{413}, S^{425}, T^{431}, T^{432}, T^{436} and T^{437}. Specifically, residue S^{425} is absolutely conserved among insect DH44 receptor sequences identified to date and may be sites for PKC phosphorylation and subsequent receptor desensitization (Hauger *et al.*, 2003). The unique presence of 10 consecutive threonine residues (T^{429}–T^{438}) in the C-terminus was confirmed by analysis *in silico* of genomic DNA. Sixteen of the 31 terminal amino acids are either serine or threonine, providing ample potential locations for GPCR kinase phosphorylation and β-arrestin recruitment (Teli *et al.*, 2005; Reiter & Lefkowitz, 2006; Oakley *et al.*, 2007).

The receptor possesses residues that within Family B GPCRs are responsible for G-protein interaction, both with the G_s- (residues T^{317}, K^{318} and E^{389}) and G_q- (residues V^{314}, L^{315} and K^{318}) mediated signaling pathways (Figure 2.1) (Huang *et al.*, 1996; Couvineau *et al.*, 2003). BLAST analysis comparing dipteran receptor sequences is summarized in Table 2.2. The *Aaeg*GPRdih1 sequence used as a BLAST query against *Aedes aegypti* whole genome sequences (WGS database, NCBI) identified several fragments of a potential paralog gene. Specific primers were designed to clone a 2791-bp partial cDNA (EU273352) from female head cDNA encoding a DH_{44} receptor hereafter referred to as *Aaeg*GPRdih2. BLAST analysis is summarized in Table 2.3.

Table 2.2   BLAST search results for *Aaeg*GPRdih1 (EU273351) among dipteran database hits.

Accession	Species	E value	Reference
AGAP005464-PA	*Anopheles gambiae*	10^{-174}	Hill *et al*., 2002; Holt *et al*., 2002
CG8422-PA	*Drosophila melanogaster*	10^{-142}	Adams *et al.*, 2000; Johnson *et al.* 2004
AGAP005465-PA	*Anopheles gambiae*	10^{-127}	Hill *et al*., 2002; Holt *et al*., 2002
CG12370-PA	*Drosophila melanogaster*	10^{-124}	Adams *et al.*, 2000; Wang *et al.*, 2004

Table 2.3   BLAST search results for *Aaeg*GPRdih2 (EU273352) among dipteran database hits.

Accession	Species	E value	Reference
AGAP005465-PA	*Anopheles gambiae*	10^{0}	Hill *et al*., 2002; Holt *et al*., 2002
CG8422-PA	*Drosophila melanogaster*	10^{-146}	Adams *et al.*, 2000; Johnson *et al.* 2004
AGAP005464-PA	*Anopheles gambiae*	10^{-144}	Hill *et al*., 2002; Holt *et al*., 2002
CG12370-PA	*Drosophila melanogaster*	10^{-119}	Adams *et al.*, 2000; Wang *et al.*, 2004

Multiple ORF alignment of cloned and predicted DH_{44} receptor sequences from Diptera indicated structural conservation throughout the receptor sequence (Figure 2.2). *Aaeg*GPRdih1 and -GPRdih2 sequences were utilized for prediction *in silico* of the respective co-ortholog encoding sequences for *Cqui*GPRdih1 (BK006347) and *Cqui*GPRdih2 (BK006348) from the genome of the Southern house mosquito, *Culex (pipiens) quinquefasciatus*; however, the exon encoding approximately 26 amino acid residues corresponding to portions of TM region IV and extracellular loop III for *Cqui*GPRdih2 could not be predicted, probably because of current incomplete identification and assembly of the *C. quinquefasciatus* genomic sequences (see *Cqui*GPRdih2 sequence in Figure 2.2; the missing region is indicated by 'X' residues at positions 237 and 238). Coincidentally, the annotated *Anopheles gambiae* GPRdih2 (AGAP005465-PA) sequence is also incorrectly predicted in the same region. However, when we used the *Aaeg*GPRdih2 sequence as a tBLASTx query of the *Anopheles gambiae* genome, a sequence was identified (LIFVGAWAIAKPFFGSVSNLEHPSKV) that has high similarity. We propose that this sequence complements the incomplete *Agam*GPRdih2 annotation from residues 322 to 348 in Figure 2.2.

```
Aedae-GPRdih1    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - M N D S S S A S    8
Culqu-GPRdih1    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - M    1
Anoga-GPRdih1    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - M A N E T    5
Drome-CG12370    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - M A D D D    5
Aedae-GPRdih2    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -    1
Culqu-GPRdih2    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -    1
Anoga-GPRdih2    M Q S C L L S A T L Q P K P Q V K P R T A V R Y Q T N R D Q T I Y V P Q K F C P T R L W T V Q M T G G I I S N S I K P S   60
Drome-CG8422     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -    1

Aedae-GPRdih1    V E S L L A L A G - - - - - - A G G D F A G S G S G D G F P P - - - - - - - - - - - M I L N E T L R D L C H Q Q N - -   49
Culqu-GPRdih1    L E S T P F W N - - - - - - A G G S G S G S G D L S Q L P F A D S M L L V - - - A S A E V N A S V E E L C L L Q R T A   51
Anoga-GPRdih1    M P T T P T A A A I A A D D G S G G S P F G E G N V T G I S V E N P S L V E G L - L A L A M N A S G A E R C R L Q Q Q A   64
Drome-CG12370    L R A L V D S L D D A S Q E D L A K V I A N F S - V D M L Q R A S A L I G A Q Q G S S G G Q L Q N R T L Q C Q Q Q Q Q R   64
Aedae-GPRdih2    - - - - - - - - - - - - - - - - - I V A D F E E L S T L S Q D L - - - - - - - - - F A E Y E N S T E L A C Y L Q A H L   33
Culqu-GPRdih2    - - - - - - - - - - - - - - - - - R V I V A D V E E F S S F P P D S D - - - - - - - - L D S Y V N S T E F K C L L E A Q L   36
Anoga-GPRdih2    G Q R T T P T T L I R L Q M D K I Q P H A S G K R L V S V A D Y D E L S S F P P E L L G A F E N S T E F E C L L R A H Q  120
Drome-CG8422     - - - - - - - - - - - - - - - - - - M S D H N H I D S V N A S G S D P L L D L H N L D G I G E S V E L Q C L V Q E H I   41

Aedae-GPRdih1    D T L F L - - - E L S - - - - - - - - - C P P L F D S I S C W P R T P P A T T A V L P C F S E F K G V Q Y D A T Q N A T R   97
Culqu-GPRdih1    E E L L T - - - D L A - - - - - - - - - C P T F F D S V S C W P R T P P A T L A I L P C M T E F K G V Q Y D S T Q N A S R  100
Anoga-GPRdih1    E E L L Q - - - D V A - - - - - - - - - C P S F F D M V S C W P R T P P G T L A V L P C F A E L K G V Q Y D S S Q N A T R  113
Drome-CG12370    E E E Q A S L E A L A S G G K R I L Q C P S F F D S V L C W P R T N A G S L A V L P C F E E F K G V H Y D T T D N A T R  124
Aedae-GPRdih2    D A Q A E I P F D G - - - - - - - - - C P V N F D S I L C W P K T P S N T W A V L P C F K E F K G V A Y D A R Q N A T R   84
Culqu-GPRdih2    D L E A G T P F D G - - - - - - - - - C P I D F D R I L C W P K T E S G A W A V L P C F K E Y N G V I Y D H S Q N A T R   87
Anoga-GPRdih2    D A Q S E T P S G G - - - - - - - - - C P V D F D R I L C W P K T D P G T W A V L P C F E E F K G V D Y D V T Q N A T R  171
Drome-CG8422     E A S T Y G N D S G H - - - - - - - - C L T Q F D S I L C W P R T A R G T L A V L Q C M D E L Q G I H Y D S S K N A T R   93

Aedae-GPRdih1    Y C N I D G T W N S F A N Y D A C K H L E L P S S D - P T L E S F I E L P I V I Y F V G Y T I S L L A L F C A V T V L V  156
Culqu-GPRdih1    Y C N I N G T W D N Y T N Y D E C Q H V G - T S A L - P E F E L H I E L P T I Y F V G Y S I S L A A L V L A V T V L V  158
Anoga-GPRdih1    F C N V D G T W D N Y T D Y D R C E H L E Q P P L - P S F E P E I E L P T L I Y F V G Y S I S L A A L V L A V A V L V  172
Drome-CG12370    F C F P N G L E I D H Y S D Y D R C H Q N S G S I P V V P D F S P N V E L P A I Y A G G Y F L S F A T L V V A L I I F L  184
Aedae-GPRdih2    Y C H S D G K W D N Y S H Y T A C H H M T E P P P D - - - - - - I V E V T S I I Y S G Y I V S L V A L S L A V I V F V  138
Culqu-GPRdih2    Y C H P N G K W D N Y S H Y T A C H H M T D P P P D - - - - - - I V E I T S I I Y Y S G Y I I S L I A L S L A V I V F V  141
Anoga-GPRdih2    Y C H P N G R W D N Y S H Y A A C H H V N E P P P D - - - - - - I V E I S S I I Y Y T G Y I L S L V A L S L A V I V F V  225
Drome-CG8422     F C H A N G T W E K Y T Y D A C A H L P A P E S V - P E F E V I V E L P T I I Y Y I G Y T L S L V S L S L A L I V F A  152

Aedae-GPRdih1    Y F K E L R C L R N T I H V N L F V T Y I M S S S L W I I L S Q Q - - L A A K Q G L V D C I F L V T L F H Y F S T T N  214
Culqu-GPRdih1    N F K D L R C L R N T I H L N L F L T Y I M S S S L W M L I L S L Q - - M T T K T E I V D C I F L V T L F H Y F S T T N  216
Anoga-GPRdih1    Y F K D L R C L R N T I H V N L F L T Y I M S S S L W I I L S L Q - - I T V K L E V A G C I F L V T L F H Y F S T T N  230
Drome-CG12370    S F K D L R C L R N T I H A N L F L T Y I T S A L L W I L T L F L Q - V I T T E S S Q A G C I T L V I M F Q Y F Y L T N  243
Aedae-GPRdih2    Y F K D L R C L R N T I H A N L F I T Y I L S A L M W I I L T L Q L S G S Q S G - I A S C V I L T L L H Y F T L T N  197
Culqu-GPRdih2    Y F K D L R C L R N T I H A N L F I T Y I L S A L M W M V I L T Q Q L S G S S G G G L T S C V I F V T L L H Y F T L T N  201
Anoga-GPRdih2    Y F K D L R C L R N T I H A N L F I T Y I L S A L M W I I L T L Q L S S G S S T G M T S C V I F V T L L H Y F T L T N  285
Drome-CG8422     Y F K E L R C L R N T I H A N L F F T Y I M S A L F W I L L L S V Q I S I R - - S G V G S C I A L I T L F H F F T L T N  210

Aedae-GPRdih1    F F W M L V E G L Y L Y M L V V Q T F S G D Y L R F W K Y S I I G W G G P L I F V G A W A I A K S F Y P Y D L - - - - M  270
Culqu-GPRdih1    F F W M L V E G L Y L Y M L V V Q T F S G D N L R F R K Y A I I G W G G P V I F V G A W A L T R P F Y L T E A T I T P T  276
Anoga-GPRdih1    F F W M L V E G L Y L Y M L V V Q T F S G D N L R F R K Y A I I G W G G P L I F V G A W A I A K P F F G S V S N - - - -  287
Drome-CG12370    F F W M F V E G L Y L Y T L V V Q T F S S D N I S F I I Y A L I G W G C P A V C I L V W S I A K A F A P H L E N - - - -  300
Aedae-GPRdih2    F F W M L V E G L Y L Y M L V V E T F S G D N L R F N M Y A A I G W G G P G V F V I M W V I A K G - - I T I S G L - - -  253
Culqu-GPRdih2    F F W M L V E G L Y L Y M L V V E T F S G D N L R F N M Y A A I G W G Y X - - - - - - - - - - - - - - - - - - - - - - -  237
Anoga-GPRdih2    F F W M L V E G L Y L Y M L V V E T F S G D N L R F N M Y A A I G W G - - - - - - - - - - - - - - K C S R A S - - - - -  327
Drome-CG8422     F F W M L V E G L Y L Y M L V V K T F S G D N L R F N I Y A S I G W G G P A L F V T W A V A K S L T V T Y S T P - - -  268

Aedae-GPRdih1    P G H P N K L E I E C S W M R E S H I D W I I Q G P S C A V L V I N L I F L L R I M W V L I T K L R S A N T V E T R Q Y  330
Culqu-GPRdih1    A E H P N K L E I E C S W M R E S H I D W I I Q G P T C T V L V I N L I F L L R I M W V L I T K L R S A N T L E T R Q Y  336
Anoga-GPRdih1    L E H P S K L E I E C S W M R E S H I D W I I Q G P S C A V L V I N L I F L L R I M W V L I T K L R S A N T V E T R Q Y  346
Drome-CG12370    - E H F N G L E I D C A W M R E S H I D W F K V P A S L A L L V N L V F L I R I M W V L I T K L R S A H T L E T R Q Y  358
Aedae-GPRdih2    - D Q T T T L E I D C S W M R E S T V D R I F Q G P V C A V L I I N L V F L I R I M W V L I T K L R S A N T V E T R Q Y  311
Culqu-GPRdih2    - - - - - X L E I D C S W M R E S V V D W I F Q G P V C A V L I I N L V F L V R I M W V L I T K L R S A N T V E T R Q Y  291
Anoga-GPRdih2    - - - - - - L E I E C S W M R E S V V D W I F Q G P V C A V L I I N L V F L I R I M W V L I T K L R S A N T V E T R Q Y  380
Drome-CG8422     - - - - E K Y E I N C P W M Q E T H V D W I Y Q G P V C A V L I I N L T F L L R I M W V L I T K L R S A N T V E T R Q Y  323

