FUNCTIONAL ELUCIDATION AND CHARACTERIZATION OF GPA2/GPB5 AND ITS RECEPTOR LGR1 IN THE MOSQUITO, *AEDES AEGYPTI*

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ABSTRACT

Glycoprotein hormones govern key aspects of physiology like reproduction, growth and development as well as metabolism in vertebrates. In 2002, a novel glycoprotein hormone formed by the heterodimeric interactions of GPA2 and GPB5 (GPA2/GPB5) subunits was discovered. Unlike other glycoprotein hormones, GPA2/GPB5 homologs are present in most vertebrate and invertebrate genomes, however its exact function remains elusive. The present set of studies aimed to elucidate the function of GPA2/GPB5 and its receptor LGR1 in the disease vector mosquito, *Aedes aegypti*. First, the tissue-specific expression and cellular localization of LGR1 was characterized to identify potential targets for GPA2/GPB5 in adult mosquitoes. LGR1 transcript and immunoreactivity was detected in gut epithelia, which supported a pre-established ionoregulatory / osmoregulatory role in the alimentary canal of adult mosquitoes. Interestingly, strong and regionalized immunoreactivity was detected in the female ovaries and male testes suggesting GPA2/GPB5 signalling may function to regulate reproduction in *A. aegypti*. In the male testes, the subcellular expression of LGR1 during spermatogenesis was characterized further, and results found LGR1 immunoreactivity specifically localized to the plasma membrane of the centriole adjunct, which contains proteins important for flagellar formation in developing spermatids. Using RNA interference, downregulation of LGR1 led to sperm defects, such as shortened flagella. Moreover, newly emerged males were characterized with significantly less spermatozoa and rendered less fertile. The GPA2 and GPB5 subunits were found to be co-expressed in the abdominal ganglia of the central nervous system in both male and female adult mosquitoes. Mammalian cell lines were then employed to produce recombinant *A. aegypti* GPA2 and GPB5 proteins and study subunit interactions, ligand-receptor and interactions and determine downstream signalling events upon LGR1 activation. Results demonstrated *A. aegypti*

GPA2/GPB5 are capable of subunit homodimerization *in vitro*. However, only tethered *A. aegypti* GPA2/GPB5, rather than individual subunits alone, were required to activate LGR1, which interestingly, appears to involve coupling to an inhibitory G protein. Together, these studies have demonstrated that signalling involving the glycoprotein hormone system is imperative for male reproductive-related processes in adult mosquitoes, a largely understudied research area, and presents novel insight to the function of its homologs in other animals.

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Supplementary:

LIST OF ABBREVIATIONS

AMP – adenosine monophosphate

AMPs – antimicrobial peptides

ADB – antibody dilution buffer

BSA – bovine serum albumin

cDNA – complimentary DNA

CG – chorionic gonadotropin

CHO – chinese hamster ovary

CKGF – cystine knot growth factor

CNS – central nervous system

CTSH – chloride transport stimulating hormone

CTP – carboxyl-terminal peptide

DAPI – 4',6-diamidino-2-phenylindole

dH_20 – de-ionized water

DIG – digoxigenin

DNA – deoxyribonucleic acid

DPBS – Dulbecco's PBS

dsRNA – double-stranded RNA

DSS – disuccinimidyl suberate

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

EGFP – enhanced GFP

F actin – filamentous actin

FISH – fluorescence *in situ* hybridization

FSH – follicle-stimulating hormone or follitropin

GFP – green fluorescent protein

Gi/o – inhibitory G protein

GPA2 – glycoprotein alpha 2

GPA2-FLAG – Flag-tagged GPA2

GPA2-His – hexa-histidine tagged GPA2

GPB5 – glycoprotein beta 5

GPB5-FLAG – Flag-tagged GPB5

GPB5-His – hexa-histidine tagged GPB5

GPCR – G protein-coupled receptor

Gs – stimulatory G protein

HEK – human embryonic kidney

hGPA2 – human GPA2

hGPB5 – human GPB5

hLHR – human LH receptor

hTSHR – human TSHR

HRP – horseradish peroxidase

IBMX – 3-isobutyl-1-methylxanthin

IPTG – ispropyl β-D-1-thiogalactopyranoside

ITP – ion transport peptide

LB – lysogeny broth

LB-amp-tet - LB media supplemented with ampicillin (100 µg ml^{-1}) and tetracycline (10 µg ml^{-1})

LDLa – lipoprotein receptor domain class A

LGR – leucine-rich repeat-containing G protein-coupled receptor

LH – luteinizing hormone or lutropin

mPRα – membrane progestin receptor-alpha

mRNA – messenger RNA

NKA - Na^+/K^+ ATPase

NSS – normal sheep serum

ORF – open reading frame

PBS – phosphate-buffered saline

PBST - PBS containing 0.1% Tween-20

PBSTB – PBST containing skim milk powder

PCR – polymerase chain reaction

PDGF – platelet-derived growth factor

PNGase F - N-glycosidase F

PVDF - polyvinylidene difluoride

qPCR – quantitative PCR

RNA – ribonucleic acid

RNAi – RNA interference

Rp49 – ribosomal protein 49

RT – room temperature

RT-qPCR – reverse transcription qPCR

S2 – schneider 2

SDS – sodium dodecyl sulfate

SEM – standard error of the mean

SIET – scanning ion-selective electrode technique

SIT – sterile insect technique

TAE – Tris base, acetic acid and EDTA

TGFβ – transforming growth factor β

TSH – thyroid stimulating hormone or thyrotropin

TSHR – TSH receptor

STATEMENT OF CONTRIBUTIONS

Chapter One

This chapter was written by D. Rocco with guidance and editorial support from Dr. J.P. Paluzzi. All experiments were performed by D. Rocco.

Chapter Two

This chapter was written by D. Rocco with guidance and editorial support from Dr. J.P. Paluzzi. Immunocytochemical experiments concerning LGR1 expression in transiently transfected CHO-K1 cells were performed by D.H. Kim. All other experiments were performed by D. Rocco.

Chapter Three

This chapter was written by D. Rocco with guidance and editorial support from Dr. J.P. Paluzzi, Dr. R.H. Nóbrega and Dr. D.C. dos Santos. Electron microscopy was performed by A.S.G. Garcia, E.L. Scudeler and Dr. D.C. dos Santos. All other experiments were performed by D. Rocco.

Chapter Four

This chapter was written by D. Rocco with guidance and editorial support from Dr. J.P. Paluzzi. All experiments were performed by D. Rocco.

Chapter Five

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CHAPTER 1:

GENERAL INTRODUCTION –

THE MOSQUITO AEDES AEGYPTI, INSECT SPERMATOGENESIS AND

GLYCOPROTEIN HORMONES IN BILATERAL METAZOANS

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1.1 *Aedes aegypti*

In common with many other blood feeding arthropods, the mosquito *Aedes aegypti* is a well known vector for a range of human diseases including dengue, yellow fever, Zika and chikungunya viruses (Vanlandingham et al. 2007; Bhatt et al. 2013; Murray et al. 2013; Sessions et al. 2013). Of the three viral diseases, dengue is the most prominent human arboviral (arthropod-borne) infection (Brady et al. 2012; Bhatt et al. 2013) with well over half a million cases of dengue reported in the Americas in 2018 alone (W.H.O. 2018). Moreover, incidence rates of dengue transmission are rapidly intensifying likely due to increased anthropogenic changes such as increased urbanization and globalization, as well as virus resistance and unresolved vector control (Brady et al. 2012; Bhatt et al. 2013; Murray et al. 2013). Mosquitoes are efficient vectors for disease because most species, like *A. aegypti*, are anautogenic, whereby the adult female feeds on the nutrient-rich blood of vertebrates for the purpose of egg development. Thus, arboviruses like dengue are easily acquired from infected blood during feeding and transmitted from host to host with each successive blood meal. Mosquito reproductive biology is tightly linked to the transmission of disease. Currently, one of the tools used to effectively suppress these disease vectors includes the sterile insect technique (SIT). SIT involves a mass production and release of sterile male mosquitoes into the wild, whereby sterilization is usually induced through chemosterilization techniques or ionizing radiation to target and elicit abnormalities in germ and somatic cell development during spermatogenesis (Helinski et al. 2009; Hassan et al. 2017). As a result, a detailed understanding of critical reproductive processes in *A. aegypti*, such as oogenesis and spermatogenesis, along with their hormonal regulation, may reveal novel strategies for decreasing vector fitness, and thus may provide a better means for mosquito vector control.

1.2 Spermatogenesis in mosquitoes

Depending on the species of mosquito, spermatogenesis predominantly occurs in late larval through to pupal developmental life stages so that adults are sexually mature and equipped to inseminate females just after adult emergence (Clements 2000). In *Anopheles culicifacies*, males were observed to copulate and inseminate females within 0-2 days of adult eclosion, however the maximum number of females inseminated occurred with males aged 3-7 days old (Mahmood and Reisien 1994). Unlike female mosquitoes that will only accept one mate at a time, male mosquitoes are considered polygynous, mating with more than one female in a given day (Clements 2000; Hellinski and Harrington 2011). To ensure a constant supply of sperm is made available, spermatogenesis continues throughout the adult stage in the male mosquito, though at a reduced rate compared to larval and pupal life stages (Hausermann and Nijhout 1975).

1.2.1 Morphology of the mosquito testis

In male mosquitoes, spermatogenesis occurs in two 'pear-shaped' testes that are each coated with a sheath of fat body. The paired testes are not always the same size nor at the same physiological state of development (Hutcheson 1972). A given testis resembles that of a follicle organized into many different compartments called cysts; each cyst is composed of an external wall that packages numerous sperm which develop in synchrony (Fig. 1-1). As a result, a single cyst will contain many sperm at a specific developmental stage (Wandall 1986; Clements 2000). From the hooked apex to the base of the testis, cysts increase in size and maturity so that smaller compartments with immature spermatogonia are found on distal ends, whereas large cysts containing mature spermatozoa are located at the base (Wandall 1986; Clements 2000) (Fig. 1-1). As maturation proceeds in the testis, spermatogonia proliferate and mature into

spermatocytes, spermatids and finally spermatozoa. Mature spermatozoa from each testis are then transported via conduits known as the vas deferens, to a common storage unit called the seminal vesicle. Upon a successful copulation event with a female, the seminal vesicle is emptied, and a new wave of spermatogenesis occurs to ensure the availability of novel sperm for a future copulation (Clements 2000; Oliva et al. 2014).

1.2.2 From spermatogonia to spermatozoa

At the very apex of the mosquito testis exists a niche of primordial germ cells that give rise to primary and secondary spermatogonia that undergo several mitotic divisions (Ndiaye et al. 1996). During these developmental stages, nuclei are round in shape, characterized with diffuse chromatin and have poorly developed organelles like the Golgi apparatus (Ndiaye et al. 1996). In a given spermatogonial cyst, cells will undergo several mitotic divisions in syncytium due to incomplete cytokinesis, a process which allows for the interconnection and sharing of cytoplasmic materials through structures known as cytoplasmic bridges (Wandall 1986). After mitosis, spermatogonia enter two meiotic divisions to form primary and secondary spermatocytes. During this stage in development, nuclear chromatin becomes more condensed, organelles are observed to be very developed and mitochondria multiply rapidly (Ndiaye et al. 1996). Spermatids are the result of the second meiotic division and, hereon, undergo drastic morphogenic changes to yield mature spermatozoa, a process known as spermiogenesis. In spermatids, five key morphological changes begin to occur; (i) the nucleus condenses and elongates from a round to elongated shape, (ii) the centriole begins to stretch into a short flagellum, (iii) a cylindrical sheath called the centriolar adjunct is formed around the centriole, at the base of the flagellum, (iv) a structure known as the nebenkern is formed by the clustering and

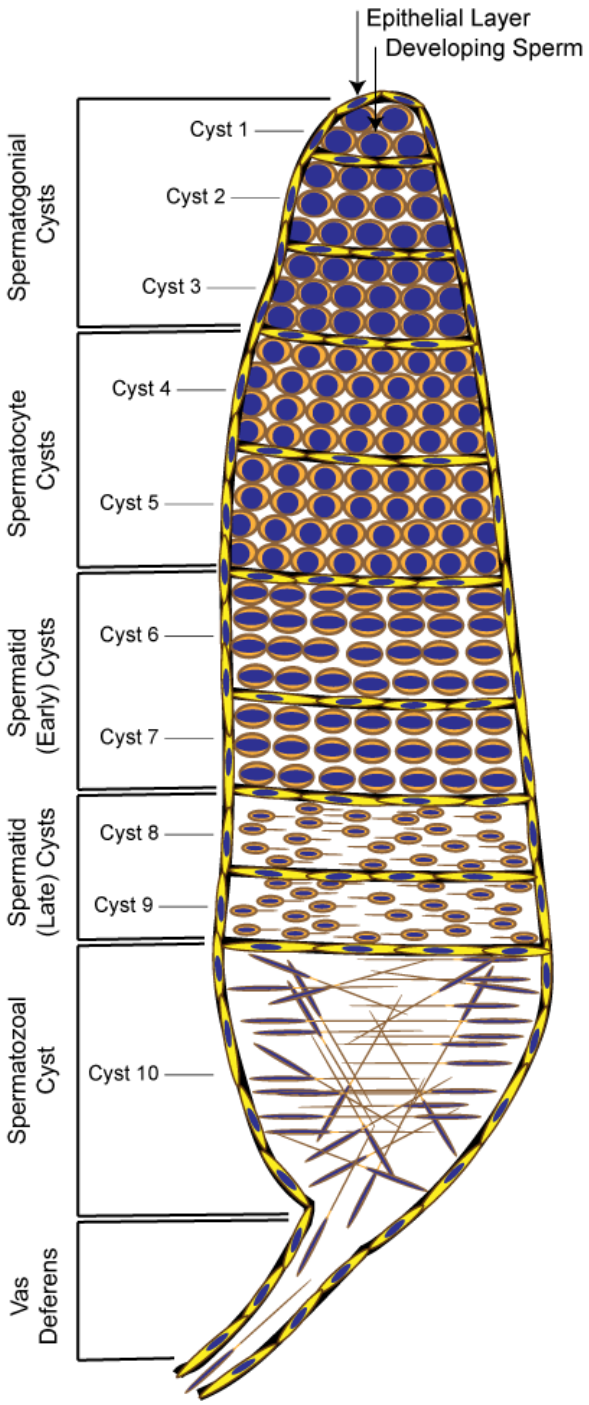

Figure 1-1: Schematic diagram demonstrating testis organization in adult *A. aegypti*. Cysts containing spermatogonia, located at the apex of the testis, grow and mature into spermatocytes, spermatids (early stages characterized with oval-shaped nuclei, late stages with more elongate shaped nuclei and the presence of short flagella), and spermatozoa at the base of the testis.

fusion of many mitochondria. The nebenkern, which stays closely associated with the nucleus and the developing flagellum, then splits into two separate entities that forms the two mitochondrial derivatives that will power flagellar movement. (v) Golgi apparatus begins to cluster on the opposite end of the nucleus to form the acrosome (Hutcheson 1972; Ndiaye et al. 1996). From late spermatids to mature spermatozoa, the nucleus becomes compact and 'needle-shaped', mitochondrial derivatives become crystallized and the acrosome, which is pyramidal-shaped in *Aedes*, becomes fully developed (Ndiaye et al. 1996).