Aedae-GPRdih1    R K A S K A L L V L I P L L G I T Y L V V I Y G P H E - G V G S Q I F A V T R A V L L S T Q G L V S L L Y C F L N Y E  389
Culqu-GPRdih1    R K A S K A L L V L I P L L G I T Y L I V I Y G P E E E G G V G S H I F A V T R A I L L S T Q G F V S L L Y C F L N S E  396
Anoga-GPRdih1    R K A S K A L L V L I P L L G I T Y L I V I Y G P V E - G V G S H I F A I T R A I L L S T Q G F V S L L Y C F L N S E  405
Drome-CG12370    Y K A S K A L L V L I P L F G I T Y L L V L T G P E Q - G I S R N L F E A I R A F L I S T Q G F F V A L F Y C F L N S E  417
Aedae-GPRdih2    R K A S K A L L V L I P L L G I T Y L V V L A A P S E - G V V S D I F A V A R A L L L S T Q G F S V S L F Y C F L N S E  370
Culqu-GPRdih2    R K A S K A L L V L I P L L G I T Y L V V L A A P A E - G V V S D I F A V A R A L L L S T Q G L S V S L F Y C F L N S E  350
Anoga-GPRdih2    R K A S K A L L V L I P L L G I T Y L V V L A A P A E - G V V S D I F A I A R A L L L S T Q G L S V S L F Y C F L N S E  439
Drome-CG8422     R K A A K A L L V L I P L F G I T Y L V L A G P S E S G L M G H M F A V L R A V L L S T Q G F S V S L F Y C F L N S E  383

Aedae-GPRdih1    V R G T L R L H Y Y R W R D E R N I R L G - - - - - I I S K H R R P T I - - - - S - - - - - - - - - - - G T E S I R I T T  430
Culqu-GPRdih1    V R Q T L R H H F Y R W R D E R N I L R P G G A S K A A N N H R R P T I - - - S K D Y S P R S - - - - R T E S I R V T T  449
Anoga-GPRdih1    V R Q T L R H H F Y R W R D E R N I L S G - - - - - K V N N N H R R P T F - - - S K D N S P R S - - - - R T E S T R L - -  453
Drome-CG12370    V R Q T L R H G F T R W R E S R N I H R N S - - - - - - S I K N R R H R A - - S K D Y S L R S - - - - R T E S L R L T S  465
Aedae-GPRdih2    V R L A L R H R L E R W R D E R N I R I G G Q V - - - - R Q R S R R F T N S G L S K E C S P R S - - - - R T E S F R P L T  422
Culqu-GPRdih2    V R L A L R H R L E R W R D E R N I R I G G Q - - - - R Q R S R R F T N S G L S K E C S P R S - - - - R T E S F R P L T  402
Anoga-GPRdih2    V R L A L R H R L E R W R D E R N I R L G G Q V - - - - R Q R S R R M E K - G P S K P E S E P E S I H H A T E S L K G F I  494
Drome-CG8422     V R N A L R H H I S T W R D T R T I Q L N Q - - - - - - - N R R Y T T K - S F S K G G G S P - - - - - R A E S M R P L T  430

Aedae-GPRdih1    T T T T T T T T S V P L I                                                                                            444
Culqu-GPRdih1    - - - - - - - T S A P L I                                                                                            456
Anoga-GPRdih1    - - - - - - - - - - V L                                                                                              454
Drome-CG12370    - - - - - - - T S P I P T G H Y E                                                                                    475
Aedae-GPRdih2    - - - - - - - - - - - - Y S K R E S C A S S A T T T T L L G P P - - V G A H G - - A Y P S T M - - - - - R G S N G A L H  461
Culqu-GPRdih2    - - - - - - - - - - - - Y S K R E S C A S S A T T T T L L G P P T L G G S H G G A N P A G I - - - - - R G S N G T L H  445
Anoga-GPRdih2    L P F S S A G T V P L T Y N K R E S C A S S A T T T T L L G P H S Y P S A M R G S N G A L H M - - - - - - - - - - - - -  542
Drome-CG8422     S Y - - - - - - - - - - Y G R G K R E S C V S S A T T T T L V G Q H A P L S L H R G S N N A L H T M P T L A A N A M S S G S  482

Aedae-GPRdih1                                                                                                                      444
Culqu-GPRdih1                                                                                                                      456
Anoga-GPRdih1                                                                                                                      454
Drome-CG12370                                                                                                                     475
Aedae-GPRdih2    L H A M A P R A I S P L M Q - - - - - G L D E N S V                                                                   483
Culqu-GPRdih2    L H T M V P R A I S P L M Q V G L F F G L V V N G Q V L                                                               473
Anoga-GPRdih2    - Q S I V P R A I S P L M Q V - R R W G E G R K S V T N Y Y P L R G A G N                                             576
Drome-CG8422     T L S V M P R A I S P L M R Q - - - - G L E E N S V                                                                   504
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Figure 2.2 Open reading frame alignment of dipteran DH$_{44}$ receptors. *A. aegypti* GPRdih1 (EU273351) and GPRdih2 (EU273352); *C. quinquefasciatus* GPRdih1 (BK006347) and GPRdih2 (BK006348); *A. gambiae* GPRdih1 (AGAP005464-PA) and GPRdih2 (AGAP005465-PA); *D. melanogaster* CG12370-PA and CG8422-PA. Sequence identifiers are to the left (longer taxon abbreviations are for clarity) and ORF residue positions are to the right of the alignment. Regions of amino acid identity among the sequences are shaded in black. BLAST analysis shows *A. aegypti* GPRdih1 is 70.2% identical to *C. quinquefasciatus* GPRdih1, 69.1% identical to *A. gambiae* GPRdih1, 54.8% identical to the *D. melanogaster* CG12370 receptor and 63.2% identical to the CG8422 receptor. *Culex* and *Anopheles* GPRdih2 sequence predictions are missing ~26 amino acid residues corresponding to portions of TM region IV and extracellular loop III, indicated by "X" residues in the *Culex* GPRdih2 sequence at positions 237 and 238.

Additional visual inspection of aligned sequences allowed the detection of conserved C-terminal motifs (Figure 2.3) differentiating the dih1 and dih2 receptors. While all the dipteran receptors share the motif identified as region I in Figure 2.3, which also contains the already alluded to conserved serine residue (S^{425} in the *Aedes aegypti* sequence), only the mosquito dih2 receptors and *Drosophila* CG8422-PA share two other well-conserved motifs (regions II and III), probably relating to their as yet undiscovered intracellular protein-protein interactions and their potential to form similar signaling complexes or 'receptosomes' (Bockaert *et al.*, 2004; Appert-Collin *et al.*, 2006). Bootstrap phylogenetic analysis (Figure 2.4) of DH_{44} receptor sequences from *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus* and *Drosophila melanogaster* indicated that the mosquito dih1 receptors form a well supported cluster most similar to the *Drosophila* CG12370-PA, known to be enriched in Malpighian tubules (Wang *et al.*, 2004; Chintapalli *et al.*, 2007). The mosquito dih2 receptors group with *Drosophila* CG8422-PA, which is apparently exclusively expressed in the nervous system (Johnson *et al.*, 2004; Johnson *et al.*, 2005; Chintapalli *et al.*, 2007) (Figure 2.4).

Because of the differential tissue expression profile observed for CG12370 and CG8422 in *Drosophila*, we asked if *Aaeg*GPRdih2 was expressed in the *Aedes aegypti* Malpighian tubules. Specific cDNA amplification reactions by RT-PCR were assembled for *Aaeg*GPRdih1 and -GPRdih2 transcripts using female head and Malpighian tubule cDNAs as template. Both transcripts are present in head tissues, but only the *Aaeg*GPRdih1 is present in sufficient abundance to be amplified from Malpighian tubule cDNA and detected by gel electrophoresis analysis (Figure 2.5).

Figure 2.3 Alignment of C-terminal sequences from predicted and cloned dipteran DH₄₄ receptors. Three motifs are indicated (I, II, III). These motifs suggest *Aedes* GPRdih1 and the *Drosophila* DH$_{44}$ receptor (CG12370-PA) are orthologs.
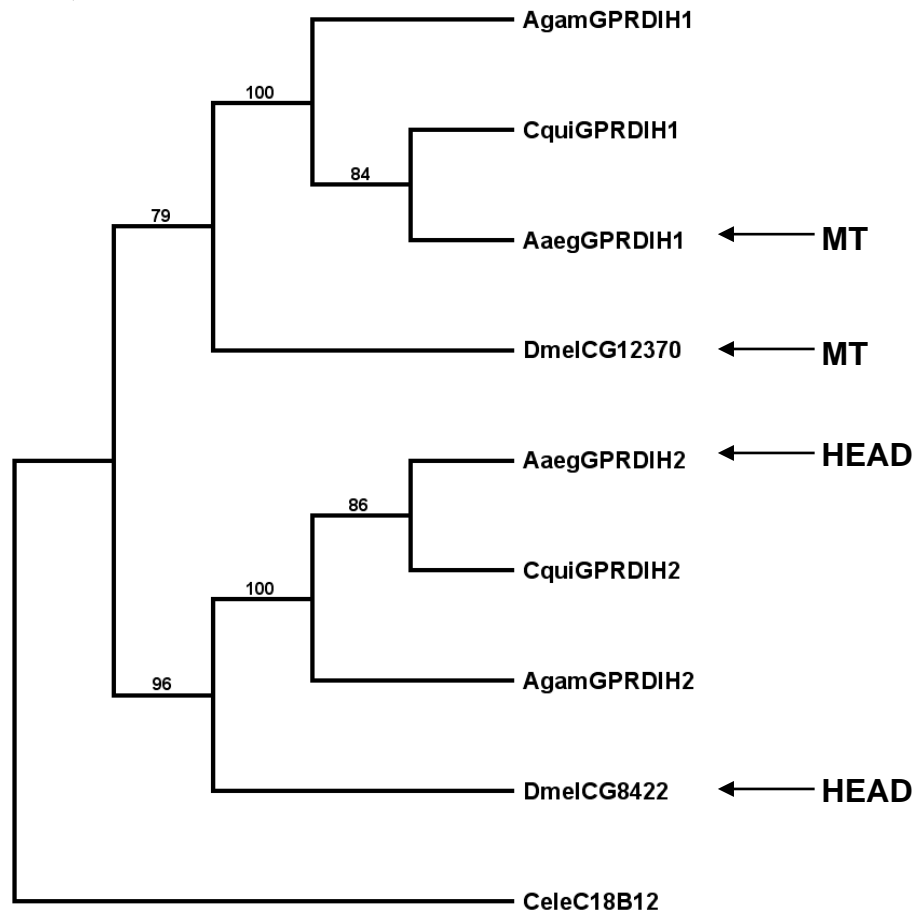
Figure 2.4   Bootstrap analysis of dipteran DH₄₄ receptor sequences. (*A. gambiae* GPRdih1 and GPRdih2; *C. quinquefasciatus* GPRdih1 and GPRdih2; *A. aegypti* GPRdih1 and GPRdih2; *D. melanogaster* CG12370 and CG8422). Tissue from which cloned sequences were obtained is indicated by arrows (MT= Malpighian tubules, H=Heads). The predicted genomic sequences for *Agam*GPRdih1 and *Cqui*GPRdih1 and the *Aedes* GPRdih1 cloned from M. tubule cDNA are similar to the CG12370 receptor from *D. melanogaster* M. tubules. The three mosquito GPRdih2 receptors cluster with *D. melanogaster* CG8422 receptor. CG8422 is expressed in head tissues (Johnson *et al.*, 2004) and not abundantly expressed in M. tubule (Chintapalli *et al.*, 2007; Wang *et al.*, 2004). The ancestral nematode *C. elegans* secretin-type receptor sequence C18B12.2 rooted the tree. Analysis used 10,000 bootstrap replications and 50% majority-rule consensus. Bootstrap values over 50% are shown at branch points.
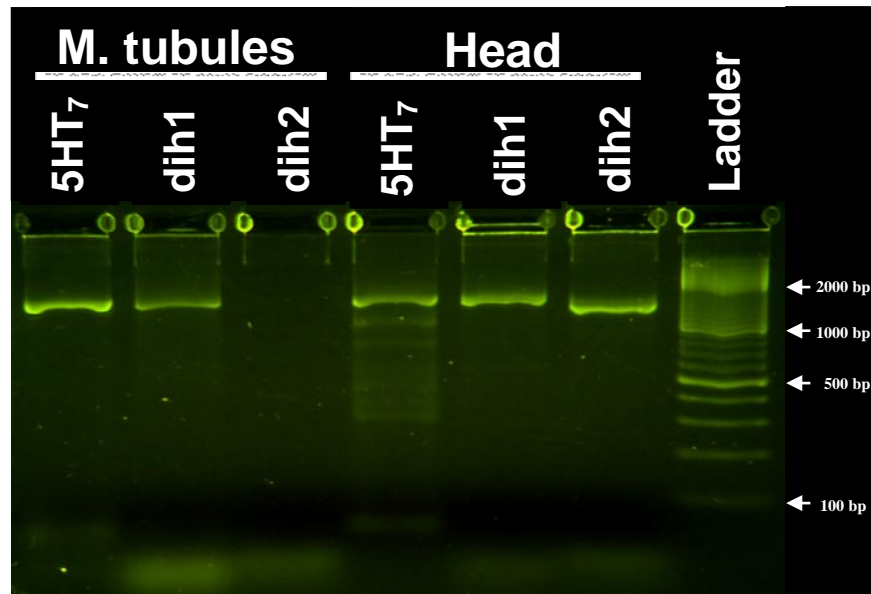
Figure 2.5   Qualitative analysis of tissue expression to verify predominance of GPRdih1 over GPRdih2 in Malpighian tubules. Amplification reactions were for three different GPCR transcripts: (1) 5HT$_7$-like as positive control, (2) GPRdih1, and (3) GPRdih2 using *Aedes* female cDNA generated from either 3-5 d pre-vitellogenic heads or 24 h vitellogenic Malpighian (M.) tubules. Products of the expected size were observed for all receptors when using head cDNA as template. Only the GPRdih1 transcript, but not GPRdih2, was present in sufficient quantity in M. tubule cDNA to yield the expected product after 35 amplification cycles. GPRdih2 amplification was not detected although products were visualized with a highly sensitive nucleic acid dye (GelStar™). The same template cDNA sample was used in all reactions from a given tissue type. The marker is a 100 bp DNA ladder, with intense bands at 500 bp, 1000 bp and 2000 bp as indicated.

Supporting QPCR analysis showed that the *Aaeg*GPRdih1 transcript is significantly more abundant than the *Aaeg*GPRdih2 transcript in Malpighian tubules, the latter transcript being barely detected (Figure. 2.6). Based on sequence analysis, tissue localization and expression abundance, we contend that *Aaeg*GPRdih1 and the *Drosophila* CG12370 receptor are co-orthologs, as are *Aaeg*GPRdih2 and the *Drosophila* CG8422 receptor.

QPCR analysis of relative *Aaeg*GPRdih1 transcript levels measured from the time of adult eclosion throughout the previtellogenic (before blood feeding) and vitellogenic (after blood feeding) periods of *Aedes aegypti* females showed that transcript abundance does fluctuate in Malpighian tubules depending upon age and physiological condition (Figure 2.7). Twenty-four hour post-eclosion transcript levels were at least double those observed immediately before blood feeding (48–72 h post-eclosion) and these differences were statistically significant. Blood feeding also significantly induced transcription of *Aaeg*GPRdih1 back to post-eclosion levels or higher, but abundance significantly declined 6 h post-blood feeding. Unexpectedly based on the observed times of female urination, we observed the most abundant transcript levels 24 h after blood feeding, when relative abundance was about five times the level in 48–72 h previtellogenic females (Figure 2.7).