1.2.3 Endocrine regulation of spermatogenesis in insects

In insects, most research until now has been geared towards understanding the hormonal regulation of female reproductive processes rather than male reproductive biology. As a result, how spermatogenesis is regulated in insects by endocrine/ neuroendocrine factors remains largely undescribed. There is evidence to support a role for ecdysteroids, a group of steroid hormones, and juvenile hormone, a sesquiterpenoid, in the regulation of insect spermatogenesis. In immature developmental life stages, ecdysteroids are produced by a pair of endocrine glands, the prothoracic glands, and govern insect development (Vafopoulou et al. 1996). In adult stages however, the predominant source of ecdysteroid production is in the gonads to regulate reproductive processes (Raikhel et al. 2005). For several male insect species, the testes serve as the site of ecdysteroid production (Loeb et al. 1982; Shimizu et al. 1985; Gelman et al. 1989; Loeb and Woods 1989; Jarvis et al. 1994; Fugo et al. 1996; Wagner et al. 1997), and function to induce the mitotic (Dumser 1980a, b) and meiotic (Takeda 1972; Yagi and Fukushima 1975; Shimizu et al. 1985; Friedlander and Reynolds 1992; Jacob 1992) divisions of spermatogonia and spermatocytes respectively. Like the ecdysteroids, juvenile hormone helps regulate

development in immature life stages, by maintaining juvenile characteristics and preventing metamorphosis, and reproduction in adults. The effects of juvenile hormone on spermatogenesis may be species-specific given that juvenile hormone accelerates spermatogenesis in the leafhopper (Reissig and Kamm 1975) and red cotton stainer (Ambika and Prabhu 1978), but inhibit spermiogenesis in the silkworm (Yagi and Fukushima 1975).

1.3 The glycoprotein hormone GPA2/GPB5 and its receptor LGR1

1.3.1 Introduction to vertebrate and invertebrate glycoprotein hormones

Glycoprotein hormones are a family of cystine-knot proteins that encompass a wide range of physiological functions in both vertebrates and invertebrates (Pierce and Parsons 1981). In mammals, the more commonly known glycoprotein hormones include follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG), which play important roles in growth and development, reproduction and metabolism. In females, FSH stimulates egg production in the ovaries (Dierich et al. 1998) and in males, stimulates inhibin production from Sertoli cells and is involved in sperm production through induction of Leydig cell maturation in the testes (Kerr and Sharpe 1985). Later stages of mammalian reproductive cycles are governed by LH and CG, which act as the triggers for follicular ovulation, aid in progesterone production and stimulate androgen production in males (Stockell Hartree and Renwick 1992). TSH is involved in the growth and differentiation of thyroid follicular cells and thyroid hormone production, that is ultimately important in regulating energy balance and metabolism (Stockell Hartree and Renwick 1992). In their biologically active states, these vertebrate glycoprotein hormones are formed by the heterodimerization of two polypeptide chains; a glycoprotein alpha subunit (GPA1) that is common to all four glycoprotein hormones, and a unique glycoprotein beta subunit (GPB1–4), which confers hormone specificity (Pierce and Parsons 1981). In addition to its heterodimeric association, the glycoprotein beta subunit of the human CG is also believed to be functionally active as a monomer or homodimer in the apoptotic actions of certain cancer cell lines (Iles et al. 2010). Two novel glycoprotein hormone subunit genes were identified following completion of the human genome and, based on the order of their discovery, these glycoprotein subunits were named GPA2 and GPB5 (Hsu et

al. 2002). In mammals, GPA2/GPB5 was named thyrostimulin since the alpha and beta subunits were found to heterodimerize and activate the TSH receptor, without stimulating the FSH and LH/CG receptors (Nakabayashi et al. 2002). To date, the physiological role of thyrostimulin has not been fully elucidated in vertebrates; however, it has been suggested that this hormone is pleiotropic as it stimulates thyroxine production (Nakabayashi et al. 2002; Okada et al. 2006), is involved in mammalian skeletal development (Duncan Bassett et al. 2015) and acts as a paracrine regulator in the mammalian ovary (Sun et al. 2010). Overall, a greater amount of research has focused on the vertebrate classic heterodimeric glycoprotein hormones and thyrostimulin (Roch and Sherwood 2014; Cahoreau et al. 2015), compared to structural homologs in the invertebrates.

Glycoprotein hormones are structurally and functionally well conserved in vertebrates spanning from the primitive hagfish to humans (Quérat et al. 2000; Park et al. 2005; So et al. 2005; Uchida et al. 2010). Moreover, homologs of the glycoprotein hormone subunit genes have also been identified in a number of invertebrates. Specifically, two types of heterodimeric glycoprotein hormones are known to exist in invertebrates, bursicon and GPA2/GPB5. In insects, the exoskeleton is critical for survival as it acts as a barrier to desiccation and infection and serves as a means of protection and structural support. During development however, the exoskeleton limits growth and so insects are required to periodically shed their exoskeleton and replace it with a new cuticle (Luo et al. 2005; Mendive et al. 2005). These complex processes during insect development are tightly regulated by at least six different hormones including bursicon (Luo et al. 2005). Bursicon is a ~30kDa neuropeptide formed by the heterodimerization of two glycoprotein subunits (burs alpha and burs beta), which are produced in the central nervous system (Dewey et al. 2004; Dong et al. 2015). The hormone is synthesized and released

into the haemolymph with the completion of ecdysis, or the shedding of the old cuticle, and aids in new cuticle formation as well as wing expansion (Honegger et al. 2008). Since the discovery of bursicon over half a century ago (Fraenkel and Hsiao 1962, 1963, 1965), much progress has been made in characterizing its structure and role in a variety of insect species (Honegger et al. 2008). In addition to its well established function in wing expansion and cuticle sclerotization and melanization (i.e. tanning), recent investigations have shown that bursicon heterodimers as well as subunit homodimers are capable of influencing antimicrobial peptide gene expression, as well as cuticular protein and chitin metabolizing genes in association with cuticular synthesis and degradation (An et al. 2008, 2012; Dong et al. 2015). As bursicon has received significant attention over the last few decades and is well characterized both structurally and functionally, the main focus herein is to summarize the relatively limited knowledge on the more novel discovered GPA2/GPB5 along with its associated receptor, LGR1, in the invertebrates.

With the ever increasing genome sequencing datasets, several invertebrate model organisms have been found to possess GPA2-like and GPB5-like genes including insects (Hsu et al. 2002; Paluzzi et al. 2014), nematodes (Oishi et al. 2009), lancelets (Dos Santos et al. 2009; Tando and Kubokawa 2009a), tunicates (Tando and Kubokawa 2009b), sea hares (Heyland et al. 2012) and bivalves (Veenstra 2010) (Fig. 1-2, 1-3). Indeed, sequence analyses of these GPA2-like and GPB5-like genes in the invertebrates, along with their deduced protein products, has confirmed their close similarity to the vertebrate glycoprotein hormone subunits of thyrostimulin, GPA2 and GPB5, rather than the subunits comprising the classic gonadotropins and TSH, which are only found in vertebrates (Hsu et al. 2002). The evolution of GPA2/GPB5 signalling systems predates the emergence of bilateral metazoans (Hauser et al. 1998; Vibede et al. 1998) indicating

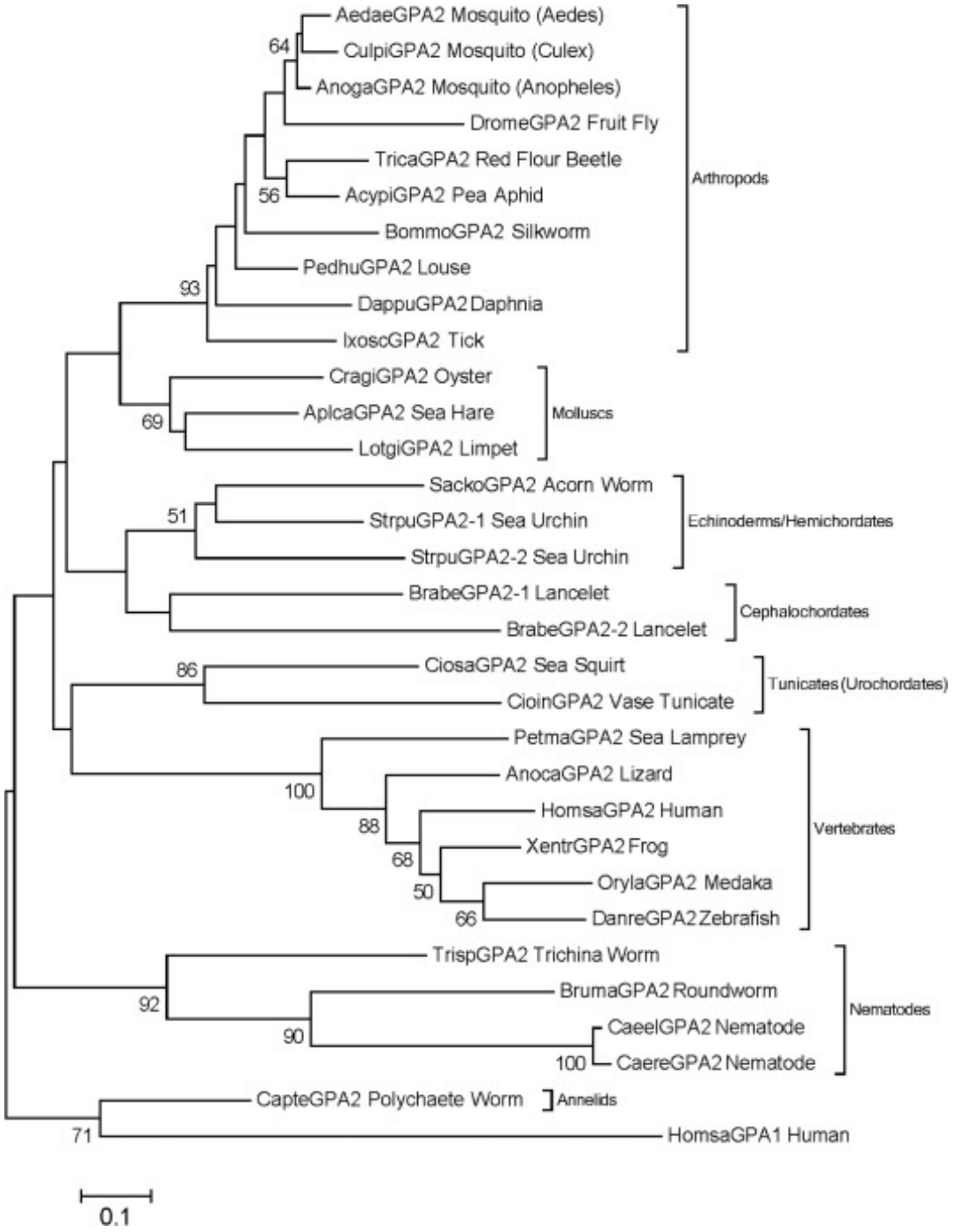

Fig. 1-2: Neighbour joining tree of GPA2-like protein homologs identified in various invertebrate and vertebrate phyla. Numbers at nodes represent bootstrap statistical significance with values less than 50% not shown. Phylogenetic analysis was carried out as previously described (Paluzzi et al. 2014). Interestingly, the predicted GPA2 amino acid sequence for the polychaete worm, *Capitella teleta*, was found to be more closely related to the human GPA1 protein sequence rather than other GPA2 amino acid sequences.

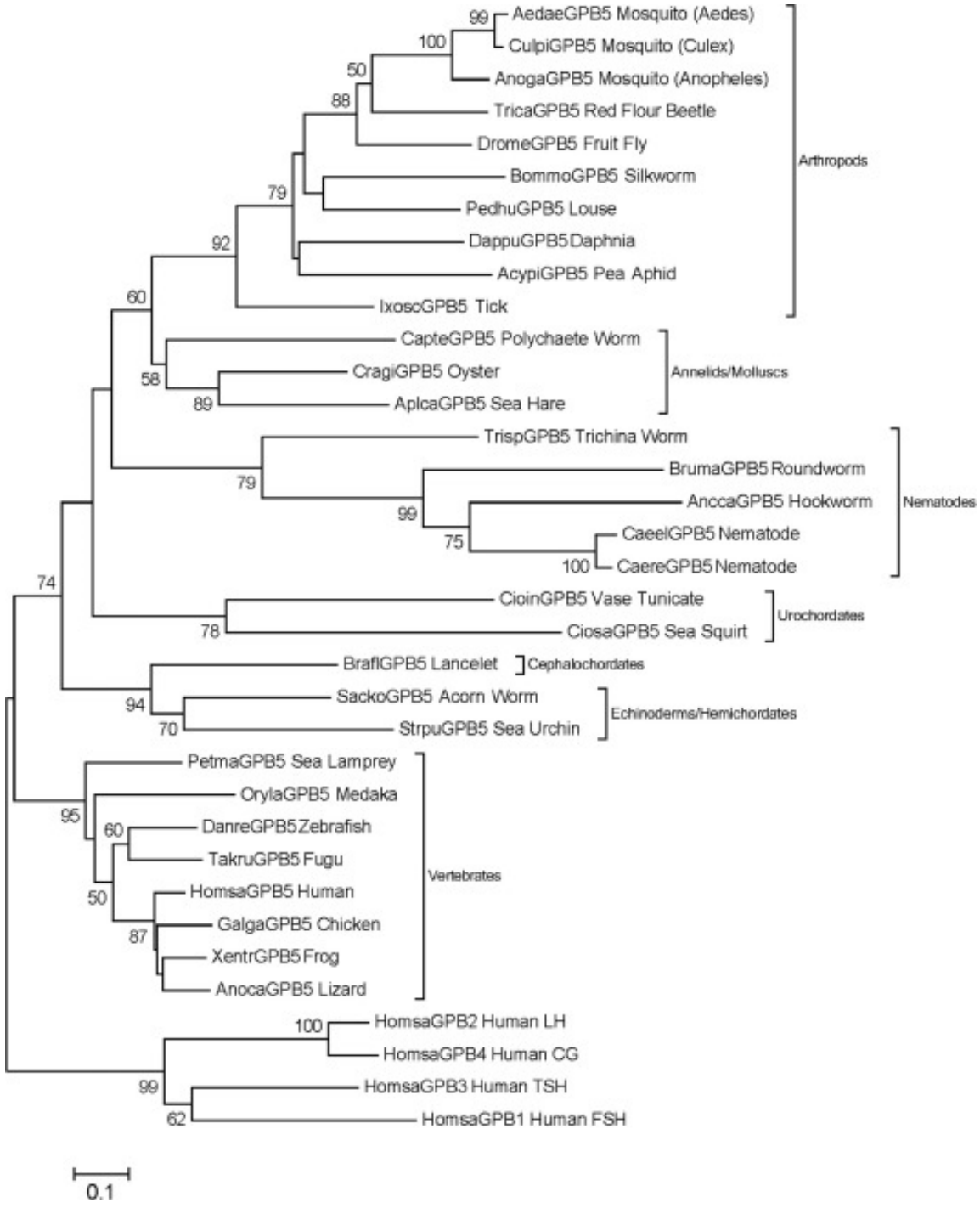

Fig. 1-3: Neighbour joining tree of various GPB5-like protein homologs identified in vertebrate and invertebrate phyla. Numbers at nodes represent bootstrap statistical significance with values less than 50% not shown. Phylogenetic analysis was carried out as previously described (Paluzzi et al. 2014).

Table 1-1: Overview of inferred physiological roles for GPA2/GPB5 in bilaterians.

Organism of study	Physiological role of GPA2/GPB5	Possible target tissues for GPA2/GPB5	References
Human	Reproduction	Ovary	Sun et al. (2010)
Mouse	i. Skeletal development	i. Bone	i. Duncan Bassett et al. (2015)
	ii. Thyroxine secretion	ii. Thyroid gland	ii. Okada et al. (2006)
Rat	i. Thyroxine secretion	i. Thyroid gland	i. Nakabayashi et al. (2002)
	ii. Reproduction	ii. Ovary	ii. Sun et al. (2010)
Nematode (*Caenorhabditis elegans*)	Neural regulation of intestinal function	Alimentary canal	Oishi et al. (2009)
Sea hare (*Aplysia californica*)	Suggested involvement in development and neural modulation	Apical sensory organ and pleural/buccal mechanosensory neurons	Heyland et al. (2012)
Fruit fly (*Drosophila melanogaster*)	i. Development	i. Prothoracic gland and salivary glands	i. Vandersmissen et al. (2014)
	ii. Hydromineral balance	ii. epithelium of alimentary canal	ii. Sellami et al. (2011)
Mosquito (*Aedes aegypti*)	Hydromineral balance	Epithelium of alimentary canal	Paluzzi et al. (2014)
Amphioxus (*Branchiostoma belcheri*)	i. Reproduction and control of thyroid hormone secretion	i. Nerve cord, gonads and endostyle	i. Tando and Kubokawa (2009b)
Amphioxus (*Branchiostoma lanceolatum*)	ii. Development	ii. Cerebral vesicle, club-shaped gland and Hatschek's pit	ii. Dos Santos et al. (2009)

that this well conserved glycoprotein hormone signalling system is evolutionarily ancient and holds critical physiological roles in the invertebrates. However, since knowledge on the function of GPA2/GPB5 in any individual organism is incomplete, this section of my thesis summarizes recent progress surrounding the GPA2/GPB5 system in bilaterians with an emphasis on terrestrial arthropods (Table 1-1 for an overview of functions of GPA2/GPB5).

1.3.2 Glycoprotein hormone receptors

Glycoprotein hormones mediate their physiological functions by binding to a subset of G protein-coupled receptors (GPCRs) called the leucine-rich repeat-containing GPCRs (LGRs). Similar to other GPCRs, LGRs are characterized with an extracellular N-terminal domain, seven transmembrane alpha helices and an intracellular carboxyl tail (Krause et al. 2012). The hallmark feature of this particular subgroup of GPCRs is their relatively large ectodomain, which is comprised of more than 320 amino acids (Vassart et al. 2004) and in the mosquito *Aedes aegypti*, for example, comprises nearly 60% of the entire protein (Paluzzi et al. 2014). The ectodomain contains several leucine-rich repeats and is responsible for the selective binding of glycoprotein hormones. The hinge region of the ectodomain functions as a structural connection between the extracellular and transmembrane domain regions and is important for basal receptor conformation and receptor activity. Although most differences among the vertebrate glycoprotein hormone receptors occur in the hinge region, LGRs are also classified according to the number of leucine-rich repeats and the occurrence or lack of a low-density lipoprotein receptor domain class A (LDLa) motif (Van Hiel et al. 2012). The invertebrate GPA2/GPB5 receptor, called LGR1, along with the mammalian glycoprotein hormone receptors belong to the Type A LGRs (Rhodopsin-type), characterized with 7–9 leucine-rich repeats and a long hinge

region (Van Hiel et al. 2012). In contrast, the Type B LGRs, which includes the bursicon receptor LGR2, features between 16 and 18 leucine-rich repeats and a short hinge region (Honegger et al. 2008; Bai and Palli 2010). The last group, Type C LGRs, are the only subclass known to contain one or more LDLa motifs (Van Hiel et al. 2012).

One strategy to delineate the unknown function of the glycoprotein hormone GPA2/GPB5 is to examine its receptor's expression profile, which could reveal target cells, tissues and/or organs. In mammals, the TSH receptor is activated by two glycoprotein hormones; TSH, whose function is well characterized in vertebrates, and thyrostimulin (the mammalian homolog of GPA2/GPB5) (Nakabayashi et al. 2002). Research has shown that expression of the TSH receptor is not exclusive to the thyroid, but instead is also found in a variety of other tissues including white and brown adipose tissue, skin, erythrocytes and lymphocytes (Williams 2011). This suggests that the TSH receptor may contribute to further physiological processes other than metabolism (Sun et al. 2010). In fact, protein expression analyses of the TSH receptor in the mammalian ovary revealed that the TSH receptor may encompass a reproductive function (Aghajanova et al. 2009). Interestingly, thyrostimulin, rather than TSH, is expressed in oocytes where it acts as a paracrine regulator on follicular cells expressing the TSH receptor (Sun et al. 2010). Indeed a recent comprehensive review highlights that the TSH receptor, along with at least two distinct heterodimeric glycoprotein hormones and possibly their individual subunits, control an array of extrathyroidal functions requiring further clarification (Kleinau et al. 2013).

Studies on the invertebrate glycoprotein hormone GPA2/GPB5 and its receptor LGR1 are even more limited than its vertebrate counterpart. Until now, only transcriptional studies on LGR1 have been performed with the majority of research being performed on the fruit fly, *Drosophila melanogaster*. Investigations centered on LGR1 transcript profiling will be discussed

herein; however, future studies should analyze LGR1 at the protein level and examine its functional characteristics as it may significantly contribute to revealing the role of GPA2/GPB5 in the invertebrates.

1.3.3 Transcriptional studies of GPA2/GPB5 and its receptor LGR1 in the invertebrates

Until the late 20th century, glycoprotein hormone receptors were believed to exist only in mammals rather than other vertebrates or invertebrates. This idea that glycoprotein hormone receptors were restricted to mammalian species alone was refuted with the identification of a novel GPCR in sea anemones that was structurally related to the mammalian glycoprotein hormone receptors LH/CG, FSH and TSH (Nothacker and Grimmelikhuijzen 1993). Soon after, the identity of yet another related receptor in molluscs (Tensen et al. 1994) confirmed the possibility that glycoprotein hormone receptors may indeed exist in invertebrates which initiated a new direction for research in comparative endocrinology. The first insect LGR to be cloned, and its expression studied, was the glycoprotein hormone receptor LGR1 in the fruit fly *D. melanogaster* (Hauser et al. 1998). However, it was not for several more years that the LGR1 receptor was deorphanized identifying its native ligand, *D. melanogaster* GPA2/GPB5, which upon receptor activation elicits a cyclic AMP signalling cascade (Sudo et al. 2005).

In an elegant study, the transcript expression patterns of the genes encoding LGR1, GPA2 and GPB5 were examined across various tissues and throughout fruit fly development (Vandersmissen et al. 2014). LGR1 transcript expression begins within 8–16h post-oviposition (Hauser et al. 1998) and gradually increases during larval development until third instar larvae, where the levels of LGR1 mRNA are at their highest. After larval eclosion, LGR1 transcripts decline in early pupal stages but increase again the last day before adult eclosion. In adults, the

relative transcript abundance of the receptor remains elevated and is higher in males than females, which suggests that the role of GPA2/GPB5 in adult *D. melanogaster* is sex-specific. Similar transcript expression patterns were also observed for the genes encoding GPA2 and GPB5 subunits. However, only the levels of GPA2 transcripts alone showed a distinct peak in expression during first instar larval stages, suggesting that heterodimerization of both subunits may not be necessary for a functional hormone in this particular stage of fruit fly development (Vandersmissen et al. 2014). Notably, however, since LGR1 is activated only by the GPA2/GPB5 heterodimer and not by either individual subunit alone (Sudo et al. 2005), there may be an alternative elusive receptor or LGR1 splice variant that responds to individual subunits when heterodimerization seems unlikely.

Tissue-specific expression analysis of the gene encoding LGR1 shows relatively high transcript abundance in osmoregulatory organs like the hindgut, salivary glands and Malpighian tubules of third instar larvae and the hindgut of adult flies (Chintapalli et al. 2007; Vandersmissen et al. 2014), and is localized to epithelial cells of the rectum in *D. melanogaster* embryo (Sellami et al. 2011). Additionally, LGR1 transcripts are more abundant in the salivary glands and reproductive tissues of adult males compared to females, further supporting a sex-specific role for GPA2/GPB5. Both subunits are expressed in the same bilateral pair of neuroendocrine cells situated within the first four abdominal neuromeres of the central nervous system (CNS) of both adults and third instar larvae (Sellami et al. 2011). Ablation of these neurons significantly impedes fly survival to adulthood, indicating that GPA2/GPB5 likely plays a critical physiological role (Sellami et al. 2011). Though not as intensely stained, expression of both subunits was also observed in cells localized within other regions of the CNS including the pars intercerebralis, suboesophageal ganglion and thoracic ganglion (Sellami et al. 2011). GPA2

transcripts are also greater in the developing and mature testes of male flies compared to female gonads, whereas GPB5 transcripts were more prominent in female reproductive tissues (Vandersmissen et al. 2014).

Based on the transcript expression profiles of the glycoprotein hormone subunits and LGR1, researchers have sought to identify potential developmental effects using RNA interference techniques (Vandersmissen et al. 2014). By knocking down LGR1 transcript expression, it was shown that only 22% of larvae underwent white pupae formation, only 1% of which progressed to adulthood. Moreover, pupae that succeeded to adulthood did so with a delay of between 2 and 4days. From these observations, it was evident that a down regulation of LGR1 mRNA significantly impacts transitional stages from larvae to pupae and pupae to adults. Knockdown of LGR1 was shown to negatively influence expression of genes involved in ecdysteroid biosynthesis as well as ecdysteroid titers during fly development, which are critical for the strict coordination of larval and pupal metamorphosis (Vandersmissen et al. 2014).

LGR1 RNAi flies were also found to have a lower tolerance to desiccation stress, indicating that the GPA2/GPB5 system may contribute to the regulation of osmoregulatory and/or ionoregulatory processes (Vandersmissen et al. 2014).The suggestion that GPA2/GPB5 may function in ionoregulation and osmoregulation has also been suggested in studies on the yellow fever mosquito, *A. aegypti* (Paluzzi et al. 2014). GPA2 and GPB5 transcripts are expressed in all post-embryonic developmental stages of A. *aegypti*. Similar to the fruit fly (Vandersmissen et al. 2014), adult males express the alpha and beta subunits 3 to 8-fold higher relative to adult females and there is higher expression in adults compared to pupa and larval stages. LGR1 mRNA transcript is enriched in osmoregulatory tissues such as the hindgut, Malpighian tubules and midgut. Its expression in different developmental stages parallels the

expression profiles of its putative ligand GPA2/GPB5, being highly enriched in adults compared to larvae or pupae, and more so in adult males compared to females (Paluzzi et al. 2014). Thus, the temporal and spatial distribution of LGR1 mRNA supports the notion that GPA2/GPB5 may play a role in iono- and osmoregulation, primarily during the life transitioning forms and adult stages of these insects. To begin investigating this potential physiological role in mosquitoes, electrophysiological techniques were used to examine if tissues that display LGR1 transcript enrichment could be influenced by treatment with a recombinant form of mosquito GPA2/GPB5. Following treatments of adult *A. aegypti* hindgut with a recombinant GPA2/GPB5, it was determined that the hormone modulates transepithelial cation flux; inhibiting sodium secretion (i.e. natriuresis) in the ileum and promoting potassium secretion (i.e. kaliuresis) in both the ileum and rectum (Paluzzi et al. 2014). Although further research is needed, these results support that GPA2/GPB5 functions as an ionoregulatory and osmoregulatory hormone in the mosquito and could play similar roles in other insects.

Genes encoding the GPA2 and GPB5 subunits are present in a number of other insect species with a completely sequenced genome including the pea aphid (Huybrechts et al. 2010), the red flour beetle (Li et al. 2008) and the silkworm (Roller et al. 2008) (Fig. 1-1, 1-2). It has been suggested that the high abundance of cysteine residues in the subunits of GPA2/GPB5 should make the cells producing this hormone easily identifiable using paraldehyde fuchsin stain (Sellami et al. 2011), which identifies neurosecretory cells rich in disulphide linkages (Buehner et al. 1979). Although informative, this would identify cells containing cysteine-rich proteins generally rather than specific GPA2/GPB5 producing cells and so additional techniques (e.g. immunohistochemistry or *in situ* hybridization) would be required for verification. Nonetheless, candidate glycoprotein hormone-producing cells have already been identified using fuchsin

staining strategies with bilateral doublets occurring in the larval stage of the dragonfly *Aeschna grandis* (Charlet 1969), in the cockroach *Periplaneta americana* (de Bessé 1967), the locust *Schistocerca gregaria* (Delphin 1965) and the walking sticks *Clitumnus extradentatus* and *Carausius morosus* (Raabe 1965). However, as the heterodimeric glycoprotein hormone in these organisms has yet to be studied, further research on these species may aid in the elucidation of GPA2/GPB5's function in invertebrates.

Studies on the model organism *Caenorhabditis elegans* have found that intestinal activity is governed by the interaction of two classes of genes; class 1 flr genes (flr-1, flr-3 and flr-4) and class 2 flr genes (flr-2, flr-5, flr-6 and flr-7) (Katsura et al. 1994; Take-Uchi et al. 1998). Of the class 2 flr gene family, flr-2 was found to have high structural homology to the mammalian GPA2, and showed expression in neurons of the head, pharynx and tail. By exposing flr-2 mutated *C. elegans* to bacterial infection, it was revealed that the GPA2 homolog is involved with antibacterial defence (Oishi et al. 2009). Mutations of *C. elegans* GPA2 also revealed it to be involved with defecation behavior, intestinal color, growth rates and dauer larva formation, which is a larval stage that appears under stressful conditions. In addition to GPA2, *C. elegans* also possesses homologous genes encoding the glycoprotein beta subunit (flr-5) as well as the glycoprotein hormone receptor (flr-6) and deletion of these genes elicits similar phenotypes as flr-2 mutations (Oishi et al. 2009).