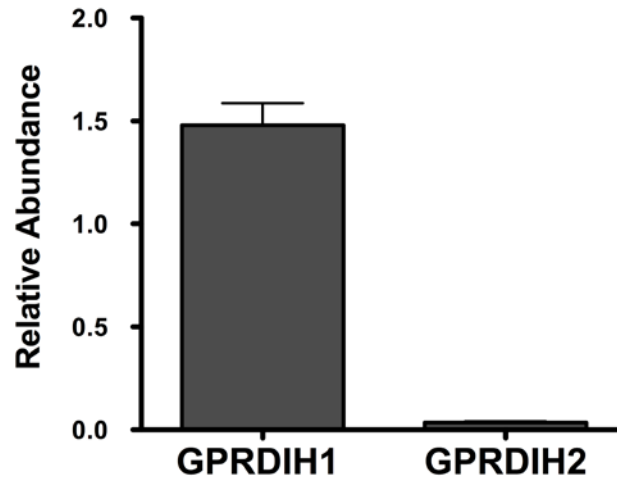
Figure 2.6 Relative abundance of *Aaeg*GPRdih1 and *Aaeg*GPRdih2 transcripts in cDNA generated from vitellogenic female Malpighian tubules estimated by QPCR. GPRdih1 is most abundant while GPRdih2 has extremely low representation in Malpighian tubule cDNA. Template cDNA was the same for both amplicons and respective transcript abundance in head cDNA (not shown) was chosen to calibrate the data. Representative data (mean ± standard error of the mean) are from three independent cDNA samples, and differences in relative abundance are significant (*p<.0001*). Normalization was using β-actin expression.

49
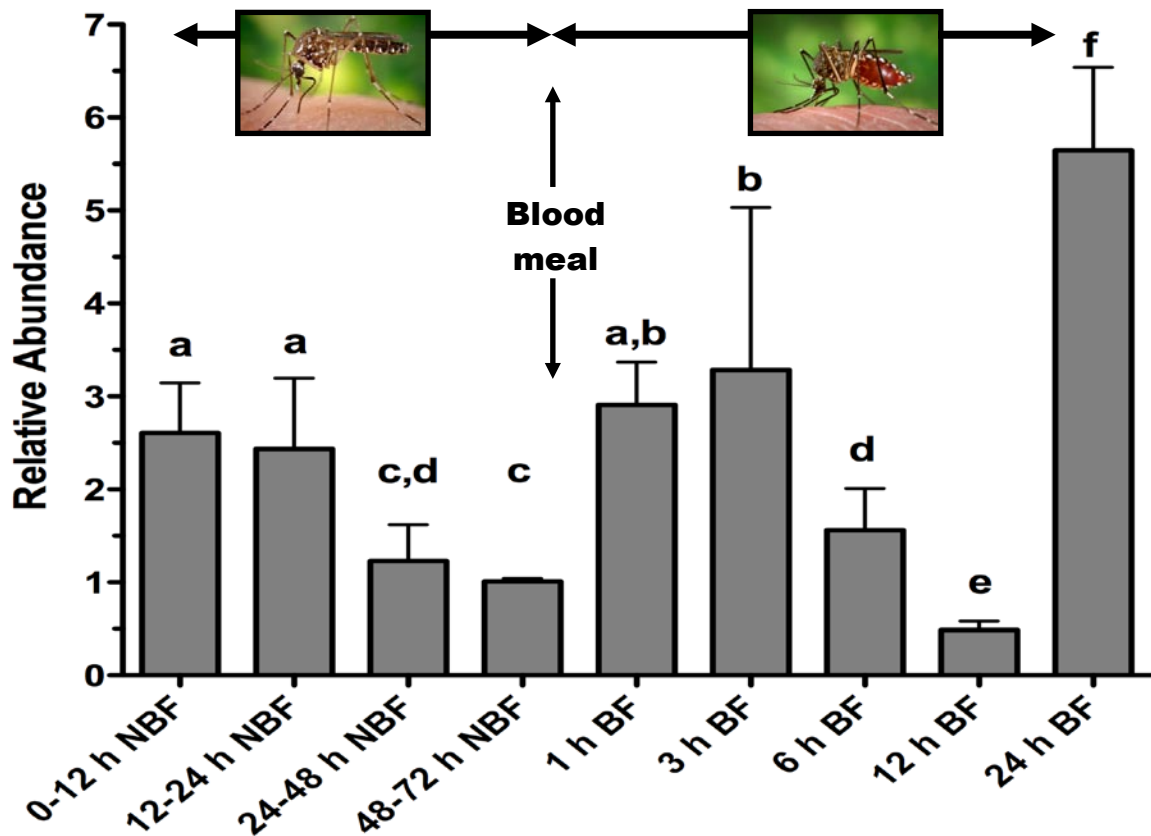
Figure 2.7   Relative abundance of *Aaeg*GPRdih1 transcript in Malpighian tubules of *Aedes aegypti* in pre-vitellogenic (non-blood fed: NBF) and vitellogenic females (blood fed: BF) by QPCR analyses. Arrows indicate previtellogenic (unfed mosquito image) and vitellogenic (fed mosquito image) time periods. Time of blood feeding is indicated. Transcript abundance (grey bars) is increased after eclosion and blood feeding, physiologically relevant times for water excretion. Highest abundance 24 h after the blood meal possibly reflects increased M. tubule function to clear nitrogenous wastes. Representative data (mean ± standard error of the mean) for each time point are from three independent experiments. ANOVA: Different letters above bars indicate relative abundance values are significantly different (*p<0.05*).

Discussion

Water excretion is tightly controlled to maintain homeostasis, both of water and ions (Pannabecker, 1995; O'Donnell & Spring, 2000; Beyenbach, 2003b; Coast, 2007). In addition to the excess water evacuated immediately upon eclosion and within the first day of adult life (Gillett, 1982; Gillett, 1983), female *Aedes aegypti* must consume a water- and sodium-rich blood meal to obtain the protein necessary for the production of eggs (Colless & Chellapah, 1960; Telang *et al.*, 2009). After taking a blood meal, the female's body weight may increase between two and four times (Stobbart, 1977), although about 40% of the water ingested with the meal is eliminated in the first hour after feeding (Williams *et al.*, 1983). This is a critical time for mosquito predation and evolution has favored a fast elimination of excess water (and weight) for flight (Roitberg *et al.*, 2003; Jackson *et al.*, 2005; Nelson & Jackson, 2006). The high rates of water excretion coincidental with the high rates of primary urine formation almost immediately upon adult eclosion and post-blood feeding imply a highly coordinated and precise hormonal control of the signaling process and downstream effector proteins for ion and water transport. Despite these obvious and extreme physiological events, there has been no global analysis of gene transcripts levels in the Malpighian tubules of *Aedes aegypti* across life stages or physiological conditions, as has been carried out with other tissues (Sanders *et al.*, 2003; Feitosa *et al.*, 2006) or Malpighian tubules in non-blood feeding dipterans (Wang *et al.*, 2004; Chintapalli *et al.*, 2007). Molecular characterization of insect Malpighian tubules, especially those from small insects such as mosquitoes, is also hindered by the low amount of tissue recoverable from each individual. In *Aedes*

aegypti the short length, 3 mm (Yu & Beyenbach, 2004), of each of the five functionally homogeneous Malpighian tubules (Beyenbach *et al.*, 1993), and low cell count per tubule, 54 ± 2 total cells (Cabrero *et al.*, 2004), require the dissection of many individuals for the detection of low abundance transcripts, such as GPCRs (Fredriksson & Schiöth, 2005). In order to better understand the mechanisms by which mosquitoes regulate Malpighian tubule water transport, we have continued the molecular characterization of the receptors involved. We cloned a Malpighian tubule cDNA for *Aaeg*GPRdih1 (EU273351) (Figure 2.1), a predicted diuretic hormone 44 (DH$_{44}$) receptor. A query of the genomic data available prior to the publication of the *Aedes aegypti* genome (Nene *et al.*, 2007) allowed the identification of ORF fragments encoding a second DH$_{44}$ receptor, *Aaeg*GPRdih2, which was subsequently cloned from *Aedes aegypti* heads (this report) (EU273352). The *Anopheles gambiae* genome also contains two genes predicted to encode DH$_{44}$ receptors (*Agam*GPRdih1: AGAP005464-RA; *Agam*GPRdih2: AGAP005465-RA) (Hill *et al.*, 2002; Holt *et al.*, 2002); however, neither has been cloned to confirm the sequence predictions.

Likewise, two putative DH$_{44}$ receptor-encoding genes have been cloned from *Drosophila melanogaster*: (1) the CG12370 transcript is present and enriched in Malpighian tubules of *Drosophila melanogaster* (Wang *et al.*, 2004; Chintapalli *et al.*, 2007) but has not been functionally characterized; and (2) the CG8422 transcript is not enriched in the fruit fly Malpighian tubule transcriptome relative to either other tissues or fly carcasses (Wang *et al.*, 2004; Chintapalli *et al.*, 2007), nor is the receptor protein detected by immuno-histochemistry of Malpighian tubules (Johnson *et al.*, 2005).

CG8422 was cloned from head cDNA and functionally characterized by expression in mammalian cells (Johnson *et al.*, 2004). In adult flies it is expressed primarily in nervous tissue (Chintapalli *et al.*, 2007).

Insect and vertebrate Family B GPCRs share a complex regulation, both with respect to the G-proteins to which they couple, and their phosphorylation sites for internalization and degradation. Similarly, the receptors *Aaeg*GPRdih1 and *Aaeg*GPRdih2 contain conserved residues in their third intracellular loops and C-terminal ends which in the human CRF receptor interact with G-protein subunits controlling both the cAMP (G_s) and inositol phosphate/Ca^{++} (G_q) signaling pathways (Huang *et al.*, 1996; Couvineau *et al.*, 2003) (Figure 2.1). While studying the effect of the CRF-like peptide from another mosquito (*Culex salinarius*) on Malpighian tubules of *Aedes aegypti*, Clark & Bradley (1998) theorized that the activation of different intracellular signaling pathways was responsible for the differential effects observed when applying low (10^{-9} mol/l) and high (10^{-7} mol/l) peptide concentrations. They found that a low concentration reduced transepithelial voltage without increasing the short-circuit current resulting in a mild diuresis, whereas a high concentration significantly stimulated fluid and ion secretion and increased the short-circuit (intracellular) current (Clark *et al.*, 1998). Additionally, activation of *Drosophila melanogaster* CG8422 expressed in human embryonic kidney (HEK)-293 cells elicited a cAMP response at nanomolar concentrations of DH_{44} (EC50 = 1.5 nM) and a calcium response at higher concentrations (EC50 = 300 nM) (Johnson *et al.*, 2004), but receptor activation by DH_{44} *in vivo* on *Drosophila melanogaster* Malpighian tubules showed no

effect on intracellular calcium levels (Cabrero *et al.*, 2002). Over expression of the CG8422 receptor in HEK-293 cells originates a skewed stoichiometry of the available G-proteins that could explain the observation of dual signaling (Maudsley *et al.*, 2005). As the population of preferred G_s subunits available for coupling to the over expressed DH_{44} receptor decreases, the likelihood of receptor coupling to the G_q subunits, of lower affinity, would increase. Based on our analysis of *Aedes aegypti* receptor sequence motifs (Figure 2.1), the ability of the DH_{44} receptors to interact with either the G_s- or G_q-mediated signaling pathways appears to be ancestral; sequence residues responsible for the interaction are also conserved in vertebrate CRF-receptors (Wietfeld *et al.*, 2004) and within the broader Family B GPCR receptor family (Huang *et al.*, 1996).

*Aaeg*GPRdih1 also contains multiple serine and threonine residues on the third intracellular loop and C-terminus, providing a multiplicity of potential sites for GPCR kinase phosphorylation and β-arrestin recruitment (Teli *et al.*, 2005; Reiter & Lefkowitz, 2006; Oakley *et al.*, 2007), and a sequence motif [-S^{425}-I^{426}-R^{427}-] implicated in PKC phosphorylation and desensitization (Hauger *et al.*, 2003). These diverse potential phosphorylation sites appear partially conserved among dipteran DH_{44} receptors (Figures 2.1 and 2.2) as well as among the greater CRF-receptor family (Teli *et al.*, 2005; Oakley *et al.*, 2007), and reflects the potential complexity in post-translational regulation in these insect receptors.

Unlike vertebrate CRF receptors (Hofmann *et al.*, 2001; Perrin *et al.*, 2001; Alken *et al.*, 2009), there is no experimental evidence for the presence of N-terminal signal sequences for any insect DH_{44} receptor identified to date. Reagan (1996) claimed

that the *Acheta domestica* DH$_{44}$ receptor possessed a signal sequence; however, no description of his prediction method was given. The 'SignalP 3.0' prediction tool (www.cbs.dtu.dk/services/) does not indicate either a signal sequence for the *Acheta domestica* receptor nor for any insect DH$_{44}$ receptor (data not shown); therefore, Reagan's claims are currently unverified. The lack of an N-terminal signal sequence indicates intracellular trafficking differences may exist between insects and vertebrates for the localization of these receptors to the cell membrane or, alternatively, that the current algorithms for prediction are biased for vertebrate signal peptide identification.

Sequence and phylogenetic analysis (Figures 2.2, 2.3, and 2.4) of dipteran DH$_{44}$ receptor transcripts clearly identify the mosquito dih2 receptors as the co-orthologs of the *Drosophila* CG8422 receptor, consistent with the cloning from head cDNA of both *Aaeg*GPRdih2 (this report) and *Drosophila melanogaster* CG8422 (Johnson *et al.*, 2005). In addition, the CG8422 transcript is apparently lacking in *Drosophila* Malpighian tubules (Wang *et al.*, 2004; Chintapalli *et al.*, 2007), while *Aaeg*GPRdih2 transcript is minimally expressed in Malpighian tubules as detected by QPCR (Figures 2.5 and 2.6). We speculate that low level GPRdih2 expression in *Aedes aegypti* Malpighian tubules may be localized in the tip cell, which is of neuronal origin in dipterans (Skaer, 2009) or perhaps the tracheolar cells (Pietrantonio *et al.*, 2000), which as part of the respiratory system, interface with the Malpighian tubules. These analyses also identify the mosquito Malpighian tubule receptor GPRdih1 as the co-ortholog of the *Drosophila* Malpighian tubule receptor encoded by CG12370. Both the *Aaeg*GPRdih1 transcript (Figure 2.5) and CG12370 (Chintapalli *et al.*, 2007) are the most abundant

DH_{44} receptor-encoding transcripts present in Malpighian tubules from their respective species, and ORF alignments reveal similarities in C-terminal protein motifs indicating conservation of intracellular interaction (Figure 2.3).