1.3.4 Current theories for GPA2/GPB5's role in invertebrates

1.3.4.1 GPA2/GPB5's role in development

The possibility that GPA2/GPB5 functions in development is a recurring theme for glycoprotein hormone studies in invertebrates. Transcript expression patterns over different life stages of the

fruit fly (Vandersmissen et al. 2014) and mosquito (Paluzzi et al. 2014) showed that GPA2, GPB5 and LGR1 are expressed throughout development, including embryonic stages for *D. melanogaster* (Vandersmissen et al. 2014). In the fruit fly, knockdown approaches using RNA interference against LGR1, the GPA2/GPB5 receptor, demonstrated significant impacts to pupal eclosion and survival, as well as hormones and genes associated with metamorphosis (Vandersmissen et al. 2014). As well, the silencing of glycoprotein hormone-like receptors in the red flour beetle, *Tribolium castaneum*, was associated with larval and pupal mortality (Bai et al. 2011). Moreover, genetic ablation of the neuroendocrine cells responsible for synthesizing GPA2 and GPB5 subunits significantly reduced the number of adult flies (Sellami et al. 2011). Recent studies conducted on the sea hare, *Aplysia californica*, have also uncovered a developmental function for GPA2/GPB5 with increased mRNA abundance in larval stages (Heyland et al. 2012). Specifically, GPA2 and GPB5 transcript abundance is increased in hatching as well as prior to and following metamorphosis. Moreover, a positive correlation between subunit transcript expression and embryonic to larval development was also noted. Interestingly, while all ganglia of the sea hare CNS were found to contain neurons co-expressing both subunits, additional neurons were identified expressing the GPA2 subunit alone (Heyland et al. 2012).

1.3.4.2 GPA2/GPB5's role in maintaining ion and water balance: There is also some evidence that GPA2/GPB5 functions in iono- and osmoregulation since transcript expression of LGR1, the GPA2/GPB5 receptor, is enriched in osmoregulatory tissues of both *D. melanogaster* (Chintapalli et al. 2007; Sellami et al. 2011; Vandersmissen et al. 2014) and *A. aegypti* (Paluzzi et al. 2014). Additionally, scanning ion-selective electrode technique (SIET), which allows investigations of epithelial preparations too small in size for implementing Using chambers or

that have spatial or temporal heterogeneity (Rheault and O'Donnell 2001), was performed on the adult mosquito hindgut and revealed that GPA2/GPB5 modulates cation transport (Paluzzi et al. 2014). Using the genetic toolbox available for *D. melanogaster*, LGR1 knockdown flies were shown to be less tolerant to dehydration stress and, in addition, water content was significantly lower in these flies when tested under desiccating conditions (Vandersmissen et al. 2014). Since the neuroendocrine cells expressing GPA2/GPB5 in *D. melanogaster* were found to be unique from cells expressing diuretic factors including corticotropin-releasing factor, calcitonin, kinin, and CAPA/periviscerokinin peptides (Sellami et al. 2011), it is possible that this hormone could function as an anti-diuretic factor as its release would require regulation distinct from the diuretic factors.

Similar to most other insects, the hindgut and Malpighian tubules of the desert locust, *S. gregaria*, play a critical role in ridding the body of excess water and ions and recycling important ions like potassium and chloride from the primary urine (Phillips et al. 1998). Chloride transport stimulating hormone (CTSH) and ion transport peptide (ITP) are two anti-diuretic hormones of the locust neuroendocrine system, important for regulating reabsorptive activity of the hindgut (Phillips and Audsley 1995). Both neuropeptides have been shown to act through the second messenger cyclic AMP to promote chloride and potassium reabsorption, and inhibit proton secretion across hindgut epithelia (Chamberlin and Phillips 1988; Phillips and Audsley 1995). Since GPA2/GPB5 also appears to mediate its effects through cyclic AMP in *D. melanogaster* (Sudo et al. 2005) and knockdown of LGR1 reduces water content in flies under desiccation stress (Vandersmissen et al. 2014), it suggests that this neuroendocrine system could hold an important role as a regulator of anti-diuresis.

1.3.5 Do GPA2 and GPB5 function as a monomer or heterodimer?

To date, there is much debate as to whether GPA2/GPB5 functions as a heterodimer or if each subunit may also function as an independent monomer. GPA2 and GPB5 subunits were found to successfully heterodimerize and activate their respective human (Nakabayashi et al. 2002) and fruit fly (Sudo et al. 2005) receptors. If the N-linked oligosaccharide chains in either subunit are removed however, hormonal secretion by HEK 293T cells is arrested and TSH receptor activation is not as effective (Okajima et al. 2008). GPA2 and GPB5 subunits that are not properly glycosylated would barely be secreted or active *in vivo* and the GPB5 subunit lacks a carboxyl tail extension that aids in dimerization, which is expected to make heterodimerization less likely (Dos Santos et al. 2009). When comparing the expression profiles of each subunit, there is indeed co-localization of GPA2 and GPB5 in cells of the human anterior pituitary (Okada et al. 2006) and rat pituitary (Nagasaki et al. 2006), each ganglion of the *A. californica* CNS (Heyland et al. 2012), as well as the fruit fly CNS (Sellami et al. 2011). However, GPA2 is also more broadly expressed in additional neurons and in a wider range of tissues than GPB5 in humans (Okada et al. 2006) and rats (Nagasaki et al. 2006). Similarly in the sea hare *A. californica*, GPA2 displays broader expression in a larger number of cells in the CNS, whereas GPB5 is more greatly expressed in pleural mechanosensory neurons (Heyland et al. 2012). To add, amphioxus GPA2 and GPB5 subunits do not share similar transcript expression patterns (Dos Santos et al. 2009; Tando and Kubokawa 2009a), in contrast to co-expression patterns observed in the fruit fly *D. melanogaster* (Vandersmissen et al. 2014) or the mosquito *A. aegypti* (Paluzzi et al. 2014). It is plausible that GPA2/GPB5 may function as both a heterodimer and as an individual set of monomers depending on the developmental stage of certain species. In *D. melanogaster* for example, GPA2 transcripts are highly enriched in first instar larval stages

whereas GPB5 transcripts remain low. Later stages of development, however, are characterized with both subunits being highly upregulated (Vandersmissen et al. 2014). As the genes encoding GPA2 and GPB5 have genomic positions in close proximity to one another for most bilateral organisms (Dos Santos et al. 2009), it could be argued that they are regulated in a similar fashion. Notably, however, the genes encoding the subunits of the classic vertebrate glycoprotein hormones are not in close genomic proximity, yet heterodimerization is critical for their proper hormonal function (Dos Santos et al. 2009).

1.3.6 Concluding remarks

The relatively novel discovered, yet evolutionarily ancient GPA2/GPB5 signalling system is thought to have evolved prior to the emergence of bilateral metazoans (Hauser et al. 1998; Vibede et al. 1998), indicating that this well-conserved system is physiologically relevant to both vertebrates and invertebrates, however its function is not well understood in any single organism to date. In mammals, GPA2/GPB5 is pleiotropic as it is believed to hold multiple physiological roles in metabolism through thyroxine production (Nakabayashi et al. 2002; Okada et al. 2006), skeletal development (Duncan Bassett et al. 2015) and reproduction (Sun et al. 2010). A pleiotropic role is also likely for invertebrates since the glycoprotein hormone signalling system plays a developmental role (Bai et al. 2011; Heyland et al. 2012; Paluzzi et al. 2014; Vandersmissen et al. 2014) and appears to function in hydromineral homeostasis (Sellami et al. 2011; Paluzzi et al. 2014; Vandersmissen et al. 2014). Genes encoding GPA2, GPB5 and LGR1-like proteins are present within the majority of vertebrate and invertebrate genomes, but for the most part have not yet been studied. Given that GPA2/GPB5 was only recently discovered, there is much to be learned. Future studies could include protein expression analyses of this particular

glycoprotein hormone signalling system as transcriptional studies may not accurately reflect physiologically relevant tissue targets and cells *in vivo*. Moreover, the next steps for future research could include reverse genetics approaches to deduce hormonal function through resultant abnormal phenotypes.

1.4 Research objectives

As reviewed in the preceding section, the glycoprotein hormone GPA2/GPB5 and its receptor LGR1 are believed to encompass a role in development and maintaining ion and water balance in invertebrates, however, its exact function remains unclear. Moreover, whether this novel glycoprotein hormone signalling system functions as a heterodimer, or as individual GPA2 and GPB5 subunits has not been studied in detail in any given organism. As a result, the primary objective of this thesis aims to elucidate the physiological function of GPA2/GPB5 and LGR1, as well as provide a more detailed understanding and characterization of its signalling in the disease vector mosquito *A. aegypti*. The following chapters outlined herein describe intriguing and novel insights to GPA2/GPB5 and LGR1 function and signalling in mosquitoes, that may aid in the functional elucidation of GPA2/GPB5 homologs in other invertebrates, and simultaneously provide a better understanding of the physiological processes that govern mosquito biology, which can contribute in the development of novel mosquito vector control strategies.

1.4.1 *The identification of prospective target tissues for GPA2/GPB5 in adult A. aegypti*

Transcriptional studies of GPA2/GPB5 and LGR1 in mosquitoes have been described throughout *A. aegypti* development, and LGR1 expression was identified in the midgut, Malpighian tubules and hindgut; where treatments with recombinant GPA2/GPB5 were found to modulate transepithelial sodium and potassium flux (Paluzzi et al. 2014). As a result, it was determined GPA2/GPB5 could act on LGR1 in tissues like the hindgut to maintain hydromineral balance, during processes like feeding, in mosquitoes (Paluzzi et al. 2014). However, a detailed understanding of the tissue-specific distribution of LGR1 expression in adult mosquitoes is unavailable, and only transcriptional studies of LGR1 expression has been performed.

Consequently, whether LGR1 expression is present in mosquito tissues other than the gut, and which cell types are involved, remains unknown. In order to hint towards a specific physiological function in a particular tissue and/or cell type for GPA2/GPB5 in adult mosquitoes, it is necessary to provide a detailed expression profile of LGR1. Given that previous studies demonstrated ionoregulatory effects of GPA2/GPB5 in the adult mosquito hindgut, it was hypothesized that LGR1 expression would be present on basolateral surfaces of epithelial tissue in the mosquito hindgut, since hormones are released into circulation to act on their target tissues. Secondly, initial studies which first identified *A. aegypti* LGR1 described a predicted molecular weight product of ~105 kDa and three putative amino-linked glycosylation sites. As a result, we also hypothesized that the heterologous expression of LGR1 and detection with a custom antibody would yield a slightly higher molecular weight product, above 105 kDa due to post translational modifications. The principal objectives of this research were as follows:

1) Elaborate a more detailed transcript-level analysis of LGR1 expression in tissues of adult male and female mosquitoes to determine tissue- and sex-specific differences;

2) Map the protein-level expression of LGR1 in different regions of the alimentary canal and reproductive tissues of male and female mosquitoes, as well as discern the tissue type and characterize the subcellular localization patterns of LGR1 protein;

3) Confirm *A. aegypti* LGR1 is post-translationally modified using heterologous systems with mammalian cell lines.

1.4.2 *Functional elucidation of GPA2/GPB5 and LGR1 in A. aegypti male reproduction*

Some of the major findings from Chapter 2 revealed moderate transcript as well as very distinct immunolocalization patterns of LGR1 expression in the testes of adult male *A. aegypti*. Though a

reproductive function has not been thoroughly deliberated in invertebrates, GPA2/GPB5 and its receptor in vertebrates, the TSH receptor, may encompass a novel paracrine system in the rat ovary to regulate gamete development (Sun et al. 2010), as discussed in Section 1.3.2. Taken together, I hypothesized that GPA2/GPB5 and LGR1 signalling would have a specific function related to the regulation of *A. aegypti* spermatogenesis. To test this hypothesis, the main objectives of this chapter were as follows:

1) Identify the cell type and characterize the subcellular localization of LGR1 expression in the testis in order to infer a more precise function associated with mosquito spermatogenesis;

2) To verify its role in spermatogenesis, knockdown LGR1 transcript expression using RNA interference techniques, and examine phenotypes associated with spermatogenesis.

1.4.3 *A detailed characterization of GPA2/GPB5 signalling*

As reviewed in Section 1.3.5, whether GPA2/GPB5 subunit heterodimerization is required to activate LGR1, or if receptor activity is elicited by independent subunits, is highly debated (Alvarez et al. 2009, Rocco et al. 2016). Furthermore, though it has been demonstrated LGR1 binds GPA2/GPB5 and activates a stimulatory G protein (Gs) pathway in the fruit fly (Sudo et al. 2005), it is not known if LGR1 binds GPA2/GPB5 and whether the signalling pathways involved upon receptor activation are conserved in other invertebrates like the mosquito *A. aegypti*. Since (i) the glycoprotein hormones FSH, LH, TSH and CG are known to activate their receptors only as subunit heterodimers, (ii) GPA2/GPB5 heterodimers activate a Gs pathway upon binding the TSH receptor in vertebrates and (iii) GPA2/GPB5 heterodimers likewise activate a Gs pathway upon binding LGR1 in *D. melanogaster*, I hypothesized GPA2 and GPB5 subunits could heterodimerize and these heterodimers would be required to activate a similar Gs pathway upon

binding LGR1 in the mosquito *A. aegypti*. GPA2 and GPB5 subunits are expressed throughout mosquito development and the transcripts of both subunits are significantly upregulated in adult stages compared to juvenile stages (Paluzzi et al. 2014). To date however, the spatial distribution of GPA2/GPB5 has not been comprehensively characterized in mosquitoes. In *D. melanogaster*, GPA2 and GPB5 subunits are expressed in bilateral pairs of neuroendocrine cells associated with the first four abdominal neuromeres in the thoracoabdominal ganglia of the central nervous system. (Sellami et al. 2011) As a result, it was hypothesized that GPA2 and GPB5 expression would be expressed in the central nervous system of adult mosquitoes, and more specifically, display a neuronal distribution pattern associated with the abdominal ganglia, which are analogous to the abdominal neuromeres in the fused thoracoabdominal ganglion in the adult fruit fly (Court et al. 2017). The main objectives of this chapter aimed to:

1) Characterize the spatial expression profile of GPA2 and GPB5 subunits in adult mosquitoes;

2) Using heterologous systems with mammalian cells, determine if *A. aegypti* GPA2/GPB5 can heterodimerize *in vitro*;

3) Elucidate whether heterodimerization is required to activate *A. aegypti* LGR1;

4) Confirm whether the activation of LGR1 elicits downstream stimulatory G protein (Gs) coupling.

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CHAPTER 2:

IMMUNOHISTOCHEMICAL MAPPING AND TRANSCRIPT EXPRESSION OF THE GPA2/GPB5 RECEPTOR IN TISSUES OF THE ADULT MOSQUITO, *AEDES AEGYPTI*

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2.1 Summary

GPA2/GPB5 is a glycoprotein hormone found in most bilateral metazoans including the mosquito, *Aedes aegypti*. To elucidate physiological roles and functions of GPA2/GPB5, we aim to identify prospective target tissues by examining the tissue- and sex-specific expression profile of its receptor, the leucine-rich repeat-containing G protein-coupled receptor 1 (LGR1) in the adult mosquito. Western analyses using a heterologous system with CHO-K1 cells, transiently expressing *A. aegypti* LGR1, yielded a 112-kDa monomeric band and high-molecular weight multimers, which associated with membrane-protein fractions. Moreover, immunoblot analyses on protein isolated from HEK 293T cells stably expressing a fusion construct of *A. aegypti* LGR1–EGFP (LGR1: 105 kDa+EGFP: 27 kDa) yielded a band with a measured molecular weight of 139 kDa that also associated with membrane-protein fractions and upon deglycosylation, migrated as a lower molecular weight band of 132 kDa. Immunocytochemical analysis of HEK 293T cells stably expressing this fusion construct confirmed EGFP fluorescence and LGR1-like immunoreactivity colocalized primarily to the plasma membrane. Immunohistochemical mapping in adult mosquitoes revealed LGR1-like immunoreactivity is widespread in the alimentary canal. Importantly, LGR1-like immunoreactivity localizes specifically to basolateral regions of epithelia and, in some regions, appeared as punctate intracellular staining, which together indicates a potential role in feeding and/or hydromineral balance. LGR1 transcript expression was also detected in gut regions that exhibited strong LGR1-like immunoreactivity. Interestingly, LGR1 transcript expression and strong LGR1-like immunoreactivity was also identified in reproductive tissues including the testes and ovaries, which together suggests a potential role linked to spermatogenesis and oogenesis in male and female mosquitoes, respectively.

2.2 Introduction

In mammals, the classic heterodimeric glycoprotein hormones include follitropin (FSH), lutropin (LH), thyrotropin (TSH) and chorionic gonadotropin (CG) (Pierce and Parsons 1981). These hormones are a family of cystine-knot proteins that play fundamental roles in metabolism, reproduction as well as growth and development. These hormones are formed by the heterodimerization of two polypeptide chains—a single glycoprotein α subunit (GPA1), which is common to all four glycoprotein hormones and a glycoprotein β subunit (GPB1-4) that is hormone-specific (Pierce and Parsons 1981; Stockell Hartree and Renwick 1992). With the availability of the human genome, two novel glycoprotein hormone subunits were identified that shared structural similarities to the classic glycoprotein subunits and accordingly were named glycoprotein α2 (GPA2) and glycoprotein β5 (GPB5) (Nakabayashi et al. 2002). GPA2 and GPB5 subunits heterodimerize (GPA2/GPB5) and is referred to as thyrostimulin in mammals since it stimulates the TSH receptor in vitro and in vivo (Nakabayashi et al. 2002). However, since its discovery, the function of GPA2/GPB5 is still not well understood in any organism to date. GPA2/GPB5 is believed to encompass multiple physiological roles in vertebrates as it stimulates thyroxine production (Nakabayashi et al. 2002; Okada et al. 2006), is involved in osteoblastic bone formation (Duncan Bassett et al. 2015) and is part of a paracrine system in the mammalian ovary (Sun et al. 2010). Nonetheless, for the invertebrates, the GPA2/GPB5 glycoprotein hormone has been suggested to possess a developmental function (Heyland et al. 2012; Paluzzi et al. 2014; Vandersmissen et al. 2014; Rocco and Paluzzi 2016) and may also play a role in hydromineral balance (Sellami et al. 2011; Paluzzi et al. 2014; Vandersmissen et al. 2014; Rocco and Paluzzi 2016).

A number of the proposed functions of GPA2/GPB5 in the invertebrates have been based upon transcript-level studies of the GPA2/GPB5 receptor (Sellami et al. 2011; Paluzzi et al. 2014; Vandersmissen et al. 2014). Glycoprotein hormone receptors are members of the leucine-rich repeat-containing G protein-coupled receptor (LGR) family. LGRs are unique relative to other G protein-coupled receptors (GPCRs) as they are characterized with a relatively large N-terminal ectodomain that contains several leucine-rich repeats important for ligand specificity (Vassart et al. 2004; Krause et al. 2012). In contrast to thyrostimulin (the mammalian homolog of GPA2/GPB5), which activates the TSH receptor in humans (Nakabayashi et al. 2002) and rat (Nagasaki et al. 2006), LGR1 is the receptor for GPA2/GPB5 in invertebrates (Sudo et al. 2005). In the fruit fly, LGR1 transcripts are expressed throughout development, detectable as early as 8–16 h post-oviposition (Hauser et al. 1998). Elevation of receptor transcripts occurs in life-transitioning stages such as the wandering third instar larval and pharate stages but decline in early pupal stages (Vandersmissen et al. 2014). Knockdown of LGR1 transcripts was shown to significantly disrupt normal metamorphosis in the fruit fly, *Drosophila melanogaster* (Vandersmissen et al. 2014). Further, flies with LGR1 knockdown exhibited lower tolerance to desiccation stress, indicating that GPA2/GPB5 is likely involved in maintaining hydromineral balance in addition to its proposed developmental role (Vandersmissen et al. 2014). Interestingly, a behavioral screen to investigate genes involved in water consumption indicated LGR1 knockdown leads to increased water intake relative to controls (Jourjine et al. 2016), suggesting a greater requirement to replenish water that is lost in the absence of this putative anti-diuretic regulator. Transcript expression studies on the mosquito *A. aegypti* (Paluzzi et al. 2014)and fruit fly *D. melanogaster* (Vandersmissen et al. 2014) have shown LGR1 enrichment in tissues critical for iono- and osmoregulation such as the hindgut. Recombinant GPA2/GPB5 applied to the

mosquito hindgut led to modulated cation transport across this osmoregulatory tissue (Paluzzi et al. 2014).

In order to further delineate the function of GPA2/GPB5, localizing expression of LGR1 at the protein level and comprehensively examining expression of the LGR1 transcript will help to confirm prospective target tissues for this glycoprotein hormone. In addition, more physiologically meaningful data can be obtained by monitoring both LGR1 transcript and protein-level expression profiles in specific regions or cell types in a given tissue. Accordingly, we sought to characterize the sex- and tissue-specific distribution patterns of LGR1 at both the transcript and protein level for the first time in the invertebrates. Further, we sought to characterize post-translational processing of the receptor using a heterologous system with mammalian cell lines expressing *A. aegypti* LGR1. As predicted in previous studies (Paluzzi et al. 2014), our results show *A. aegypti* LGR1 is a membrane-bound protein that migrates slightly higher than the predicted ~105 kDa likely as a result of glycosylation. We determined that LGR1 transcript expression and LGR1-like immunoreactivity is localized to a variety of regions along the alimentary canal in adult mosquitoes. Moreover, LGR1 transcript and LGR1-like immunoreactivity was observed in reproductive tissues of both males and females, suggesting LGR1 and its ligand, GPA2/GPB5, may have pleiotropic actions (i.e., exhibit more than one physiological role) in *A. aegypti*. LGR1 expression associated with the alimentary canal suggests a role in feeding, digestion and/or hydromineral balance. Further, LGR1 expression identified in reproductive tissues, including regionalized immunoreactive staining in the testes and ovaries, indicate a possible role in spermatogenesis and oogenesis in males and females, respectively.

2.3 Materials and methods

2.3.1 Experimental animals

Adult *A. aegypti* were obtained from an established laboratory colony in the Department of Biology, York University. Larvae were hatched from semi-desiccated eggs that were oviposited onto Whatmann Filter Papers (GE Bioscience) in plasticware containers containing room-temperature (RT) distilled water and fed daily with several drops of a 2% liver powder:brewers yeast mixture (1:1). Pupae were isolated from larval containers and transferred to small beakers containing distilled water and placed into hardwood-framed cages lined with mosquito netting. Mosquitoes were kept on a 12:12 h light:dark cycle and adults were fed using large Petri dishes containing 10% sucrose solution fitted with cotton ball wicks. Females were blood fed using an artificial feeding system comprising an inverted plastic cup containing warm water (∼37 °C) and sheep blood in Alsever's solution (Cedarlane Laboratories, Hornby, ON, Canada) aliquoted into an adjacent microchamber that was sealed with parafilm.

2.3.2 LGR1 transcript expression profile determined by RT-qPCR

Various gut regions and reproductive tissues from adult male and female *A. aegypti* 4 days post-eclosion were dissected in nuclease-free Dulbecco's phosphate-buffered saline (PBS) (pH = 7.3) (Wisent, St. Bruno, QC, Canada) after being briefly anesthetized with CO_2 at RT and placed on ice to minimize activity. Tissues were dissected and transferred immediately into chilled RNA Lysis Buffer containing beta-mercapto-ethanol (1:100 dilution) aliquoted in microcentrifuge tubes, vortexed and frozen at −20 °C. The next day, samples were left to thaw at RT and total RNA was purified using the PureLink RNA Micro Kit that included an on-column DNase I treatment to remove any contaminating genomic DNA (Life Technologies, Burlington, ON,

Canada). Total RNA samples were quantified using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Reverse transcription was then performed using 50 ng of total RNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Mississauga, ON, Canada). Samples were then diluted 5-fold with molecular grade nuclease-free water (Wisent Corporation, St. Bruno, QC, Canada). These cDNA samples were then used to determine LGR1 transcript abundance using PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) and quantified on a StepOnePlus Real-Time PCR System (Applied Biosystems) following conditions previously described (Gondalia et al. 2016). Primers used for LGR1 transcript quantification were described previously (Paluzzi et al. 2014) and overlap exon–exon boundaries to prevent genomic DNA amplification. Data was normalized to three stably expressed reference genes; ribosomal protein 49 (GenBank accession: AY539746), 40S ribosomal protein L8 (GenBank accession: XM_001657661) and 60S ribosomal protein S18 (GenBank accession: XM_001660270) according to the ΔΔCt method as described previously (Paluzzi et al. 2014). Quantitative measurements were performed using three technical replicates per plate and 4–6 biological replicates for each of the studied tissues.

2.3.3 Wholemount immunohistochemistry

To analyze the spatial distribution patterns of LGR1-like immunoreactivity across different regions of the mosquito alimentary canal and reproductive tissues, adult male and female *A. aegypti* less than 1 week post-eclosion were collected and wholemount immunohistochemistry was performed. Mosquitoes were briefly anesthetized with CO_2 and dissected at RT in PBS. Whole gut and reproductive tissues from adult males and females were then transferred to 4% paraformaldehyde fixative for 1 h at RT, rinsed once with PBS and then incubated for 1 h at RT

with 4% Triton X-100, 10% normal sheep serum (NSS) (v/v) and 2% BSA (w/v) prepared in PBS. After three 10-min washes with PBS, whole gut and reproductive tissues were then incubated for 48 h at 4 °C with gentle agitation in primary antibody solutions made up in 0.4% Triton X-100, 2% NSS (v/v) and 2% BSA (w/v) in PBS. Treatments included a custom affinity-purified rabbit polyclonal primary antibody (0.5 μg ml^{-1}; Genscript, Piscataway, NJ, USA) against *A. aegypti* LGR1 using an antigen (SINFRPDEQQDGPA) located in the N-terminal ectodomain, residues 449–462 (Paluzzi et al. 2014). This region had a high antigenic score for optimal antibody production and shares no sequence similarity (Fig. 2-S1) with other leucine-rich repeat containing GPCR identified or predicted in dipterans such as *D. melanogaster*, *Anopheles gambiae* and *Aedes aegypti* (Hauser et al. 1998; Nishi et al. 2000; Hill et al. 2002; Luo et al. 2005; Nene et al. 2007; Van Loy et al. 2008; Van Hiel et al. 2012). Treatments also included detection of P-type Na^+/K^+ ATPase alpha subunit (2.8 μg ml^{-1}; Developmental Studies Hybridoma Bank product 'a5') (Lebovitz et al. 1989), which was shown previously to primarily label basolateral membranes in the mosquito gut (Patrick et al. 2006). Finally, control treatments involved pre-incubated *A. aegypti* LGR1 primary antibody solution containing 400:1 peptide antigen:antibody (mol:mol) that served as a negative control. Both experimental and control primary antibody solutions were made and incubated at 4 °C overnight prior to their application to adult mosquito tissues. Following primary antibody treatments, tissues were washed three times for 10 min each with PBS, samples were then incubated overnight at 4 °C with Cy3-labelled goat anti-rabbit secondary antibody (1:200 dilution; Sigma Aldrich, Oakville, ON, Canada) and either Cy2-labeled sheep anti-mouse secondary antibody (1:500 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or 0.165 μM Alexa Fluor 488-conjugated phalloidin (Life Technologies) in 10% NSS made up in PBS. Tissues were mounted

Figure 2-S1: Epitope mapping of an *A. aegypti* LGR1-targeted custom primary antibody and sequence comparison to other LGR sequences in mosquito (*Aedes aegypti* and *Anopheles gambiae*) and fly (*Drosophila melanogaster*).

on cover slips with mounting media containing 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) to visualize cell nuclei and analyzed using a Lumen Dynamics X-Cite™ 120Q Nikon fluorescence microscope (Nikon, Mississauga, ON, Canada). For some preparations, confocal optical sections of wholemounts (obtained using an Olympus BX-51 laser-scanning confocal microscope) were used to determine LGR1-like cellular distribution patterns in gut tissues, as well as regionalization of LGR1-like staining in reproductive tissues. All images were processed using ImageJ.

2.3.4 Generation of A. aegypti LGR1-EGFP fusion construct

A fusion construct was prepared involving the full open reading frame of *A. aegypti* LGR1 and enhanced green fluorescent protein (EGFP) coding sequences that were amplified from previously available plasmid vectors (Paluzzi et al. 2014). Conditions for PCR involved using 1 ng of the corresponding vector as template for amplification with Q5 High Fidelity DNA Polymerase (New England Biolabs, Whitby, ON, Canada), following manufacturer recommended conditions. Primer sequences included LGR1-for-NotI (5′-GCGGCCGCCACCATGAGCGTATGTAGTG-3′), LGR1-rev-EcoRI (5′-GAATTCCAGAAAAGTTTCACTTTCCTG-3′), EGFP-for-EcoRI (5′-GAATTCGGTACCATGGTGAGCAAGGG-3′) and EGFP-rev-NotI (5′-GCGGCCGCAGATCTACTTGTACAGCTCGTCC-3′). The amplicons were then purified using a PureLink PCR purification kit (Life Technologies), A-tailed to facilitate T/A cloning as described previously (Gondalia et al. 2016), cloned into pGEM T vector (Promega, Madison, WI, USA) and base accuracy was confirmed by Sanger sequencing of plasmid clones (The Centre for Applied Genomics, Sick Kids Hospital, Toronto, ON, Canada). The products were then prepared to incorporate EGFP at the 3′ end of the LGR1 coding sequence to yield an LGR1 protein with EGFP fused on the C-terminus, which involved a series of restriction enzyme digestion reactions. Firstly, sequencing plasmid clones were digested with EcoRI (Promega) over

a 30-min incubation at 37 °C, followed by a heat-inactivation step at 80 °C for 5 min and reaction was purified with a Purelink purification kit (Life Technologies). Next, to improve fusion product generation, EcoRI-digested LGR1 plasmid DNA was then further digested with PvuI (New England Biolabs) that cleaves pGEM T vector only and the reaction was incubated at 37 °C for 1 h and purified as stated above. Lastly, both EGFP and LGR1 plasmid clones were next digested with the NotI (Anza™ cloning system; ThermoFisher Scientific, Whitby, ON, Canada) for 1 h and 20 min at 37 °C and digested products were run on a 1% agarose-TAE gel stained with ethidium bromide and desired DNA products correlating to LGR1 or EGFP digested sequences were gel-extracted and purified with PureLink gel extraction kit (Life Technologies). Purified digest products were quantified and then ligated with T4 DNA Ligase to pcDNA3.1+ mammalian expression vector that was also digested with the NotI restriction enzyme (as above) and dephosphorylated with alkaline phosphatase to prevent formation of non-recombinants (Anza™ cloning system; ThermoFisher Scientific). Following ligation, an aliquot of the reaction was used to transform laboratory-grown competent DH5α-strain *Escherichia coli* cells and recombinant bacteria were grown on ampicillin-selective LB-agar plates. Recombinant bacterial colonies were screened by colony PCR using standard Taq DNA polymerase (Life Technologies) to confirm the presence of the appropriate insert size and correct directionality as well as verify base accuracy by Sanger sequencing (The Centre for Applied Genomics). A midiprep plasmid purification (Life Technologies) was then performed on selected recombinant bacterial colonies carrying pcDNA3.1+ with the LGR1–EGFP construct in the correct orientation.