We hypothesized that the relative abundance of the *Aaeg*GPRdih1 transcript in *Aedes aegypti* Malpighian tubules varies according to the age and physiological state of the female given that: (1) the *Aaeg*GPRdih1 is indeed the most abundant receptor transcript in the *Aedes aegypti* Malpighian tubule; (2) the DH_{44} receptor contributes to the production of primary urine; and (3) the diuretic need of female mosquitoes is not constant (Cabrero *et al.*, 2002; Beyenbach, 2003b; Coast *et al.*, 2005). Therefore, using QPCR we verified that the relative abundance of the *Aaeg*GPRdih1 transcript in Malpighian tubules significantly increased paralleling known periods of high diuresis in the previtellogenic and vitellogenic stages (Figure 2.7).

In newly emerged female *Aedes aegypti*, Gillett observed two periods of water excretion: immediately upon eclosion, and again 11–17 h after eclosion (Gillett, 1982; Gillett, 1983). During this first day the female to eliminates excess water and/or nitrogenous wastes remaining from the larval stages (von Dungern & Briegel, 2001a), but because blood feeding has yet to occur, there is no physiological demand for differential cation loss to maintain homeostasis. This corresponds to the high *Aaeg*GPRdih1 transcript abundance observed during the first day of adult life (Figure 2.7). Upon blood feeding, three different phases of urination are observed in female *Aedes aegypti*: peak phase (within 10 min of feeding), post-peak phase (10 min to 50 min) and late phase (50 to 120 min), with the most significant water loss during the

immediate peak phase (Williams *et al.*, 1983). Ion composition of the urine varies during these three postprandial phases with greatest natriuresis (Na^+ elimination) during the peak phase, with potassium excretion delayed until the post-peak and late-peak phases. The pattern of water and ion loss indicates that multiple control mechanisms act simultaneously during the postprandial period (Williams *et al.*, 1983). Using blood-fed females, Coast (2001b) continuously measured water loss from individual mosquitoes and observed a pattern of water excretion similar to Williams *et al.* (1983). From isolated Malpighian tubules, Coast *et al.* (2005) also observed that the calcitonin-like peptide (DH_{31}) stimulated sodium transport preferentially over potassium ions in both *Anopheles gambiae* and *Aedes aegypti*, whereas the CRF-like peptide (DH_{44}) nonspecifically signaled transport of both cations. While hemolymph concentrations of these peptides have not been determined, the observations by Williams *et al.* and Coast (2005) suggest that after a blood meal, DH_{31} is released immediately into circulation, stimulating the rapid loss of sodium ions and excess water. Somewhat delayed DH_{44} release after blood feeding maintains the elevated, but decreasing, water loss while conserving sodium ions. We observed that *Aaeg*GPRdih1 transcript expression doubled after blood feeding compared to expression levels at 48–72 h in non-fed animals (Figure 2.7). Although it remains to be determined if the highest DH_{44} receptor transcript pools measured are for receptor protein synthesis or alternatively reflect new transcript synthesis for replacement of the depleted transcript pool, we favor the first possibility because the high levels of receptor transcript after 1 h of blood feeding are consistent with a delayed release of DH_{44}, further suggesting that the DH_{44} receptor is involved in

the post-peak and late phase of diuresis. Interestingly, the highest transcript abundance was observed at 24 h post-feeding, a point when there is no observed increase in water loss from the mosquito. This perhaps reflects the need to transport large quantities of nitrogenous waste from the digestion of the blood-meal into the Malpighian tubules (Briegel & Lea, 1975; Cole & Gillett, 1979; Briegel, 1980; Van Handel & Klowden, 1996; von Dungern & Briegel, 2001b) with the hindgut then acting to reabsorb and recycle the water. Female mosquitoes also begin eliminating the indigestible hematin portion of the blood meal 24 h post-feeding (Cole & Gillett, 1979) without additional water loss. The abundance of *Aaeg*GPRdih1 transcript in Malpighian tubules 24 h after taking a blood-meal suggests an important yet-uninvestigated role for DH_{44} signaling in the elimination of nitrogenous waste.

The release of diuretic hormones has been demonstrated immediately after eclosion in locusts (Audsley *et al.*, 1997b) and during and after feeding, such as serotonin release in the bug *Rhodnius* (Lange *et al.*, 1989), CRF-like diuretic hormone in the locust (Audsley *et al.*, 1997a) and diuretic hormones in the mosquito *Anopheles freeborni* (Nijhout & Carrow, 1978). Ours is the first study to show temporal variation of a diuretic hormone receptor transcript in a target tissue from an arthropod, specifically a blood-feeding insect, providing evidence that receptor biology (transcriptional expression) in the Malpighian tubules may be linked to diuretic hormone levels during peaks of diuresis after eclosion and feeding.

Experimental Procedures

Nomenclature

Rules and conventions for genetic features, gene products and the role of species designations in gene names were as proposed in VectorBase for Family Culicidae (aaegypti.vectorbase.org/Docs; under Naming Conventions). Similar nomenclature is accepted for *Anopheles gambiae* GPCRs (Hill *et al.*, 2002) and used for *Aedes aegypti* genome supplementary information (Nene *et al.*, 2007).

Mosquito rearing, dissection and cDNA synthesis

Aedes aegypti L. (Diptera: Culicidae) (Rockefeller strain) larvae were reared at 27°C (Shapiro & Hagedorn, 1982). Larvae were fed high protein Purina ONE® Natural Blends dog food (Nestle Purina PetCare, St. Louis, MO, USA); density was kept low to maximize adult size. Adults were provided 10% sucrose solution for feeding *ad libitum*. Malpighian tubules from 50 previtellogenic 3–5-dayold females were dissected under phosphate-buffered saline (PBS) and placed into RNAlater® (Ambion, Austin, TX, USA) RNA stabilization solution. mRNA was isolated using the DynaBeads® mRNA Direct kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer's specifications. The final elution volume was 20 μl; other details as in Pietrantonio *et al.*, 2005. cDNA concentration was considered as relative 'tissue equivalents' per unit volume for all experiments. To ensure reverse transcription of full-length cDNA, RNA ligase-mediated (RLM) cDNA synthesis was with the GeneRacer™ Kit (Invitrogen) as per the manufacturer's protocol. cDNA was similarly synthesized using mRNA extracted

(DynaBead® kit) from heads of 50 3–5-day-old, previtellogenic females, and Malpighian tubules from 24 h vitellogenic females. Females were blood-fed on an anesthetized mouse following NIH guidelines (Approved Animal Use Protocol TAMU 2007-141).

AaegGPRdih1 cloning

A pair of nested, degenerate sense primers (P133 and P134; Table 2.1) were designed for 3′ RACE which corresponded to the TM region II (residues 169–179 in the final *Aaeg*GPRdih1 sequence) and TM region V (residues 308–317), respectively (Figure 2.1), of insect DH_{44} receptors (*Drosophila melanogaster*: CG8422, CG12370; *Anopheles gambiae*: AGAP005464-RA; AGAP005465-RA; *Manduca sexta*: U03489; *Acheta domestica*: U15959). All rapid amplification of cDNA ends (RACE) reactions contained 1 μl Advantage® 2 Polymerase Mix (Clontech, Mountain View, CA, USA), enzyme buffer to 1× and 0.2 μM of each dNTPs. For 3′ RACE, a 50 μl primary reaction was assembled as follows: to 2.5 μl (1 Malpighian tubules equivalent) of diluted GeneRacer™ Malpighian tubules cDNA was added 0.6 μM P133 and 0.3 μM GeneRacer™ 3′ adaptor primer. After initial heating at 94°C for 3 min, amplification was for 45 cycles (94°C 30 s, 65°C 60 s, 72°C 90 s) with final incubation at 72°C for 5 min. A 50 μl secondary reaction was assembled by adding 1 μl primary reaction to 0.4 μM P134 and 0.2 μM GeneRacer™ 3′ nested adaptor primer. Amplification was as above accept that the time for the extension step was 120 s. Products were visualized by agarose electrophoresis with GelStar™ dye (Lonza Group Ltd, Basel, Switzerland). A

1900-bp band was purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA), and cloned using the TOPO TA Cloning® Kit (Invitrogen). Plasmid DNA was prepared using the QIAprep® Spin Miniprep Kit (Qiagen). Plasmid inserts were confirmed by *EcoR*I restriction analysis, and sequenced as detailed below. Sequence similarity to known DH_{44} receptors was confirmed by BLAST analyses. Specific antisense primers, P144 and P143, were designed based on this 3′ RACE product sequence (Table 2.1). For 5′ RACE, a primary asymmetric reaction was assembled using 2.5 μl of the same Malpighian tubules cDNA to which was added 0.4 μM Primer P144. Final volume was 49 μl. After an initial incubation at 94°C for 3 min, asymmetric amplification proceeded for 10 cycles (94°C 30 s, 64°C 60 s, 72°C 120 s) before the addition of 0.2 μM GeneRacer™ 5′ adaptor primer for 45 additional cycles, with final incubation at 72°C for 5 min. A secondary nested reaction combined 1 μl primary reaction with 0.4 μM Primer P143 and 0.2 μM GeneRacer™ 5′ nested adaptor primer in 50 μl volume. Amplification was for 45 cycles (94°C 30 s, 64°C 60 s, 72°C 120 s), prior to a final 72°C extension step for 5 min. A 1900-bp product was purified and cloned as above. Sequencing analyses identified a large sequence overlap between the 3′ RACE and 5′ RACE clones, confirming that they were obtained from the same cDNA. The amplification product corresponding to the full length ORF is shown in Figure 2.5.

AaegGPRdih2 cloning

A partial ORF for *Aaeg*GPRdih2 was predicted based on the sequence present in the *Aedes aegypti* genome. Based on this, a specific sense primer, P197F (encompassing amino acid residues 69–82 in the final GPRdih2 sequence), was designed (Table 2.1). All RACE reactions contained 1 µl Advantage® 2 Polymerase Mix (Clontech), enzyme buffer to 1X and 0.2 µM of each dNTPs. 3′ RACE reactions were in 50 µl containing 1 µl (0.4 head equivalent) diluted GeneRacer™ head cDNA, 0.4 µM P197F and 0.4 µM GeneRacer® 3′ adaptor primer. Amplification proceeded for 40 total cycles using 'Touch Down': 94°C for 3 min initial denaturation, followed by 20 cycles of 94°C for 30 s, 72°C (decreasing 0.5°C/cycle) for 60 s and 72°C for 120 s; then following 20 cycles of 94°C for 30 s, 65°C for 60 s and 72°C for 120 s. The reaction was then incubated at 72°C for 5 min. Products were visualized by gel electrophoresis, cut from the gel, cleaned and cloned as above. Plasmid DNA was prepared, and inserts were confirmed by restriction analysis as above. The 2130-bp DNA 3′ RACE product was sequenced from both directions as detailed below, with identity confirmed by comparison with the available genomic prediction. For 5′ RACE, specific antisense primers P197R (complementary to P197F) and P206 (encompassing the stop codon and a portion of the 3′ UTR) (Table 2.1) were designed and RACE carried out in a 50 µl amplification reaction containing 1 µl (0.4 head equivalent) diluted GeneRacer™ head cDNA to which was added 0.4 µM GeneRacer® 5′ adaptor primer and 0.4 µM P206 primer. After initial denaturation and enzyme activation at 94°C for 3 min, amplification was for 40 cycles (94°C 30 s, 62°C 60 s, 72°C 120 s) with a final extension step at 72°C for 5 min. Gel

electrophoresis analysis revealed no products of the expected size, so a secondary nested reaction was assembled using 1 µl of a 1:100 dilution of the previous reaction as template to which was added 0.4 µM GeneRacer® 5′ nested adaptor primer and 0.4 µM P197R primer. The amplification procedure was as for the primary reaction except the annealing temperature was raised from 62°C to 66°C. Products were visualized, cut, gel extracted and cloned as above. Plasmid DNA was prepared and inserts were confirmed as above. The 5′ RACE 909-bp DNA insert was sequenced as below and a region of identical DNA sequence overlap with the 3′ RACE clone confirmed this sequence as part of the same cDNA.

Sequence analyses

All sequencing reactions used ~400 ng DNA template and proceeded for 50 cycles using ABI Big Dye® (Applied Biosystems, Foster City, CA, USA) with other details as described (Pietrantonio *et al.*, 2005). Sequence data were analyzed using the DNASTAR software suite (DNASTAR Inc., Madison, WI, USA). The BLAST search algorithms at NCBI (www.ncbi.nlm.nih.gov/) and VectorBase (www.vectorbase.com) were used to identify DH_{44} receptor-like sequences from other organisms. Protein domain searching was with PFAM (www.sanger.ac.uk/cgi-bin/Pfam) (Finn *et al.*, 2006). Transmembrane region predictions were made using TMHMM (www.cbs.dtu.dk/services/).   Potential glycosylation sites and kinase-specific phosphorylation sites located within the *Aaeg*GPRdih1 sequence were predicted with 'NetNGlyc' and 'NetPhosK' (www.cbs.dtu.dk/services/), respectively. Potential N-

terminal signal peptides were investigated using 'SignalP 3.0' (www.cbs.dtu.dk/services/) (Bendtsen *et al.*, 2004). Sequences were aligned using MEGALIGN (DNASTAR) and CLUSTALW (align.genome.jp/). Bootstrap analysis was performed using PAUP version 4.0b10 (Swofford, 2002) with 10,000 bootstrap replications and 50% majority-rule consensus. Alignment gaps were treated as missing data. Trees were rooted using the *Caenorhabditis elegans* C18B12.2 secretin-type receptor (Harmar, 2001). Sequence predictions for *Culex quinquefasciatus* GPRdih1 and GPRdih2 were obtained from the WGS database (NCBI) and the CpipJ1.0_5 gene assembly (www.vectorbase.org) by using *Aaeg*GPRdih1 and *Aaeg*GPRdih2 as query sequences with the tBLASTn algorithm to identify genomic DNA fragments with the highest similarities. Genomic DNA fragments encoding potential ORFs were imported into MAPDRAW (DNASTAR) and translated in all reading frames. tBLASTn results were manually mapped to the MAPDRAW output, and potential exon−intron boundaries were confirmed using the splice site prediction tool of the Berkeley *Drosophila* Genome Project (www.fruitfly.org/seq_tools/splice.html). Identified ORF-containing exons were manually arranged using EDITSEQ (DNASTAR) and translated *in silico*.

Tissue expression analysis of AaegGPRdih1 and -GPRdih2 transcripts

To determine the presence or absence of GPRdih1 and GPRdih2 transcripts in *Aedes aegypti* heads and Malpighian tubules, amplification reactions were assembled using tissue-specific cDNA templates and unique pairs of specific primers (Table 2.1). Reactions contained either 1 μl (0.4 head equivalent) diluted GeneRacer™ head cDNA

prepared from 3–5-day-old, previtellogenic females or 5 μl (0.4 Malpighian tubule

equivalent/μl) diluted GeneRacer™ Malpighian tubule cDNA prepared from 24 h

vitellogenic females, to which was added 0.2 μM dNTPs (each), 0.6 μM sense primer,

0.6 μM antisense primer, 1X enzyme buffer and 1 μl Advantage® 2 Polymerase Mix

(Clontech). Primer pairs corresponded to sequences encompassing the start (sense) or

stop (antisense) codons of each transcript (Table 2.1). The amplification of another

GPCR transcript (5HT$_7$-like receptor) known to be expressed in heads and in tracheolar

cells attached to Malpighian tubules (Pietrantonio *et al.*, 2000; Lee & Pietrantonio, 2003)

was used as a positive control. As the *Aaeg*GPRdih2 sequence is incomplete on the 5′

end, P197F was used as the sense primer. After initial heating at 94°C for 3 min,

amplification was for 35 cycles of 94°C 30 s, 62°C 60 s, 72°C 90 s, with a final

extension at 72°C for 5 min. Amplification products were visualized on a 1% agarose

gel, 0.5× TBE, stained with GelStar™ dye (Lonza) and digitally documented using the

Foto/Analyst® Investigator photographic system with Image J 1.34s software (Fotodyne,

Hartland, WI, USA).

Amplicon design for quantitative PCR

Using Primer Express® software (Applied Biosystems), primers P180 and P181

(Table 2.1) were designed to amplify a 69-bp amplicon corresponding to the sequence

between TM regions V and IV of *Aaeg*GPRdih1. Primers P203 and P204 (Table 2.1)

were designed to amplify a 67 bp region of the *Aaeg*GPRdih2 transcript corresponding

to the terminal intracellular domain. There was no sequence similarity between the

amplicons. Optimal primer concentrations (900 nM each) were determined according to ABI directions (SYBR® Green PCR Master Mix protocol) using as template M. tubule (GPRdih1) or head cDNA (GPRdih2). β-actin gene expression was used to normalize all experimental results, with amplicon primers synthesized as reported (Zhu *et al.*, 2003) and used at 900 nM each.

Time course analysis of AaegGPRdih1 abundance

Malpighian tubules from 50 females (~250 Malpighian tubules; about 11 μg total RNA) for each of the following nine time points were dissected into RNAlater® (Ambion) stabilization solution and stored at 4°C. Previtellogenic period: 0–12, 12–24, 24–48 and 48–72 h post-eclosion. Vitellogenic period: 1, 3, 6, 12 and 24 h post-blood feeding. Dissections were repeated in triplicate for each time point. In order to avoid any potential variability from circadianicity, the tissues for each time point were dissected at various times of the day and in no particular order. Blood-fed females were allowed to feed for 30 min and to rest for 30 additional minutes prior to the start of dissections. Total tissues dissected were approximately 7000 Malpighian tubules from 1350 female mosquitoes. For each time point, three independent cDNA synthesis reactions were from the three separate tissue isolations. For each synthesis, Malpighian tubules were pelleted by centrifugation, and mRNA was extracted (DynaBead® kit); beads were reconditioned for an additional extraction from the same homogenate resulting in a final eluted volume of 20 μl mRNA which was stored at –80°C. Single-stranded cDNA was prepared for every isolated mRNA sample using the SuperScript™

III First-Strand Synthesis System (Invitrogen) using oligo-$(dT)_{20}$ as per the kit instructions.

Quantitative PCR

Reactions using SYBR® Green PCR Master Mix (Applied Biosystems) were assembled for three replicate cDNAs for every time point as follows: to 60 μl SYBR® Green reagent was added 6 μl (~30 Malpighian tubule equivalents) cDNA and 10.8 μl water. This volume was equally divided (38.4 μl each) for estimation of the *Aaeg*GPRdih1 and β-actin transcripts, respectively. To each aliquot was added either 10.8 μl each of *Aaeg*GPRdih1 amplicon primers P180 and P181, or 10.8 μl each of β-actin amplicon primers for a final volume of 60 μl. On a 96-well MicroAmp® plate (Applied Biosystems), three wells (20 μl) were loaded for each template-primer combination. QPCR was performed for 45 cycles (95°C 15 s, 60°C 60 s) using the 7300 RT-PCR System (Applied Biosystems). Relative abundance for each transcript was calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method and Sequence Detection Software v1.3.1 (Relative Quantification Study Application; Applied Biosystems) according to the manufacturer's directions, with statistical analysis using SPSS v12.0.1 for Windows (SPSS Inc, Chicago, IL, USA). The 48–72 h previtellogenic data point was used as the reference ratio (calibrator) for the time course analysis (Figure 2.7). For time course analysis, the null-hypothesis that there were no differences among sample means with respect to transcript relative abundance was rejected and differences among sample means were tested by one-way analysis of variance (ANOVA) with Bonferroni

correction (Figure 2.7). Significance was established as $P < 0.05$. For QPCR supporting analysis of expression in specific tissues (Figure 2.6), relative transcript abundance was determined using respective amplicon primers for *Aaeg*GPRdih1 and *Aaeg*GPRdih2 (Table 2.1). Abundance of the respective transcripts in head cDNA (not shown), which was similar for both receptors, was set as the calibrator. Calibrators were set according to ABI protocols . Dissociation curve analysis indicated that the QPCR amplicons were specific for their respective cDNAs (data not shown). Graphs were prepared using GraphPad Prism v4.0 (GraphPad Software Inc., San Diego, CA, USA).

CHAPTER III

INVESTIGATING THE PHYSIOLOGICAL FUNCTION OF

GPRdih1 IN MALPIGHIAN TUBULES OF THE

YELLOW FEVER MOSQUITO *Aedes aegypti* (DIPTERA: CULICIDAE)

Introduction

The insect Malpighian tubule is a recognized model for epithelial transport, osmoregulation and excretion (Maddrell, 1969; O'Donnell & Maddrell, 1984; O'Donnell & Maddrell, 1995; Dow, 2009). Further understanding of Malpighian tubule function is now potentially enhanced with the discovery that exogenous double-stranded RNA can be used to alter endogenous, post-translational gene expression. With the discovery and description of RNA interference (RNAi) in animals (Fire *et al.*, 1998), the regulatory cascades controlling and coordinating Malpighian tubule function, and the contribution made by any single gene to the physiology of the whole organism can now be more fully elucidated. Exploitation of RNAi is especially useful for the many genes of unknown function, the disruption of which may have a recognizable phenotype (Dow, 2007; Downward, 2009). Coupled with a functional assay, RNAi can be exploited in non-model organisms to screen for gene function without creating transgenic animals.

Applications using RNAi have been successfully used to knock down gene expression in tissues of blood-feeding dipterans including the tsetse fly, *Glossina*

morsitans, midgut (Walshe *et al.*, 2009), the sand fly, *Lutzomyia longipalpis,* fat body (Sant'Anna *et al.*, 2008), *Aedes aegypti* fat body (Zhu *et al.*, 2003; Blitzer *et al.*, 2005; Scaraffia *et al.*, 2008), ovaries (Brown *et al.*, 2008) and midgut (Franz *et al.*, 2006), and *Anopheles gambiae* midgut, fat body, hemocytes (Blandin *et al.*, 2002), salivary glands (Boisson *et al.*, 2006), and oenocytes (Lycett *et al.*, 2006). Several investigators have used RNAi to study Malpighian tubule function in mosquitoes. In *Anopheles gambiae* Lycett and colleagues (2006) observed knock down of a cytochrome P450 reductase (CPR) gene expressed in midgut and Malpighian tubule principal cells; however, since their RNAi experiments used pooled midgut and Malpighian tubule tissues, it is not clear if both tissues contributed to the observed decrease in CPR translation. Using transgenic methods RNAi has been shown to be effective in Malpighian tubules of *D. melanogaster* (Torrie *et al.*, 2004; Day *et al.*, 2006; Huang *et al.*, 2005; Yang *et al.*, 2007). The first unequivocal demonstration that non-transgenic RNAi is effective in mosquito Malpighian tubules was made by Scaraffia and colleagues (2008) who were able to block urea synthesis after a blood meal by targeting a Malpighian tubule urate oxidase (Scaraffia *et al.*, 2008). The amenability of Malpighian tubules to functional study with RNAi-based methods will greatly enhance our understanding of the regulatory and signaling cascades controlling the function of these vital organs.

In adult females of *Aedes aegypti*, the Malpighian tubules are responsible for the removal from the hemolymph to the alimentary canal of (1) water and metabolic wastes retained from the larval-pupal stages (Romoser *et al.*, 2000; von Dungern & Briegel, 2001a) and (2) excess water and ions obtained as a result of consuming a hypo-osmotic

blood meal (Williams *et al.*, 1983). These two periods of functionality are accompanied by a high rate of urination; however, the Malpighian tubules also serve to transport nitrogen waste into the alimentary canal without a subsequent high rate of urination. Females of *Aedes aegypti* require a blood meal in order to obtain the amino acids and energy requirements for the synthesis of egg proteins (Briegel, 1985; Zhou *et al.*, 2004). Blood meal digestion and protein deamination results in large quantities of toxic ammonia, which may be temporarily converted to glutamine or proline for short term storage (Scaraffia *et al.*, 2005), but which is ultimately catabolized via the uricotelic or ureotelic enzymatic pathways, producing uric acid or urea respectively. Little research has addressed the functional coordination among fat body, alimentary canal and Malpighian tubules for digestion and waste elimination of the protein portion of the blood meal. Nitrogen waste elimination (defecation) begins within one day of blood feeding, and a coordinated function among several tissues including the Malpighian tubule is suggested by the up regulation 24 h after blood feeding of a diuretic hormone receptor (GPRdih1) (Jagge & Pietrantonio, 2008) and transcripts for some of the enzyme components of the uricotelic/ureotelic pathways (von Dungern & Briegel, 2001b; von Dungern & Briegel, 2001a; Scaraffia *et al.*, 2005; Scaraffia *et al.*, 2008). Additionally, the indigestible, iron-containing hematin portion of the blood meal is expelled from the hindgut 24-48 h post-feeding (Cole & Gillett, 1979; von Dungern & Briegel, 2001a). Defecation of nitrogen waste, hematin, and water from the alimentary canal is directly controlled by the terminal abdominal ganglion (Van Handel & Klowden, 1996) which innervates the hindgut (Curtin & Jones, 1961) further indicating neuronal coordination

among diverse tissues and behaviors including reproduction, feeding, digestion, diuresis, and defecation.

G protein-coupled receptors (GPCRs) are seven transmembrane-spanning proteins which transduce an extracellular signal via conformational changes in their tertiary structure into an intracellular signal. Signaling by GPCRs is one mechanism by which multiple tissues and behaviors are coordinated. Four classes of identified diuretic hormones (cardioacceleratory peptide CAP2b; insect kinins; Calcitonin-like diuretic hormone 33; Corticotropin releasing factor-like diuretic hormone 44) regulate Malpighian tubule function in the Malpighian tubules of *Aedes aegypti*. RNAi targeting these receptors could shed light on the pivotal physiological role of these proteins in the regulation of diuresis, and additionally the potential role they may also have in the coordination of multiple behaviors. Indeed systematic gene knockout of sixty neuropeptide and neurotransmitter GPCRs in the nematode *Caenorhabditis elegans* identified receptors with critical function in reproduction and locomotion (Keating *et al.*, 2003). RNAi was also used to comprehensively characterize eight potential serotonin GPCRs (Family A) in *C. elegans* (Carre-Pierrat *et al.*, 2006). Among insects RNAi has been used to show the necessity for a GPCR in the formation of the blood-brain barrier in *Drosophila melanogaster* (Schwabe *et al.*, 2005); however, specific dsRNA targeting of the arginine vasopressin-like (AVPL) receptor (Family A GPCR) in the red flour beetle *Tribolium castaneum* resulted in a decrease of receptor transcript but no observable phenotype (Aikins *et al.*, 2008), suggesting redundancy in receptor function. To date there has been only one report of the successful generation of RNAi in

Malpighian tubules of *Aedes aegypti* by injection of dsRNA. Scaraffia and colleagues (2008) triggered the RNAi cascade by injection of dsRNA targeting urate oxidase expression which is critical for the processing of uric acid to urea. The transcript for urate oxidase (UO) is up-regulated approximately 24 h after a blood meal; however, homologous dsRNA introduction reduced UO transcript abundance at 24 h post-feeding by 96% with a simultaneous whole-body uric acid accumulation. The authors neglected to highlight an additional impact of their work, namely that the Malpighian tubules of *Aedes aegypti* are capable of dsRNA uptake, proving that RNAi methods using exogenous dsRNA can be used to study Malpighian tubule function.

To demonstrate that RNAi can be an effective tool for the study of Malpighian tubule function in females of *Aedes aegypti* and to further our understanding of the signaling cascades coordinating Malpighian tubule function, I attempted to decrease transcript abundance for the diuretic hormone 44 (corticotropin-like) hormone receptor (GPRdih1).

Results

The purpose of this research was to determine if a corticotropin releasing factor-like diuretic hormone 44 receptor in Malpighian tubules of *Aedes aegypti* is amenable to physiological study using RNA interference techniques. A GPRdih1 cDNA clone (*Aaeg*GPRdih1; EU273351) previously identified (Jagge & Pietrantonio, 2008) and a clone for the <u>e</u>nhanced <u>g</u>reen <u>f</u>luorescent <u>p</u>rotein (EGFP; Accession #U55763) (Cormack *et al.*, 1996) were used as templates for DNA amplification using gene specific primers (*GPRdih1*: P156-P166; *EGFP*: P164-P165; Table 3.1) flanked with the T7 RNA polymerase recognition sequence. From the resulting amplification products, high quality double-stranded RNA (dsRNA) corresponding to a 675 bp fragment of the GPRdih1 transcript and 611 bp region of EGFP were generated *in vitro* using the MEGAscript™ RNAi Kit (Ambion) (Figure 3.1). To attempt to knock down the GPRdih1 transcript in Malpighian tubules, ~400 ng respective dsRNA was introduced into 12-24 h post-eclosion females by intrathoracic injection (Figure 3.2).

Table 3.1 DNA primers used in PCR to generate the templates for double-stranded RNA synthesis. Each of the primers incorporates the T7 promoter minimal sequence at the 5' end. The T7 minimal primer is shown for reference.