2.3.5 Transiently transfected mammalian cell lines

To drive transient expression of LGR1 or EGFP in mammalian Chinese Hamster Ovary (CHO-K1) cells, transfection involved pBudCE4.1 mammalian expression vector containing EGFP or the full open reading frame of *A. aegypti* LGR1 (Paluzzi et al. 2014), including a Kozak translation initiation sequence as previously described (Paluzzi et al. 2010). To drive transient expression of the fusion construct in human embryonic kidney (HEK 293T) cells, transfection involved the pcDNA3.1+ containing the LGR1–EGFP sequence (described above). Mammalian cell lines were grown and maintained in a water-jacketed incubator at 37 °C, 5% CO2, and were transfected at 80–90% confluency with Lipofectamine LTX transfection reagent (Life Technologies) using a 3:1 transfection reagent to DNA (μL:μg) ratio. Approximately 48 h post-transfection, cells were either examined for LGR1-like expression using immunocytochemical analysis or prepared for protein quantification, electrophoresis and western blot analyses.

2.3.6 Generation of HEK 293T cell line stably expressing LGR1-EGFP fusion construct

To promote genomic incorporation of the LGR1–EGFP fusion construct from pcDNA3.1+ into HEK 293T cells, recombinant plasmid DNA was first linearized with a PvuI restriction enzyme (New England Biolabs) following recommended conditions. Cells maintained in 6-well tissue culture plates were then transfected at ~80% confluency with the linearized plasmid, using Lipofectamine LTX transfection reagent [3:1 transfection reagent to DNA construct (μL:μg) ratio]. Approximately 48 h post-transfection, HEK 293T cells were maintained on 900 $\mu g \mu L^{-1}$ geneticin for 15 days until antibiotic resistance was evident through the presence of clonal colonies of cells. Cells that survived the antibiotic treatment were then examined for EGFP fluorescence in 96-well tissue culture plates using a EVOS FL Auto live-cell imaging system (Life Technologies). Cells growing stably under antibiotic selection and that showed strong

EGFP fluorescence were selected as candidates for stable lines expressing the LGR1–EGFP fusion protein and were subsequently validated using immunocytochemistry and western blot analyses.

2.3.7 Immunocytochemistry

Transiently transfected CHO-K1 and untransfected or stably transfected HEK 293T cells were first grown to 60–70% confluency in 6-well tissue culture plates containing cover slips treated with poly-L-lysine to promote cell attachment. Cells were then washed with DPBS and fixed with 3% paraformaldehyde fixative in DPBS for 30 min at RT. Following fixation, cells were washed three times with DPBS and incubated with 1% glycine in DPBS for 10 min. To facilitate membrane permeabilization, cells were treated with 0.01% Triton X-100 in DPBS for 10 min at RT, washed once with DPBS and blocked for 1 h at RT under constant mixing using antibody dilution buffer (ADB) comprising 10% goat serum (v/v), 3% bovine serum albumin (w/v), 0.05% Triton X-100 (w/v) in DPBS. Cells were then incubated overnight at 4 °C under gentle agitation with either 1 μg ml^{-1} of the affinity-purified primary antibody targeted against *A. aegypti* LGR1 (prepared in ADB) or pre-incubated *A. aegypti* LGR1 primary antibody solution containing 400:1 peptide antigen:antibody (mol:mol) that served as a negative control. Both experimental and control antibodies were prepared 1 day prior and kept at 4 °C under constant mixing. Following overnight treatment of cells with LGR1 antibody alone or LGR1 antibody preabsorbed with the LGR1-specific antigen, cells were washed for 30 min with DPBS before and after a 1-h incubation with 1:500 dilution of Cy3-labelled goat anti-rabbit secondary antibody (Sigma-Aldrich) under constant mixing. To confirm LGR1-like staining was membrane-associated in CHO-K1 cells, a co-detection with 1 μg mL^{-1} mouse monoclonal α-

tubulin antibody (GenScript) was utilized post-LGR1 primary and secondary antibody incubations, as a determinant for cytosolic protein detection. CHO-K1 cells were then incubated with a Cy2-conjugated sheep anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) in ADB (1:100 dilution). Nuclei of CHO-K1 cells were stained with 5 μg/mL DAPI in DPBS and images acquired using a Lumen Dynamics X-Cite™ 120Q fluorescence microscope (Nikon). Untransfected and stably transfected HEK 293T cells were mounted on glass slides containing DAPI (5 μg/mL) made up in DPBS/glycerol (1:1) to stain nuclei and imaged using a Zeiss LSM 700 confocal microscope. All images were processed using imageJ.

2.3.8 Preparation of protein for western blot analyses

Protein for western blot analyses was purified from stably expressing cell lines or transiently expressing cell lines 48 h post-transfection. Cells were dislodged using DPBS containing 5 mM EDTA (Life Technologies) for 15 min and were centrifuged at 300g for 5 min. Cell pellets were sonicated for 3–5 s in PBS with 1× FastBreak Cell Lysis Reagent (Promega) and 1:100 protease inhibitor cocktail (Sigma Aldrich). Cell lysates containing total cellular proteins were then collected for protein analysis. In order to separate cytosolic and membrane proteins, a membrane protein extraction kit was used (ThermoFisher Scientific) following recommended guidelines with minor modifications including 1:100 protease inhibitor cocktail (Sigma Aldrich) in both the permeabilization and solubilization buffers. Final protein concentrations were calculated with a microscale Bradford assay in 96-well plates, using bovine serum albumin standard (Bio-Rad Laboratories) and quantified using a Synergy 2 Multi-Mode Microplate Reader (BioTek).

To test for LGR1 glycosylation, samples were treated with N-glycosidase F (PNGase F) (New England Biolabs) following manufacturer guidelines with the only modification being that samples were not heated at 100 °C prior to PNGase F treatment.

2.3.9 Western blot analyses

To resolve proteins, an 8% SDS-polyacrylamide gel was used for electrophoresis under reducing conditions. Protein samples, prepared with beta-mercaptoethanol (1:100) and standards were migrated initially at 100 V for 30 min and subsequently at 120 V for 90 min before being transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher, Rockford, IL, USA) using a wet transfer system at 100 V for 75 min. Following transfer, PVDF membranes were blocked for 1 h at RT in PBS containing 0.1% Tween-20 (BioShop, Burlington, ON, Canada) and 5% skim milk powder (PBSTB) and incubated on a rocking platform overnight at 4 °C with PBSTB containing 0.5 μg ml^{-1} custom primary antibody against *A. aegypti* LGR1, a rabbit polyclonal anti-GFP primary antibody (1 μg ml^{-1}) (Novus Biologicals Canada, Oakville, ON, Canada), or 0.25 μg ml^{-1} of a mouse monoclonal alpha tubulin primary antibody (Genscript) for a loading control. The next day, membranes were washed for 1 h with PBS containing 0.1% Tween-20 (PBST), changing the wash buffer every 15 min. Blots were then incubated with PBSTB containing goat anti-rabbit HRP conjugated secondary antibody (1:6000 dilution for LGR1 transfected cells, or 1:1000 dilution for LGR1–EGFP transfected cells) (Life Technologies) or anti-mouse HRP conjugated secondary antibody (1:8000), for 1 h at RT and subsequently washed with several buffer changes for 1 h with PBST. Lastly, blots were drained of excess PBST, incubated with Clarity Western ECL substrate and images were acquired using

a ChemiDoc MP Imaging System (Bio-Rad). Exact molecular weight measurements and data analysis were performed using Image Lab 5.0 software (Bio-Rad).

2.4 Results

2.4.1 Tissue-specific expression profile of LGR1 transcript

Selected gut regions and reproductive tissues of adult mosquitoes were examined for LGR1 transcript expression and compared between males and females to determine potential sex-specific roles for GPA2/GPB5 in adult *A. aegypti*. Expression of LGR1 transcript was observed in all tissues studied, including the midgut, Malpighian tubules and hindgut of adult mosquitoes (Fig. 2-1). Notably, greater transcript abundance was observed in the anterior midgut compared to the posterior midgut region. Modest expression of LGR1 was also observed in the diverticula, as well as reproductive tissues including the testes, ovaries and accessory reproductive tissues (Fig. 2-1). In almost all tissues studied, greater LGR1 transcript expression was observed in adult females, with significantly greater expression in the hindgut and accessory reproductive tissues relative to corresponding male tissues (Fig. 2-1).

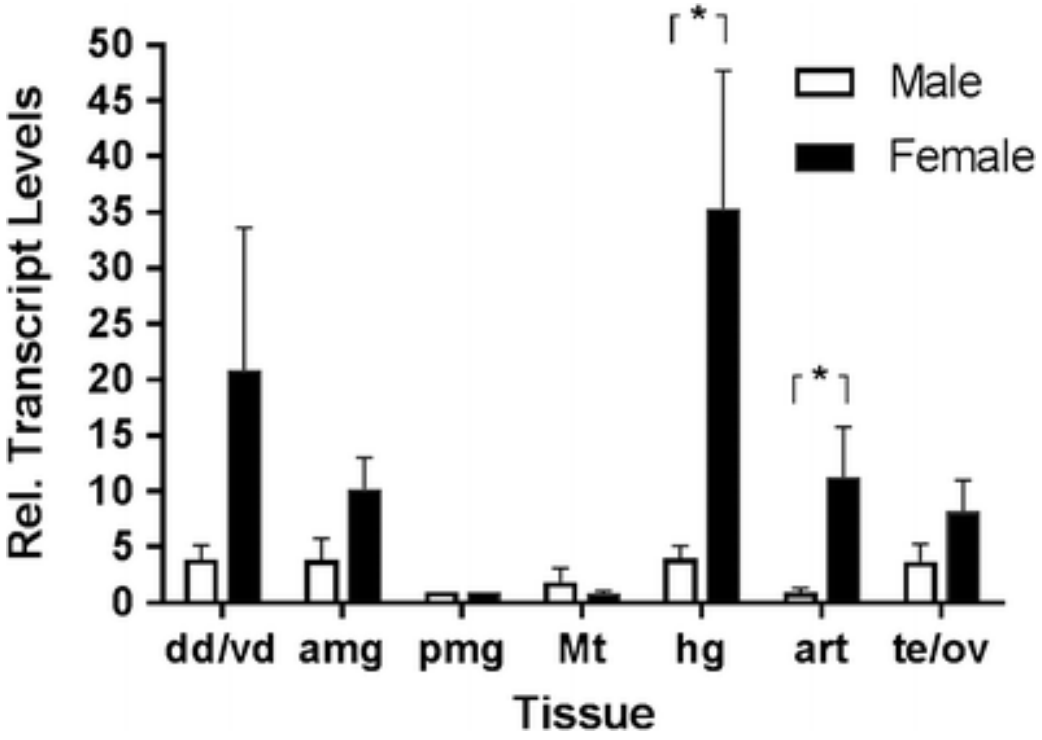

Figure 2-1: Tissue distribution of LGR1 transcript in 4-day-old adult *Aedes aegypti* tissues determined by qRT-PCR. LGR1 transcript was detected in all tissues studied of both male and female mosquitoes. Females showed greater expression of LGR1 transcript in all tissues except the Malpighian tubules, with significantly greater expression in the hindgut and accessory reproductive tissues. Mean ± SEM of 4–6 biological replicates for various gut regions and reproductive tissues. Transcript abundance is shown relative to that observed in the posterior midgut (=1). Student's t test (*$P < 0.05$) was used to determine sex-specific differences in each examined tissue. dd dorsolateral, vd ventral diverticula, amg anterior midgut, pmg posterior midgut, Mt Malpighian tubules, hg hindgut, art accessory reproductive tissues, te testes, ov ovaries.

2.4.2 Spatial and cellular distribution patterns of LGR1-like immunoreactivity

Wholemounts of reproductive tissues from adult male and female mosquitoes with preincubated LGR1 control antibody showed no immunoreactive staining (Fig. 2-2a, c). In experimental treatments, however, robust LGR1-like immunoreactivity was observed in the ovaries and testes (Fig. 2-2b, d). Moreover, LGR1-like immunoreactivity in the testes was greater in intensity in more distal regions, compared to proximal regions of the testes (Figs. 2-2d, 2-3b). LGR1-like immunoreactivity was not observed in other male reproductive tissues such as the accessory glands and seminal vesicles (Fig. 2-2d). Notably, however, LGR1-like immunoreactive staining in females was evident in the lateral and common oviducts (Fig. 2-2b). In the ovaries, confocal optical sections revealed some co-localization of LGR1-like staining with the P-type Na^+/K^+ ATPase (NKA) within outer membranes of the follicular epithelium (Fig. 2-3d). In addition, LGR1-like immunoreactivity was observed intracellularly within follicular cells and nurse cells localized within the follicles of female mosquitoes (Fig. 2-3d). Staining was absent in reproductive tissues in controls treated with preincubated antibody (Fig. 2-3a, c).

LGR1-like immunofluorescence was also observed throughout the alimentary canal of both male and female adult mosquitoes. LGR1-like immunoreactivity was evident in a variety of alimentary canal tissues including the Malpighian tubules (Fig. 2-4b), hindgut (Fig. 2-4d), anterior midgut and diverticula (Fig. 2-5b), as well as the posterior midgut (Fig. 2-5d) with no obvious differences between male and female tissue preparations. Notably, however, differences in LGR1-like immunoreactivity between distinct regions of the gut were observed. Specifically, LGR1-like immunoreactivity examined in the diverticula, which originate at the junction between the anterior midgut and foregut of adult *A. aegypti*, showed stronger staining in the ventral diverticulum compared to limited immunoreactivity associated with the paired

dorsolateral diverticula of both males and females (Fig. 2-5b). Staining was abolished in all gut regions in controls treated with preincubated antibody (Figs. 2-4a, c, 2-5a, c).

To examine whether LGR1-like immunoreactivity localizes to gut epithelia or muscle associated with the alimentary canal (or both), we processed tissues with the custom LGR1 antibody together with phalloidin, which detects filamentous actin (F-actin) that is enriched in muscle tissue (in addition to its cytoskeletal association in most eukaryotic cells). LGR1-like immunoreactivity does not co-localize with phalloidin staining of F-actin as is exemplified in the anterior midgut and diverticula (Fig. 2-6a), posterior midgut (Fig. 2-6b), ileum and rectum of the hindgut (Fig. 2-6c) and Malpighian tubules, the latter of which exhibit enhanced LGR1-immunoreactive staining associated with the nuclei of principal cells (Fig. 2-6d). Similarly, phalloidin-staining of F-actin in female and male reproductive tissue did not co-localize with LGR1-like immunoreactivity (Fig. 2-6e, f). These results indicate that LGR1 expression is associated with an alternate tissue type associated with the gut and reproductive tissues—the most logical next step was to confirm if LGR1-like immunoreactivity is associated with epithelia.