| Designation	Sequence	Orientation
For dsRNA-dih1 template preparation		
T7-P156	5'TAATACGACTCACTATAGGGAATTAGTCTCCCGGAGTCGAGTATGAATG3'	Sense
P166-T7	5'TAATACGACTCACTATAGGGCTCCTTAAAGTAAACTAGAACGGTGAC3'	Anti-sense
For dsRNA-EGFP template preparation		
T7-P164	5'TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTCAGCGTGTC3'	Sense
P165-T7	5'TAATACGACTCACTATAGGGTCACGAACTCCAGCAGGACCATGTGATC3'	Anti-sense
T7 Minimal	5'TAATACGACTCACTATAGGG3'	
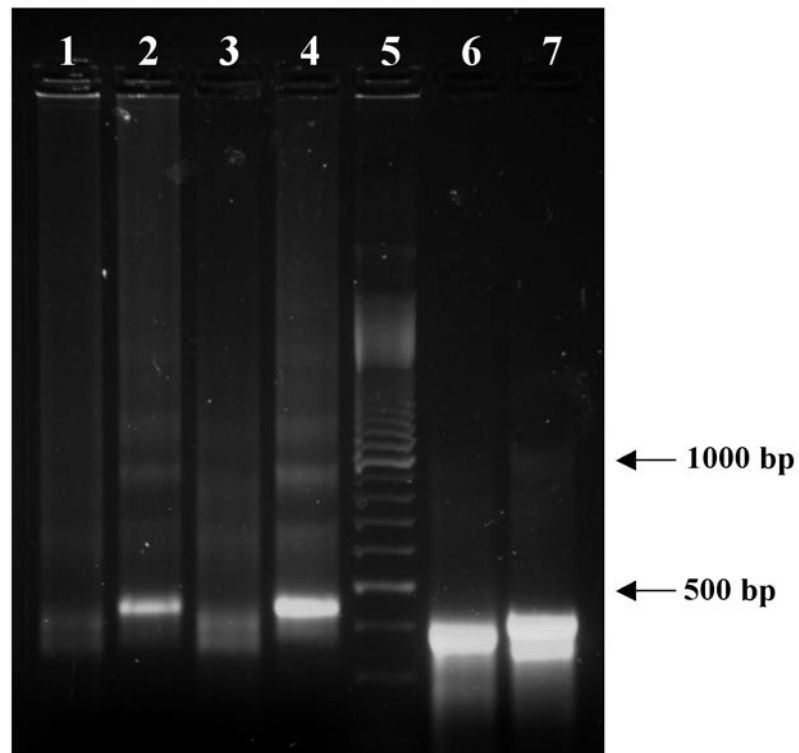
Figure 3.1 Results of the *in vitro* synthesis of dsRNA for GPRdih1 and EGFP. Synthesis of dsRNA was for two different templates: enhanced green fluorescent protein (Lane 1: raw synthesis, Lane 2: product after annealing, Lane 6: purified product) and Malpighian tubule DH_{44} receptor transcript (Lane 3: raw synthesis, Lane 4: product after annealing, Lane 7: purified product). Lane 5 is a 100 bp DNA size standard. Notice that dsRNA quality and concentration increases greatly after annealing and purification. The appearance of two bands in lane 7 is an artifact of the quantity of the dsRNA visualized on the gel. Note: purified dsRNA migrates approximately 200 bp faster than would be expected when compared to a DNA standard.

Figure 3.2 Intrathoracic injection of a female of *Aedes aegypti*. A cold-anesthetized female of *Aedes aegypti* immediately prior to dsRNA intrathoracic injection using a Femtotip® sterile injection capillary needle (Eppendorf). Females anesthetized with cold had a lower control mortality than those anesthetized using carbon dioxide (data not shown).

Mosquitoes were segregated according to injection treatment (Figure 3.3) and allowed to recover for 4 days. After recovery females of all treatments were blood-fed by exposure to an anesthetized mouse (Approved Animal Use Protocol TAMU 2007-141) according to NIH protocols. Mosquitoes were allowed to feed for 30 minutes after which their Malpighian tubules were dissected for RNAi evaluation. Since GPRdih1 transcript abundance is up regulated by blood-feeding (Jagge & Pietrantonio, 2008), I believed a decrease in transcript abundance due to RNAi effect would be more easily observed in tissues dissected within 1 hour after blood feeding. The relative abundance of GPRdih1 transcript in Malpighian tubules of all treatments was determined by real-time QPCR four days after treatment. In three independent experiments, the introduction of GPRdih1-specific dsRNA had no observable effect upon the abundance of endogenous GPRdih1 mRNA transcript when observed four days after injection and 1 h after blood-feeding (Figure 3.4).
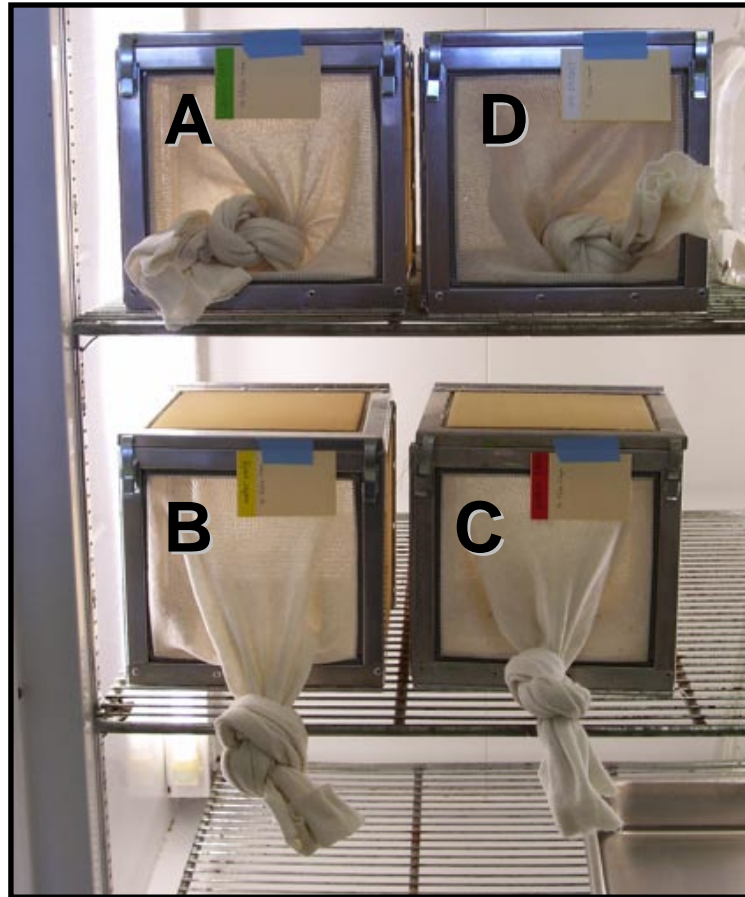
Figure 3.3 Cages containing females of *Aedes aegypti* subjected to control and injection treatments.
(A) dsRNA-EGFP injected, (B) water injected, (C) dsRNA-GPRdih1 injected, and (D) non-injected females were maintained inside a humidified insect rearing chamber. Insects were kept at 27°C and maintained a light/dark cycle of 16:8 hours. Mortality was determined four days after injection for all injected treatments. Remaining mosquitoes were then blood fed for 30 minutes and Malpighian tubules were dissected.

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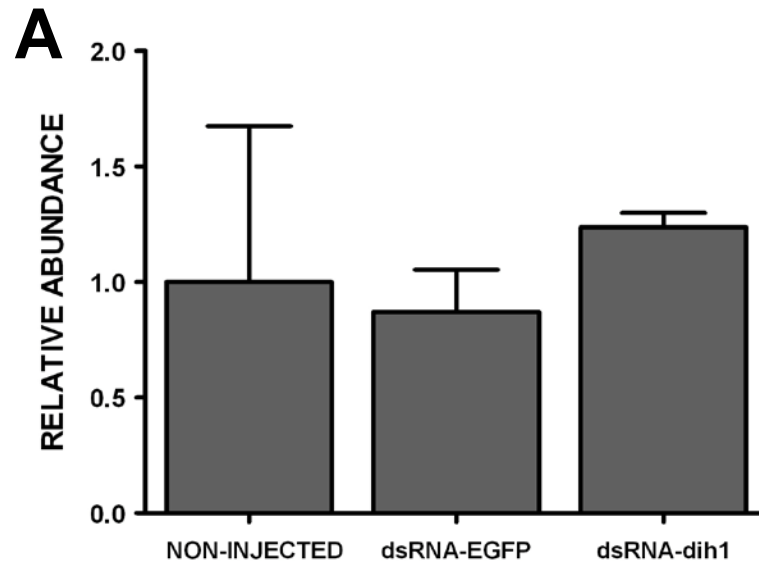
Figure 3.4   Determination of GPRdih1 transcript relative abundance in Malpighian tubules four days after dsRNA treatment and thirty minutes after a blood meal.   QPCR was performed to determine the relative abundance of GPRdih1 transcript in Malpighian tubules of females of *Aedes aegypti* four days after dsRNA injection and thirty minutes after blood-feeding.   For three independent replicates, three treatments were no injection, dsRNA-EGFP or dsRNA-dih1 (A,B) or water, dsRNA-EGFP or dsRNA-dih1 (C).   No decrease in GPRdih1 transcript abundance was observed for any of the three independent replicates. The large apparent increase in GPRdih1 transcript abundance (B) is likely due to a high degree of variability among individual mosquitoes.
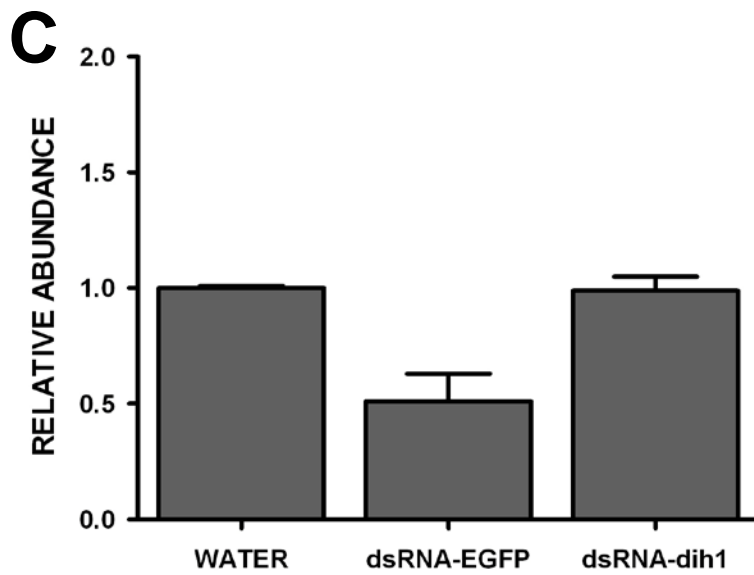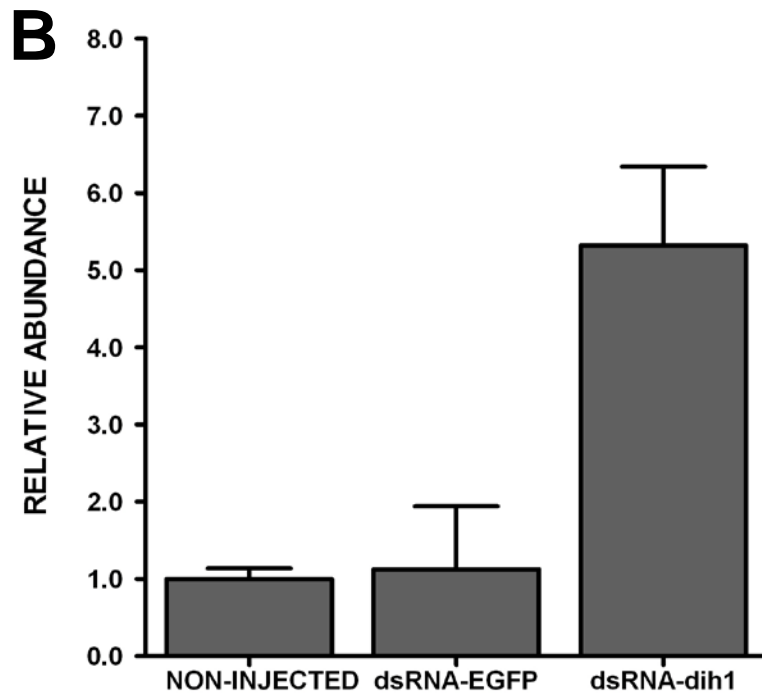
Figure 3.4   *continued*

GPRdih1 transcript relative abundance is elevated 24 h after taking a blood meal (Jagge & Pietrantonio, 2008). This may correspond to increased demand for alimentary canal transport of nitrogen waste after blood meal digestion and metabolism. To determine if GPRdih1 has a function with regard to nitrogen waste excretion, female mosquitoes were intrathoracically injected and monitored for the onset and duration of excretion of the indigestible portion of the blood-meal, which begins approximately 24 h after blood feeding (Cole & Gillett, 1979). Introduction of GPRdih1-specific dsRNA had no effect on the observed onset or rate of defecation (determined by counting excreta droplets) when animals were blood-fed four days after injection (Figure 3.5).

Discussion

I attempted to use RNA interference to investigate the role of the CRF-like DH_{44} receptor (GPRdih1) in *Aedes aegypti* female Malpighian tubule functionality; however the introduction of GPRdih1 specific dsRNA did not result in a decrease of endogenous dih1 transcript (Figure 3.4), nor had any observable effect upon the number of excreta droplets counted for 30 hours beginning 24 hours after blood-feeding (Figure 3.5). RNAi methods have been adapted to study gene function in mosquitoes; however, Boisson and colleagues (2006) showed that the quantity of dsRNA used to demonstrate an RNAi effect in some tissues was insufficient when used in other tissues. Using *Anopheles gambiae, Anopheles stephensi* and *Aedes aegypti* adults expressing the green fluorescence protein (GFP), they showed that injection of 140 ng dsRNA-GFP was sufficient to silence gene expression in midgut cells and ovaries; however, the
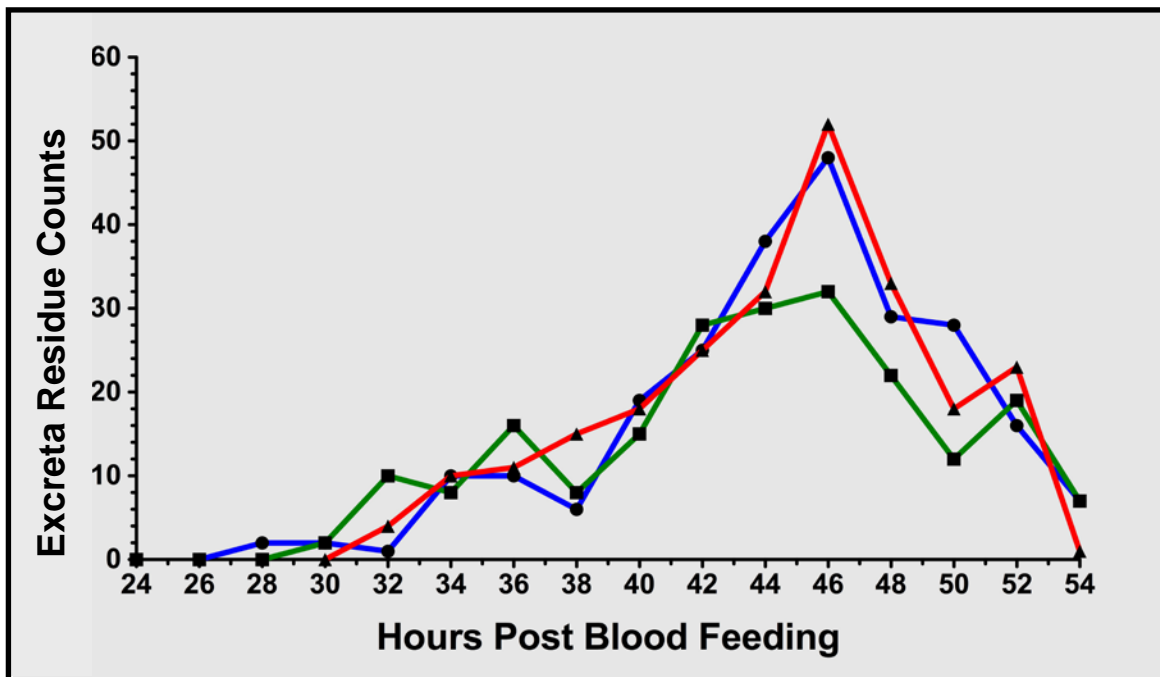
Figure 3.5   Defecation onset determination in dsRNA treated females of *Aedes aegypti*.   Treated females (●, blue line: no injection; ■, green line: dsRNA-EGFP injection; ▲, red line: dsRNA-dih1 injection) were blood fed to repletion four days after injection and placed into glass vials. The time of appearance and count of excreta residues was monitored beginning 24 h post-blood feeding. There was no difference among treatments with regard to the time of appearance of or number of excreta droplets observed.

observed knockdown of salivary gland GFP expression was successful only after injection of 1600 ng dsRNA, approximately ten times more dsRNA (Boisson *et al.*, 2006). They also showed that an 80% reduction in expression of two endogenous genes was observed only after injection of 840 ng and 2300 ng per female, demonstrating that the RNAi effectiveness of dsRNA is both tissue and gene-specific. Malpighian tubules of *Aedes aegypti* are amenable to study using RNAi (Sacaraffia *et al.*, 2008). By knocking down expression of urate oxidase, which converts uric acid to allatonin in the urea-synthesis pathway, treated animals had an accumulation of uric acid (Scaraffia *et al.*, 2008). Injection of newly emerged females with 250 ng of urate oxidase-specific dsRNA and recovery for 4 days prior to blood-feeding was sufficient to reduce the urate oxidase transcript 96% 24 h after blood-feeding.

The failure of my experiments to show post-transcriptional silencing of GPRdih1 was likely because the quantity of dsRNA introduced was insufficient to decrease transcript abundance in Malpighian tubules and/or that the incubation time from injection to analysis was insufficient for an effective RNAi response. The amount of dsRNA I introduced into female mosquitoes (~400 ng) was lower than shown by Boisson *et al* (2006) (~840–2300 ng) to effectively knockdown salivary gland genes. Kwon and Pietrantonio (personal communication) have recently shown that injection of 1500 ng dsRNA for another Malpighian tubule GPCR decreased transcript abundance only 30-40%. RNAi targeting GPCRs in *Aedes* Malpighian tubules may require large amounts of dsRNA to show a decrease in transcript. Additionally, mosquitoes injected with dsRNA-dih1 were allowed to recover for 4 days prior to blood-feeding and

analysis. Scaraffia *et al* (2008) reported an injection regime similar to my experiment where injected female mosquitoes were allowed to recover for 4 days before blood-feeding prior to dissection; however Kwon and Pietrantonio (personal communication) demonstrated that the most effective RNAi effect targeting another Malpighian tubule GPCR is observed more than four days after dsRNA injection. The potential role of GPRdih1 in defecation was suggested by a preliminary experiment where dsRNA-dih1 treatment appeared to delay the start of defecation when blood-feeding commenced four days after injection. Though exposure to dsRNA was for an additional two days, further investigation showed that the onset of defecation was no different in dsRNA-dih1 treated and control animals (Figure 3.5).

Experimental Procedures

Double-strand RNA (dsRNA) synthesis

Sense (P156; nucleotides -195 to -166 on Figure 2.1) and antisense (P166; nucleotides 454 to 481 on Figure 2.1) primers flanking a 675 bp fragment of the mosquito *Aaeg*GPRdih1 sequence and sense (P164; nucleotides 69 to 92 of pEGFP-C1 clone, Accession #U55763) and antisense (P165; nucleotides 649 to 695 of pEGFP-C1 clone, Accession #U55763) primers flanking a 611 bp fragment of the enhanced green fluorescent protein from the jellyfish *Aequorea victoria* (Cormack *et al.*, 1996) were designed with T7 minimal promoter overhangs (5'-TAATACGACTCACTATAGGG-3'; Ambion, Austin, TX, USA) (Table 3.1). To prepare the DNA templates for dsRNA-EGFP and dsRNA-dih1 synthesis, 100 μl amplification reactions contained 1 μl Taq

polymerase (Promega; Madison, WI, USA), enzyme buffer to 1X, 0.4 µM of each dNTPs, 0.2 µM each sense and antisense primer, and approximately 4 ng of plasmid template. For dsRNA-EGFP template synthesis, template was the EGFP cDNA in the PinPoint™ vector (Promega) generously provided by Dr. Craig Coates (TAMU). For dsRNA-dih1 template synthesis, template was a cDNA corresponding to the 5'UTR and ORF of *Aaeg*GPRdih1 in the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) (Jagge & Pietrantonio, 2008). After initial heating at 94°C for 3 min, amplification was for 45 cycles (94°C 30 s, 67°C 30 s, 72°C 60 s) with final incubation at 72°C for 5 min. Products were visualized by agarose electrophoresis with GelStar™ dye (Lonza Group Ltd, Basel, Switzerland), cut from the agarose gel, and purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA). For each purified product, a 100 µl secondary reaction contained 1 µl Taq polymerase (Promega), enzyme buffer to 1X, 0.4 µM of each dNTPs, 0.4 µM T7 minimal primer (Table 3.1), and approximately 100 ng of purified product. Products were visualized by agarose electrophoresis with GelStar™ dye (Lonza Group Ltd, Basel, Switzerland), and the resulting products were phenol/chloroform extracted, ethanol precipitated, and DNA resolubilized in 50 µl nuclease-free water. The final concentration for each was approximately 130 ng/µl. Amplification and purification were repeated as needed to generate sufficient DNA template for dsRNA synthesis. Using respective T7-flanked DNA templates (~3 µg) and the MEGAscript™ RNAi Kit (Ambion), dsRNA synthesis reactions were scaled 3X with respect to the manufacturer's instructions and proceeded at 37°C overnight in a thermocycler as per the manufacture's protocol. After synthesis, reactions were

denatured at 75°C for 5 min and allowed to cool to room temperature. All dsRNA was column purified as per directions and eluted in 200 μl nuclease-free water (Zhu *et al.*, 2003). After determining the nucleic acid concentration, samples were concentrated to ~4 μg/μl at room temperature in a vacuum drier.

Mosquito rearing, injection, and dissection

     Aedes aegypti L. (Diptera: Culicidae) (Rockefeller strain) larvae were reared at 27°C (Shapiro & Hagedorn, 1982) with a light cycle of 16:8 hours. Larvae were fed high protein Purina ONE® Natural Blends dog food (Nestle Purina PetCare, St. Louis, MO, USA); density was kept low to maximize adult size. Batches of young females aged 12-24 h post-eclosion were exposed to 4°C to knockdown and placed in a glass dish on ice prior to injection. Each batch contained 15-20 females to minimize anesthetization time. Anesthetized females were intrathoracically injected using Femtotip® sterile injection capillaries with the FemtoJet® programmable microinjector (Eppendorf, Westbury, NY, USA) with either ~100 nl of either dsRNA-EGFP or ~100 nl dsRNA-dih1. Anesthetized but non-injected females were also maintained as negative controls. After treatment females were placed in cages (1 ft^3), with untreated males (two males for each female) to recover and mate. Adults were provided 10% sucrose solution for feeding *ad libitum*. After four days recovery, mortality was less than 20% for all treatments and less than 5% for cold-treated but non-injected females. After the recovery period, female mosquitoes were exposed for 30 min to an anesthetized mouse (Approved Animal Use Protocol TAMU 2007-141). After 30 min blood feeding,

females were rested for an additional 30 min. Malpighian tubules from 60-65 females of each treatment were dissected into RNA*later*™ RNA stabilization solution (Ambion) at room temperature. Exposure to the anesthetized mouse, blood feeding and dissection were completed for each experimental treatment prior to the blood feeding of the next treatment. This regime of experimental treatment (dsRNA injection and control treatments) of females, recovery, blood feeding and dissection of ~180 females (~60 females per treatment) was considered as a single experimental block. Two experimental blocks were performed in this manner, and a third experimental block was identical except the negative control females received an injection of ~100 nl water, but otherwise treated as above.

cDNA preparation

For each cDNA synthesis, Malpighian tubules in RNA*later*™ (Ambion) were pelleted by centrifugation, and tissues were processed for mRNA extraction using the DynaBead® kit (Invitrogen); magnetic beads were reconditioned for an additional extraction from the same homogenate resulting in a final eluted volume of 20 μl mRNA, which was stored at -80°C. Single-stranded cDNA was prepared for the entirety of each isolated mRNA with the SuperScript™ III First-Strand Synthesis System (Invitrogen). Briefly, to each 20 μl mRNA isolation (~300 Malpighian tubule equivalents) was added 2 μl 50μM oligo(dT)$_{20}$ with incubation at 65°C for 5 min and then on ice for 1 min. A 20 μl final volume cDNA synthesis mix was created in a separate tube by combining 4 μl reverse transcription buffer, 8 μl 25 mM MgCl$_2$, 4 μl 100 mM dithiothreitol, 2 μl (80

U) RNaseOUT™ RNase inhibitor, and 2 μl (400 U) SuperScript™ III reverse transcriptase. The 20 μl synthesis mix was added to the 22 μl mRNA/primer mix and incubated at 50°C for 50 min followed by an enzyme inactivation step of 85°C for 5 min. The synthesized cDNA was then incubated at 37°C for 20 min with 2 μl RNase H in order to degrade any residual mRNA. The final volume of each synthesis was 43 μl.

Amplicon design for quantitative PCR

Using Primer Express® software (Applied Biosystems), primers P180 (bases 927 to 946; Figure 2.1) and P181 (bases 976 to 995; Figure 2.1) were designed to amplify a 69-bp amplicon corresponding to the sequence between TM regions V and IV of *Aaeg*GPRdih1 (Table 2.1). Optimal primer concentrations (900 nM each) were determined according to ABI directions (SYBR® Green PCR Master Mix protocol) using as template Malpighian tubule cDNA (5 equivalents per well). Analysis indicated that the optimized QPCR amplicon was specific as determined by dissociation curve analysis (not shown). β-Actin gene expression was used to normalize all experimental results, with amplicon primers synthesized as reported (Zhu *et al.*, 2003) and used at 900 nM each.

Quantitative Real-Time PCR (QPCR)

Reactions using SYBR® Green PCR Master Mix (Applied Biosystems) were assembled for cDNAs from every injection treatment as follows: to 60 μl SYBR® Green reagent was added 6 μl (~60 Malpighian tubule equivalents) cDNA and 10.8 μl water.

This volume was equally divided (38.4 µl each) for estimation of the *Aaeg*GPRdih1 and β-actin transcripts, respectively. To each aliquot was added either 10.8 µl each of *Aaeg*GPRdih1 amplicon primers P180 and P181, or 10.8 µl each of β-actin amplicon primers for a final volume of 60 µl. On a 96-well MicroAmp® plate (Applied Biosystems), three wells (20 µl) were loaded for each template-primer combination. QPCR was performed for 45 cycles (95°C 15 s, 60°C 60 s) using the 7300 RT-PCR System (Applied Biosystems). Relative abundance for each transcript was calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method and Sequence Detection Software v1.3.1 (Relative Quantification Study Application; ABI) according to the manufacturer's directions. Statistical analysis used SPSS v12.0.1 for Windows (SPSS Inc, Chicago, IL, USA). The non-injected control data point or the water injected control data point was used as the reference ratio (calibrator) for the analysis. The null-hypothesis that there were no differences among GPRdih1 transcript relative abundances as a result of dsRNA injection was not rejected. Significance was established as $P < 0.05$. Dissociation curve analysis indicated that the QPCR amplicons were specific for their respective cDNAs (data not shown). Graphs were prepared using GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA).

Defecation monitoring

Twenty-five vitellogenic mosquitoes (24 h post-feeding) of each of three injection treatments (dsRNA-EGFP, dsRNA-dih1, non-injected) detailed above were placed into flat-bottomed glass vials (5 females/vial, five vials total per treatment) and

monitored for the appearance and quantity of brown excreta residues after blood-feeding. Access to 10 % sucrose-soaked cotton was provided and each vial was capped with a dry cotton plug. Excreta residues were counted in each vial every two hours beginning 24 h after initial blood feeding and continuing until 54 h after blood feeding. No absolute measures of excreta content were performed. Excreta counts from all vials per treatment were pooled. Graph was prepared using GraphPad Prism v5.0 (GraphPad Software Inc.).

CHAPTER IV

CONCLUSIONS

This dissertation provides significant novel contributions to the understanding of mosquito Malpighian tubule physiology, biology of a diuretic hormone 44 receptor, and the relationship of insect diuretic hormone 44 receptors to the greater corticotropin-releasing factor receptor family.

The description in this dissertation of the *Aedes aegypti* corticotropin releasing factor-like diuretic hormone 44 receptor (GPRdih1) is the first molecular characterization of a diuretic hormone 44 receptor from any mosquito. This characterization is important for the understanding of mosquito renal physiology and overall coordination of Malpighian tubule function with other physiological processes. A cDNA for a paralog receptor cDNA (GPRdih2) was isolated from female head tissues, but the precise role for that receptor is unknown in mosquitoes. Though these two receptors are related and likely share the same ligand, C-terminal motif differences and tissue-specific expression profiles suggest that they interact with different intracellular signaling cascades in their respective tissues.