To validate LGR1-like staining was associated with gut epithelial tissue, LGR1-like immunoreactivity patterns were assessed throughout individual epithelia and compared to the distribution of NKA, since previous studies demonstrated this to be a useful marker for gut epithelia of adult *A. aegypti* (Patrick et al. 2006) and it is localized primarily to basolateral membranes. In many tissues examined, LGR1-like immunoreactivity was associated with intracellular punctate staining with weak or absence of staining in the basolateral membrane as observed in proximal principal cells of the MTs (Fig. 2-7a, b), where NKA staining was clearly evident in basolateral membrane. In stellate cells, LGR1-like immunoreactivity also appeared as

intracellular punctate-like staining but was nonetheless absent in control treatments where the LGR1 antibody was pre-absorbed with peptide antigen (see “Materials and methods”). Interestingly, LGR1-like immunoreactivity exhibited track-like staining in both cell types of the Malpighian tubules (Fig. 2-7c, d). Comparatively, LGR1-like staining was substantially weaker in stellate cells where NKA immunoreactivity was strongly localized. Optical sections of the anterior and posterior midgut were chosen as representatives for other structures of the alimentary canal, where either basolateral or intracellular LGR1-like immunoreactivity was observed. The type of staining, whether punctate intracellular, or solely basolateral, was dependant on the area of focus within the tissue under examination. Distinct and strong basolateral LGR1-like immunoreactivity was observed in sections of the anterior midgut (Fig. 2-7e, f). Moreover, similar to principal cells of the Malpighian tubules (Fig. 2-7a, b), intracellular punctate-like staining of LGR1 was prominent in the posterior midgut (Fig. 2-7e–h).

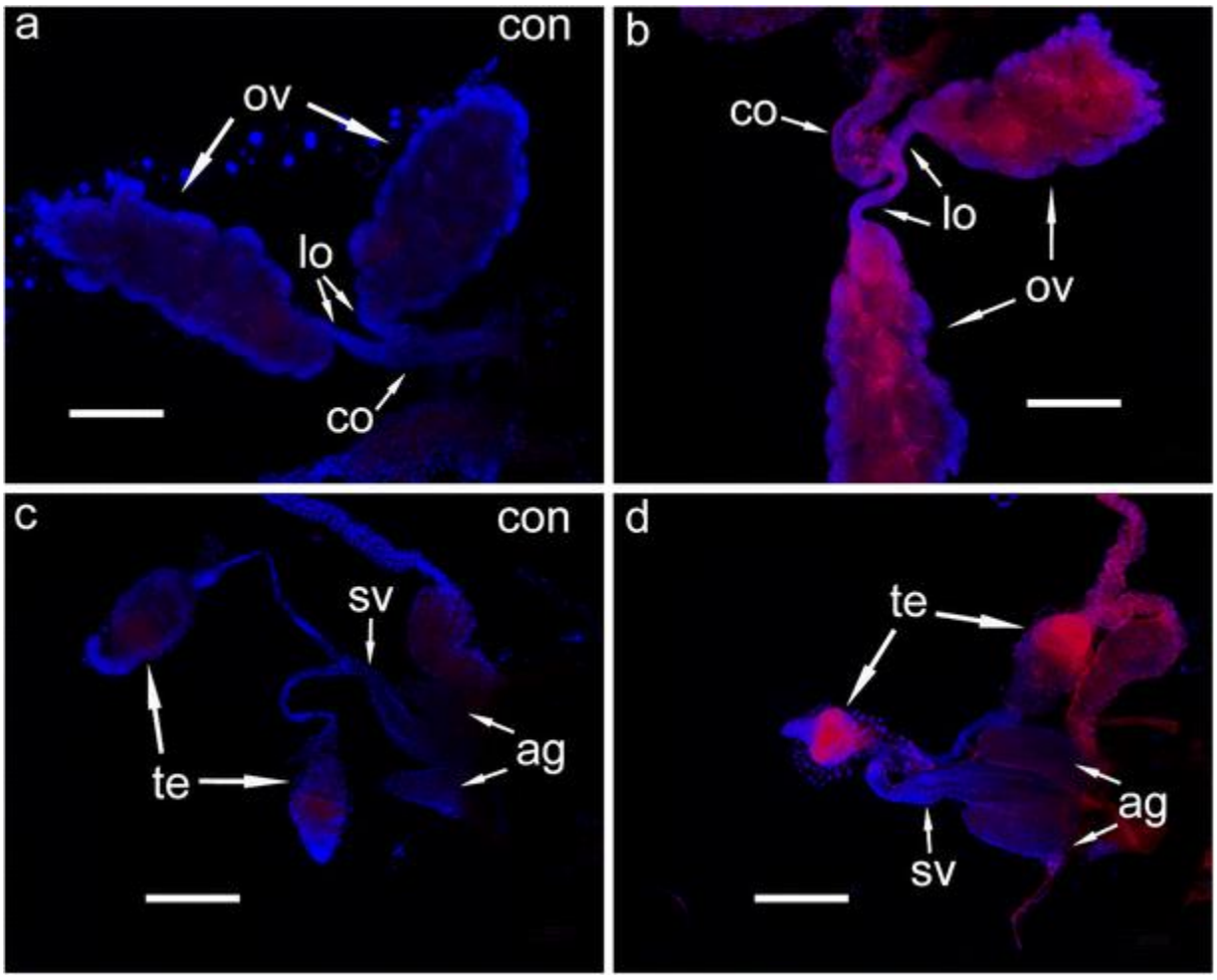

Figure 2-2: Immunolocalization of LGR1 (*red*) and DAPI (*blue*) in the male and female reproductive tissues of adult *A. aegypti*. Control treatments with preincubated primary antibody (**a**, **c**) and preparations showing LGR1-like immunoreactivity in the ovaries (*ov*) and testes (*te*) of experimental groups (**b**, **d**). LGR1-like immunoreactivity was noted in the lateral (*lo*) and common (*co*) oviducts; however, the seminal vesicle (*sv*) and accessory glands (*ag*) (**b**, **d**) associated with the male reproductive system did not display LGR1-like immunoreactivity. *Scale bars* 100 μm

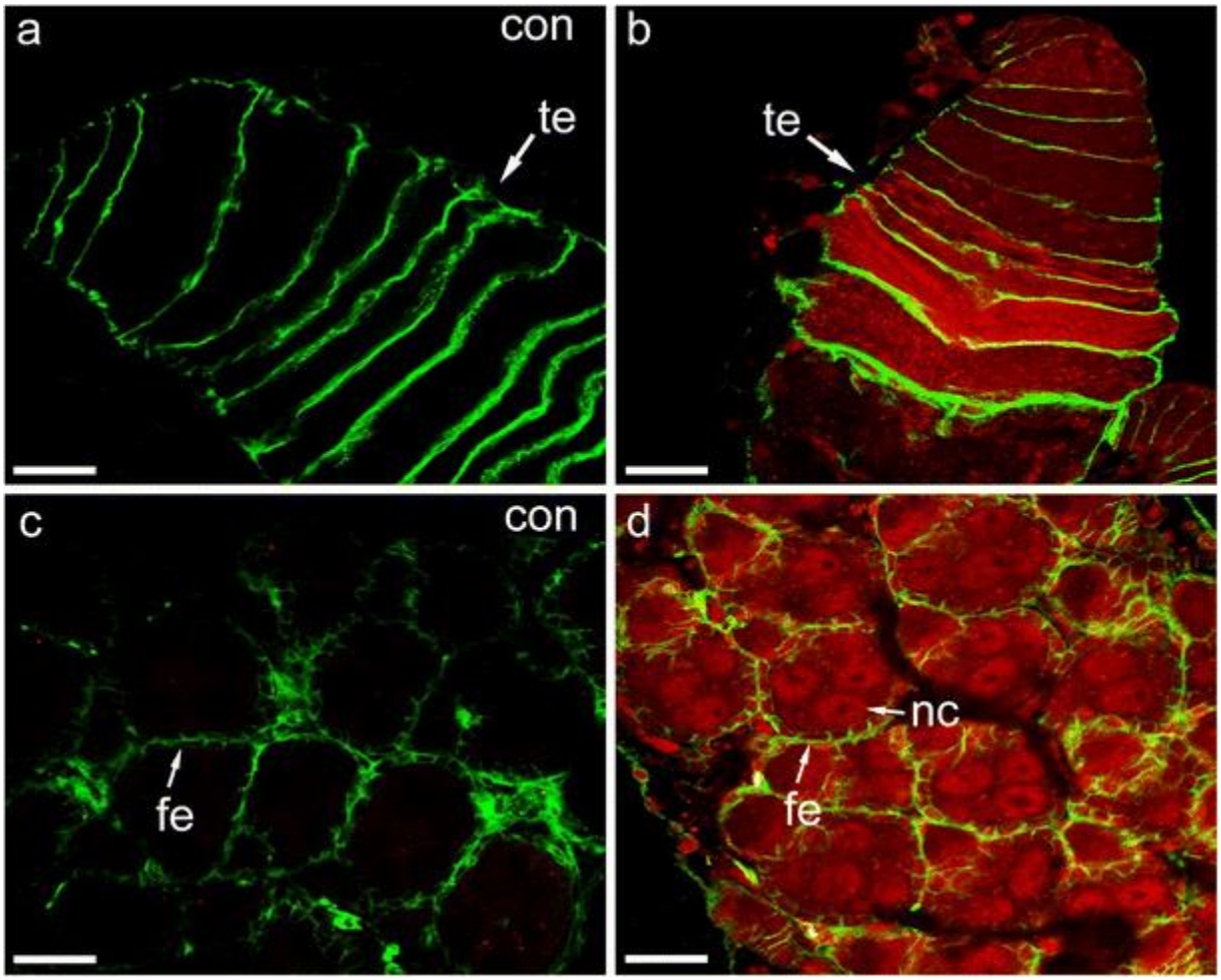

Figure 2-3: Immunolocalization of LGR1 (*red*) and P-type Na^+/K^+ ATPase (*green*) in optical sections of the testes (**a**, **b**) and ovaries (**c**, **d**) of adult *A. aegypti*. Relative to control-treated groups (**a**), LGR1-like staining is regionalized in more distal regions of the male testes (*te*) of experimental groups (**b**). Relative to the control ovaries (**c**), LGR1-like immunofluorescence is detected in nurse cells (*nc*) associated with follicles and some co-localization of LGR1-like staining with the P-type Na^+/K^+ ATPase is observed in follicular epithelia (*fe*) (**d**) of previtellogenic-staged females. *Scale bars* 50 μm

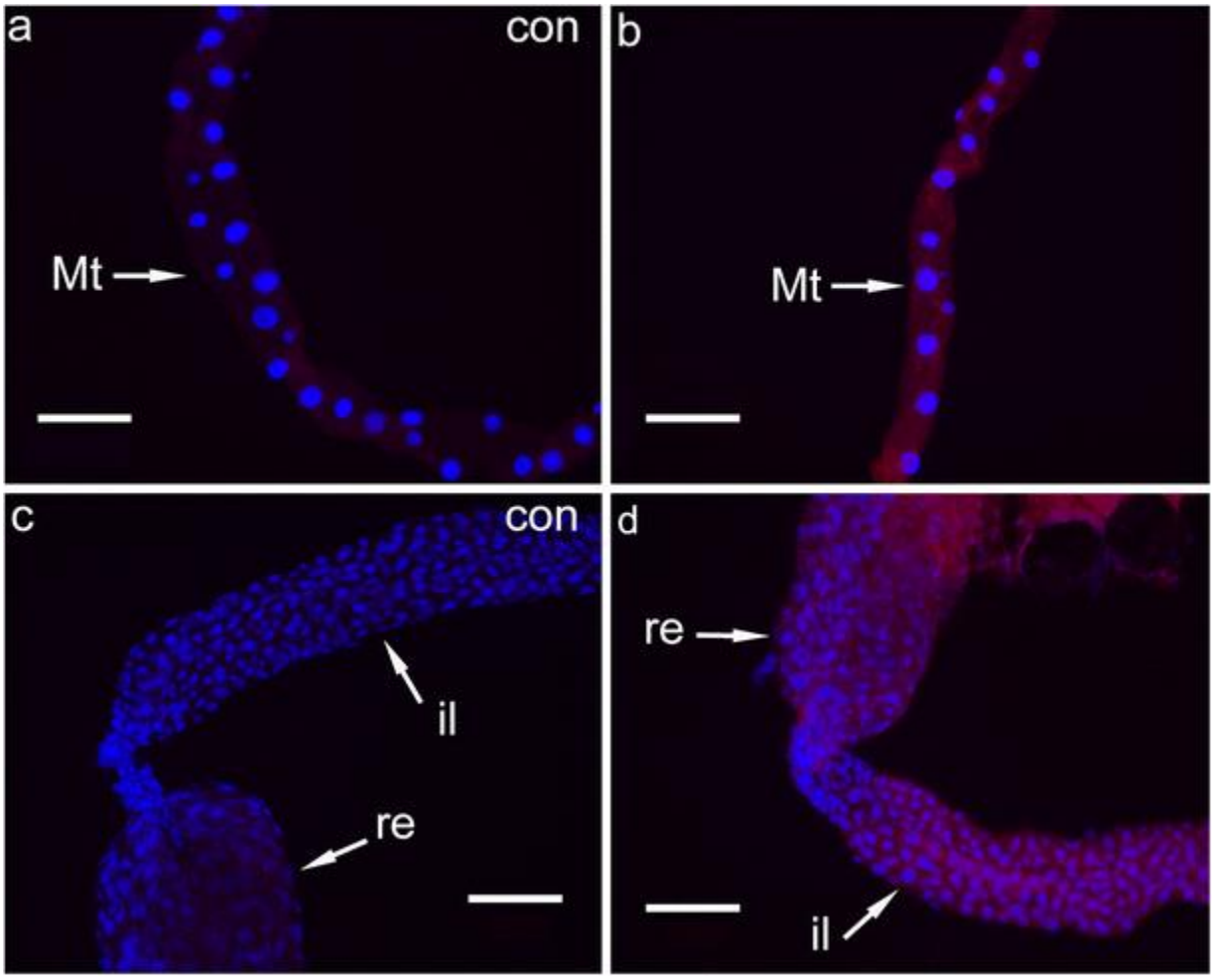

Figure 2-4: Immunolocalization of LGR1 (*red*) and DAPI (*blue*) in female Malpighian tubules and the hindgut of adult *A. aegypti*. Relative to control groups (**a**, **c**), there was LGR1-like immunoreactivity evident in the Malpighian tubules (*Mt*), ileum (*il*) and rectum (*re*) of the hindgut of experimental groups (**b**, **d**). Similar results were observed for male mosquito tissues (not shown). *Scale bars* 100 μm