Identifying these receptor cDNAs in *Aedes aegypti* also allowed the use of several bioinformatics tools and online databases to predict the ortholog GPRdih1 and GPRdih2 open reading frames from another mosquito, *Culex quinquefasciatus*, and to clarify the identity of the receptors in a non-blood feeding dipteran, *Drosophila*

melanogaster. Partial sequences for two CRF-like hormone receptors were predicted from the malaria mosquito, *Anopheles gambiae*, as a result of sequencing the *Anopheles* genome, but those sequences have not yet been confirmed by molecular characterization. Comparing all these sequence data allowed the discovery of several sequence motifs conserved between these dipteran receptors and the CRF receptor family in vertebrates. For example, human CRF receptor can couple to both the cAMP and calcium intracellular signaling cascades (Huang *et al.*, 1996; Couvineau *et al.*, 2003). The amino acid residues responsible for dual signaling potential are conserved in the *Aedes* dih1 and dih2 receptors (residues V^{314}, L^{315}, T^{317}, K^{318} and E^{38}; Figure 2.1). This provides a mechanism to explain the observation by Clark and Bradley (1998) that varying concentrations of mosquito CRF-like peptide activated both the cAMP and calcium cascades in *Aedes aegypti* Malpighian tubules. These data from mosquitoes suggest the intriguing possibility that all insect CRF-like receptors retain the potential for dual signaling and may further explain the observation that a single hormone can have multiple target site effects. I also show for the first time that the *Aedes* dih2 receptor shares conserved C-terminal motifs with other dipteran dih2 receptors which may represent evolutionarily conserved domains for the assembly of multi-protein signaling complexes.

My research is the first to show in any mosquito that the GPRdih1 is the predominant diuretic hormone 44 receptor transcript in the Malpighian tubules; the GPRdih2 cDNA cannot be detected by RT-PCR indicating it is in lower abundance than a serotonin receptor transcript known to be present only in the Malpighian tubule-

associated tracheolar cells (Pietrantonio *et al.*, 2001). Both dih1 and dih2 receptor transcripts are present in head tissues. The differential expression of these receptors established in this dissertation (one in Malpighian tubules, both in heads) most likely indicates the presence of a feedback mechanism regulating hormone release from the nervous system and Malpighian tubule function, and also points to the functional coordination of the Malpighian tubule with other organs in the adult female. Future work should establish the full tissue expression profile of GPRdih1 including potential expression of the receptor transcript in midgut, hindgut, salivary glands and ovaries.

Elevated urination has been observed in female mosquitoes during the post-eclosion and postprandial periods. I provide for the first time evidence that GPRdih1 biology fluctuates in tandem with periods of elevated urination suggesting a common regulatory mechanism between hormone release and receptor function in the target tissue. Though the causal factor for this relationship is currently unknown, searching the promoter regions in the genomic DNA of both the hormone and receptor genes may reveal regulatory elements and transcription factor binding sites common to both genes. GPRdih1 transcript abundance during the first 24 hours after adult emergence (during post-eclosion diuresis) and the first three hours after blood-feeding (after postprandial diuresis) is approximately double the transcript abundance in 2-3 day non-fed females; however it is currently unknown if this increase in transcript abundance translates to an increase in receptor protein abundance during these periods of increased urination. Additionally I provide the first indirect evidence to suggest that GPRdih1 has a role in the excretion of nitrogenous waste which results from blood meal digestion. GPRdih1

transcript peak abundance in Malpighian tubules is observed in females 24 hours after blood feeding when the transcript is increased over 5 times compared to non-fed females. Though diuresis in Malpighian tubules is continuous, there is no observable urination by female mosquitoes 24 hours after consuming a blood meal. Twenty-four hours after blood feeding, a female mosquito begins to excrete waste products from protein metabolism while simultaneously conserving water and ions. Any potential increased rate of diuresis needed to move metabolites into the hindgut via the Malpighian tubules is likely masked by a concomitant recycling of water by the hindgut into the hemolymph. Though observed urination has been considered representative of increased Malpighian tubule activity, my evidence suggests that urination rate alone may not be an accurate proxy for diuresis. Functional assays should be designed with this consideration.

The revolution in medicine and physiology sparked by the discovery of RNA interference is akin to the revolution in molecular biology with the advent of the polymerase chain reaction (PCR) (Dykzhoorn & Lieberman, 2005; Eeles *et al.*, 1992). With a minimum of sequence information and in the absence of mutants, a gene knockdown or knockout phenotype can potentially be generated for any gene within an organism. With the demonstration that exogenous dsRNA is able to decrease endogenous gene function in mosquito Malpighian tubules (Scaraffia *et al.*, 2008), physiologists now have an additional tool with which to investigate tubule function. Unfortunately, I was unable to show an RNAi effect on GPRdih1 transcript abundance in Malpighian tubules from female *Aedes aegypti* when analyzed by QPCR. This was

likely the result of the introduction of insufficient quantities of target dsRNA and insufficient time for RNAi to proceed before data collection. My work did however help to refine the methodology for the introduction of dsRNA into adult females by intrathoracic injection. Injection soon after eclosion resulted in high mortality rates (> 50%) while injection of one day old females resulted in lower rates (10-20%). I also demonstrated that ~400 ng of exogenous dsRNA specific for a GPCR with a post-injection incubation of 4-5 days was insufficient to observe RNAi in Malpighian tubules.

Additional experiments are needed to validate GPRdih1 as a potential target for mosquito control. For example, the circulating levels before and after feeding of diuretic hormone 44 has not been measured for any dipteran (Coast & Garside, 2005). Understanding the precise hormone concentration is hampered by the small size of the mosquito but this is critical to the understanding of the regulation of Malpighian tubule function. It may be possible to develop peptide and small molecule non-peptide antagonists for GPRdih1 as has been accomplished for the corticrotropin releasing factor receptor in humans (Zhang *et al.*, 2003; Hoare *et al.*, 2006; Mesleh *et al.*, 2007). The therapeutic importance of CRF-mimicking drugs has been proven in humans (Chalmers *et al.*, 1996; Grigoriadis *et al.*, 2001; Hoare *et al.*, 2006), suggesting that continued study of the Malpighian tubule CRF-like receptors will not only further our understanding of insect renal physiology, but may result in the development of safe, novel pesticides for those mosquito species that are vectors of several deadly human pathogens.

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APPENDIX

GPRDIH2 CLONED FROM HEAD CDNA FROM FEMALES OF *Aedes aegypti*

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                          TGAATTGCACCGAGTTTGACGAGGGAGCCTGGGAAAGAAAAGAAAAAATACGGTCAGGAGGTCGTCGGTCCAGCAGACATA    -81
GTGGCGGACTTCGAGGAGCTAAGCACCCTATCGCAGGATCTCTTCGCAGAGTACGAGAACAGCACCGAGCTAGCATGTTACCTGCAGGCG     90
 V   A   D   F   E   E   L   S   T   L   S   Q   D   L   F   A   E   Y   E   N   S   T   E   L   A   C   Y   L   Q   A     30
                                                                                        ▲
CACCTGGATGCACAAGCCGAGATTCCATTCGATGGCTGCCCGGTCAACTTTGACAGCATCCTGTGCTGGCCGAAGACGCCAAGCAATACC    180
 H   L   D   A   Q   A   E   I   P   F   D   G   C   P   V   N   F   D   S   I   L   C   W   P   K   T   P   S   N   T     60
                                             ▲                                 ▲
TGGGCGGTGCTTCCATGCTTCAAGGAGTTCAAGGGCGTCGCTTATGATGCACGCCAGAACGCCACCCGCTACTGCCATTCGGACGGTAAG    270
 W   A   V   L   P   C   F   K   E   F   K   G   V   A   Y   D   A   R   Q   N   A   T   R   Y   C   H   S   D   G   K     90
                 ▲                                                                 ▲
TGGGACAACTACAGCCACTATACAGCCTGCCATCACATGACAGAACCACCGCCGGACATAGTCGAGGTCACATCGATCATCTACTACTCC    360
 W   D   N   Y   S   H   Y   T   A   C   H   H   M   T   E   P   P   P   D   I   V   E   V   T   S   I   I   Y   Y   S    120
                         ▲                                                              _____
GGATACATCGTTAGCCTAGTTGCGTTAAGTCTCGCTGTGATAGTTTTTGTCTACTTCAAGGATTTACGTTGTTTGCGTAATACGATCCAC    450
 G   Y   I   V   S   L   V   A   L   S   L   A   V   I   V   F   V   Y   F   K   D   L   R   C   L   R   N   T   I   H    150
_____
GCAAATCTGTTCATCACCTACATTCGTCCGCCCTGATGTGGATTATCATTCTGACGCTGCAGCTCTCCGGCAGTCAGAGTGGCATCGCT    540
 A   N   L   F   I   T   Y   I   L   S   A   L   M   W   I   I   I   L   T   L   Q   L   S   G   S   Q   S   G   I   A    180
_____
AGTTGTGTAATACTCGTTACCTTGCTGCACTACTTCACGCTGACCAACTTCTTCTGGATGCTGGTCGAAGGACTCTATCTGTACATGTTG    630
 S   C   V   I   L   V   T   L   L   H   Y   F   T   L   T   N   F   F   W   M   L   V   E   G   L   Y   L   Y   M   L    210
         _____
GTTGTGGAAACGTTTTCCGGCCGATAACCTACGATTCAATATGTATGCGGCCATCGGGTGGGGAGGACCCGGAGTATTTGTGATTATGTGG    720
 V   V   E   T   F   S   G   D   N   L   R   F   N   M   Y   A   A   I   G   W   G   G   P   G   V   F   V   I   M   W    240
                                 _____
GTCATTGCCAAGGGCATCACCATTTCCGGTCTGGATCAGACGACCACGCTGGAAATCGACTGTTCCTGGATGCGAGAATCGACCGTGGAT    810
 V   I   A   K   G   I   T   I   S   G   L   D   Q   T   T   T   L   E   I   D   C   S   W   M   R   E   S   T   V   D    270
_____
CGGATCTTCCAGGGGCCCGTGTGTGCGGTGCTCATCATCAATCTAGTGTTTCTCATACGAATCATGTGGGTATTAATAACCAAACTCCGT    900
 R   I   F   Q   G   P   V   C   A   V   L   I   I   N   L   V   F   L   I   R   I   M   W   V   L   I   T   K   L   R    300
         _____
TCGGCCAACACCGTCGAAACGCGTCAGTACCGGAAAGCATCGAAAGCCCTGTTGGTCCTGATCCCGCTGCTTGGCATCACCTACCTGGTG    990
 S   A   N   T   V   E   T   R   Q   Y   R   K   A   S   K   A   L   L   V   L   I   P   L   L   G   I   T   Y   L   V    330
                                             _____
GTCCTGGCCGCACCGTCCGAGGGTGTCGTGAGTGACATTTTCGCCGTCGCTCGGGCACTGCTACTCAGCACGCAGGGCTTTTCGGTGTCG   1080
 V   L   A   A   P   S   E   G   V   V   S   D   I   F   A   V   A   R   A   L   L   L   S   T   Q   G   F   S   V   S    360
_____                         _____
CTGTTCTACTGCTTCCTCAACTCGGAAGTCCGCCTGGCCCTGCGGCACCGGCTGGAACGCTGGCGAGACGAGCGCAATATCCGGATAGGT   1170
 L   F   Y   C   F   L   N   S   E   V   R   L   A   L   R   H   R   L   E   R   W   R   D   E   R   N   I   R   I   G    390
_____
CAAGTCCGACAACGAAGTCGCCGATTCACCAACTCTGGCCTTTCGAAGGAATGCTCGCCTCGCTCTAGAACTGAAAGTTTCCGGCCACTA   1260
 Q   V   R   Q   R   S   R   R   F   T   N   S   G   L   S   K   E   C   S   P   R   S   R   T   E   S   F   R   P   L    420
ACCTACAGCAAGCGCGAAAGCTGTGCATCTTCAGCAACTACGACCACCCTGCTGGGACCTCCGGTGGGGGCGCATGGTGCCTATCCGAGT   1350
 T   Y   S   K   R   E   S   C   A   S   S   A   T   T   T   T   L   L   G   P   P   V   G   A   H   G   A   Y   P   S    450
ACGATGCGTGGCTCCAATGGTGCTCTCCATCTGCATGCAATGGCACCGAGAGCCATCAGTCCGCTGATGCAGGGTCTGGACGAGAACTCG   1440
 T   M   R   G   S   N   G   A   L   H   L   H   A   M   A   P   R   A   I   S   P   L   M   Q   G   L   D   E   N   S    480
GTCTAAGTGCAAATTCATCATCGTCACCACCATTCGGGAACTTCTCTTTCTGTTTGACTTCAGCTAGCCTAATCTAACGAATCTCTTCAC   1530
 V   -                                                                                                                481
AGGTTTTTTTCATGGTCAAATCAATGTCTTGTACGCTTCATGCTGTAAGTTAACTTTGCCTATAGGATATAACTCAAAAAGGGAAACGAA   1620
ATGAGATTTGTATATGAAAATATCTGATAGTGTATGGAATTTCTTGTTGGATCATCGTACCGAGCTGTGTGCATAAATTAGGACAATTAT   1710
ATTTTCGAGAAGAAATATTTAAGATTGTTACCATTCTGTTGTACAGCCTGATCGGAGCCTAACTAGCCTAAAGGAAAAACGTACCGATTT   1800
GCATACTTTTAACTAAATTCATCGGTATATGTACAAAGAAAGACTTTTTAATTGGGGAAATGTTTTTGCTAACGAATATTTATGAAGCTC   1890
GAAAAGTCTTGTGTCAGTTTAGTAATGAATAGATTTACAGAACATGGAACGCGATGAAGCTCTTGCAAGGTACATCAATAGCTGAAATGC   1980
AACAGCAAAAAAACGAAAGCACAAAACTTTCCTCTATCTCTATAAATGTTGATGTCAGGTAAAATTGTACACTATCTGTAACAAATACA   2070
CACACAATTTCTTTACAAGTTATTTTTTCGATGATATAAATATAATGTCAACTAATATGGAAAAAAAAAAAAAAA   2146
```

Figure A-1 GPRdih2 clone identified from head cDNA from females of *Aedes aegypti*.
(GenBank accession number EU273352) This sequence should be used in connection with Table 2.1 to determine specific primer locations as designed for PCR and QPCR analysis. Predicted seven transmembrane regions are underlined (◆━━━◆). Conserved N-terminal cysteine residues are indicated (▲: C^{26}, C^{43}, C^{52}, C^{66}, C^{85}, C^{100}). The initiator codon in this image is "GTG" but this codon has not been verified as the true start codon. Several 5' UTR transcript variants, all with the same predicted open reading frame, were identified but are not reported in this dissertation.

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Winner, President's Prize Runner-Up. 2007 Entomological Society of America Annual Meeting. San Diego, CA. Diuretic hormone receptor transcripts in Malpighian tubules from females of the mosquito *Aedes aegypti* (Diptera: Culicidae).

Recipient, J. "Gus" Foyle Memorial Scholarship. Oct 2007. TX Mosquito Control Assn.

Winner, First Place. 2007 Dept. of Entomology Graduate Student Forum, GPRdih1 transcript regulation in female Malpighian tubules of the mosquito *Aedes aegypti* (Diptera: Culicidae).

Winner, Second Place. 2006 Dept. of Entomology Graduate Student Forum. Interfering with Diuretic Hormone (DH_{44}) receptor expression in the mosquito *Aedes aegypti*.

Competitive Research Assistantship, 2003-2004. Dept. of Entomology, Texas A&M University.