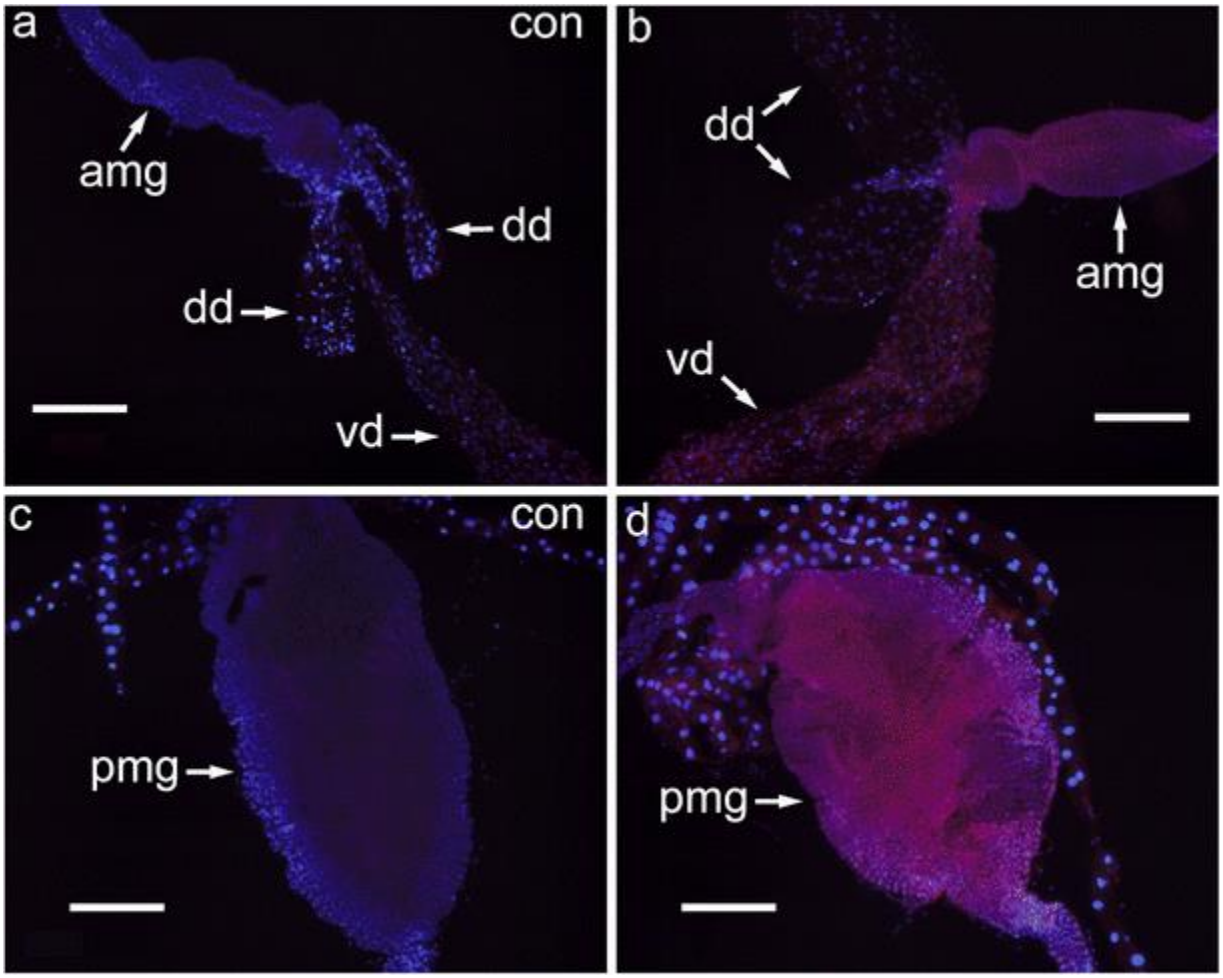

Figure 2-5: Immunolocalization of LGR1 (*red*) and DAPI (*blue*) in the female midgut and diverticula of adult *A. aegypti*. Relative to control groups (**a**, **c**), there was LGR1-like immunofluorescence present in the ventral diverticulum (*vd*), anterior midgut (*amg*) and posterior midgut (*pmg*) of experimental groups (**b**, **d**). Similar results were observed for male mosquito tissues (not shown). Dorsolateral diverticula (*dd*) varied in its LGR1-like immunoreactivity relative to controls for both sexes. Notably, however, this tissue exhibited only limited LGR1-like immunoreactivity (**a**, **b**) for the majority of preparations examined. *Scale bars* (**a**, **b**) 100 μm, (**c**, **d**) 200 μm

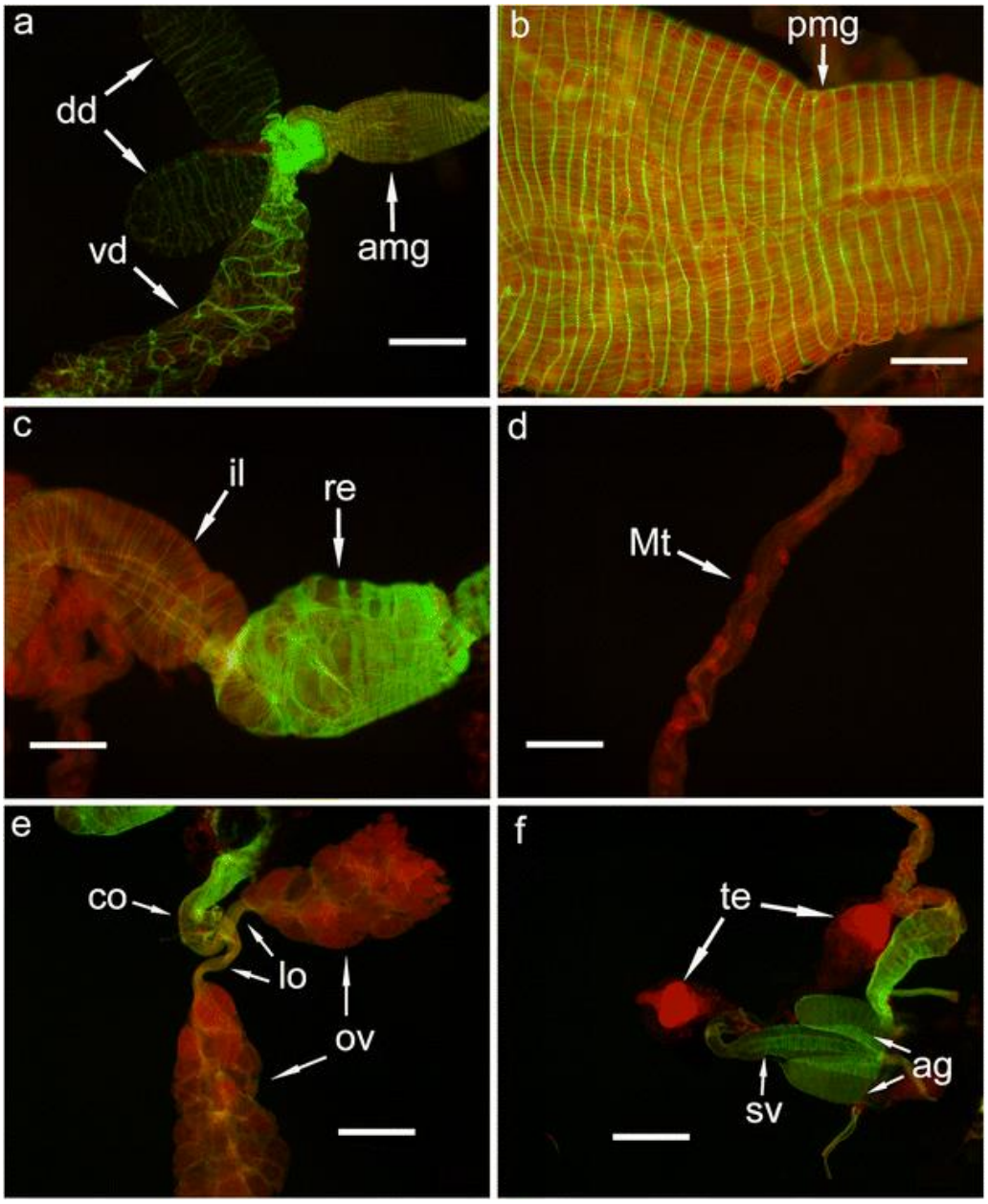

Figure 2-6: Immunolocalization of LGR1 (red) and phalloidin-stained F-actin (green) associated with muscle in female gut as well as reproductive tissues of both sexes of adult *A. aegypti*. Co-localization of LGR1-like immunoreactivity and phalloidin-stained muscle was not observed in (**A**) the anterior midgut (amg), dorsolateral diverticula (dd) and ventral diverticulum (vd), (**B**) posterior midgut (pmg), (**C**) ileum (il) and rectum (re) of the hindgut, (**D**) Malpighian tubules (Mt), (**E**) ovaries (ov) and (**F**) testes (te), seminal vesicles (sv) and accessory glands (ag). Scale bars are 100 µm.

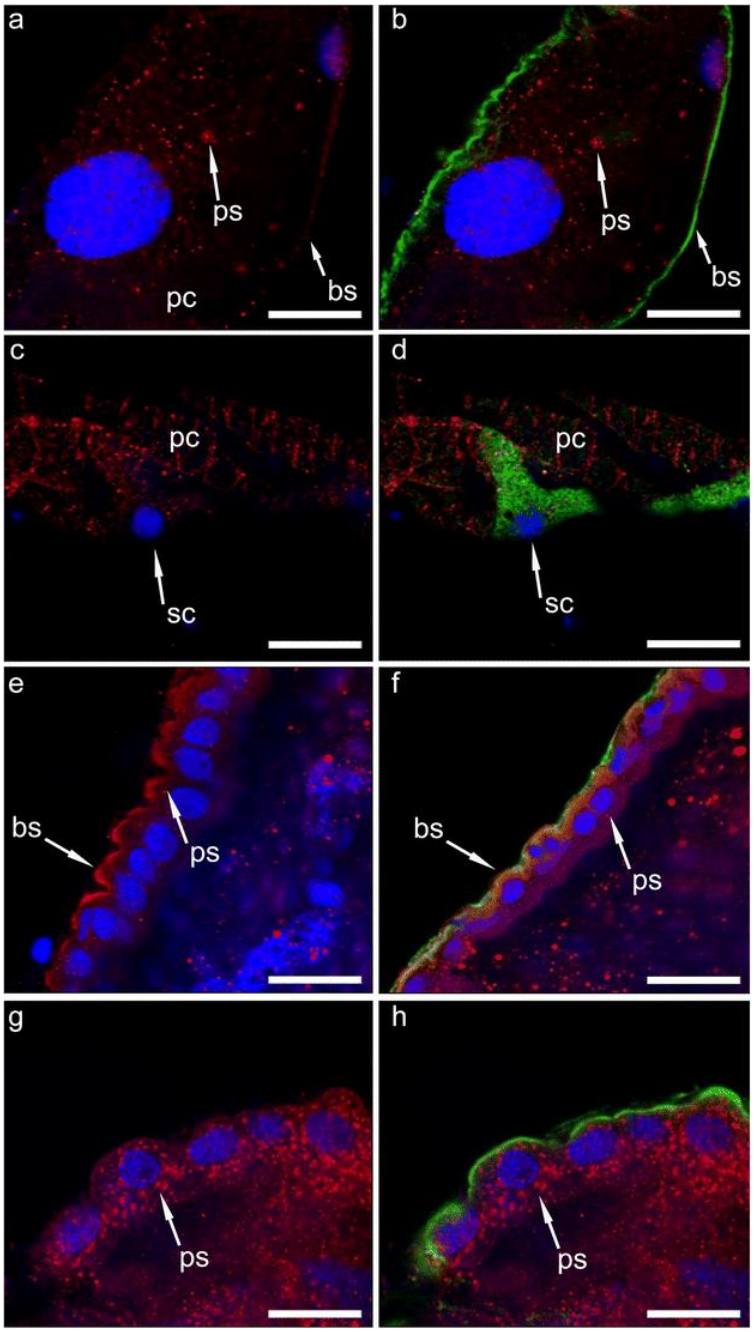

Figure 2-7: Immunolocalization of LGR1 (*red*), DAPI (*blue*) and P-type Na^+/K^+ ATPase (*green*) in optical sections of the Malpighian tubules and midgut from adult female *A. aegypti*. LGR1-like immunoreactivity was observed in both the principal cells (*pc*) (**a**, **b**) and stellate cells (*sc*) (**c**, **d**) of the proximal and distal Malpighian tubules, respectively. Basolateral staining (*bs*) and punctate staining (*ps*) of LGR1-like expression was observed in all tissues, including cells of the Malpighian tubules (**a**–**d**) and the anterior (**e**, **f**) and posterior (**g**, **h**) midgut. Similar results were observed in male *A. aegypti* tissues (not shown). *Scale bars* 20 μm

2.4.3 Western blot and immunocytochemical analyses of transiently transfected CHO-K1 cells

Protein extracts were isolated from CHO-K1 cells transiently expressing *A. aegypti* LGR1 or EGFP and incubated with the custom primary antibody against the *A. aegypti* LGR1 ectodomain antigen sequence (see "Materials and methods"). A band size of 112 kDa along with high-molecular weight multimers (>250 kDa) were detected in protein samples isolated from LGR1-transfected CHO-K1 cells; however, these bands were absent in lanes loaded with protein from EGFP-transfected and untransfected control cells (Fig. 2-8a). Similar high-molecular-weight multimers >250 kDa have been detected in other studies with the thyroid stimulating hormone receptor; however, upon treatments with DTT and heating, less mature, monomeric forms developed (Mizrachi and Segaloff 2004; Latif et al. 2010). Analogous treatments were performed with LGR1-containing samples but multimerization appeared to be unaffected (data not shown). To determine whether LGR1 is cytosolic or membrane-associated, we isolated membrane and cytosolic protein fractions of untransfected and LGR1-transfected CHO-K1 cells. We found that multimerization of the receptor protein was detected only in membrane protein isolations from LGR1-transfected cells, though the monomeric band size of 112 kDa was not detected (Fig. 2-8b). Moreover, cytosolic fractions and protein isolations from untransfected CHO-K1 cells did not show either the monomeric or multimeric LGR1-positive bands (Fig. 2-8b), indicating that LGR1 is membrane-associated under transiently expressed conditions in the heterologous system. Unlike samples that were simply lysed and sonicated (Fig. 2-8a), the high-molecular weight multimers of LGR1 show two distinct LGR1-positive bands of >250 kDa, rather than a single smeared band (Fig. 2-8b). Non-specific bands were identified in protein samples from all treatment groups including controls (Fig. 2-8a). Moreover, lanes with protein isolated from the cytosolic fractions of untransfected and LGR1-transfected cells yielded similar bands at 88.6

kDa, whereas membrane fractions resulted with similar bands of 67.4 kDa, indicating sub-optimal performance and non-specific binding of the LGR1-targeted primary antibody under reducing conditions used in the western analysis (Fig. 2-8b).

Immunocytochemical analysis of LGR1 transfected CHO-K1 cells supports that the receptor is membrane-associated since LGR1-like immunofluorescence was not co-localized with alpha tubulin, used as a cytosolic marker as a component of the microtubule network (Fig. 2-9a–d). LGR1-like immunoreactivity was localized to perinuclear regions in some cells, while in other cells, staining was associated with the plasma membrane (Fig. 2-9a–d) of LGR1 expressing CHO-K1 cells.

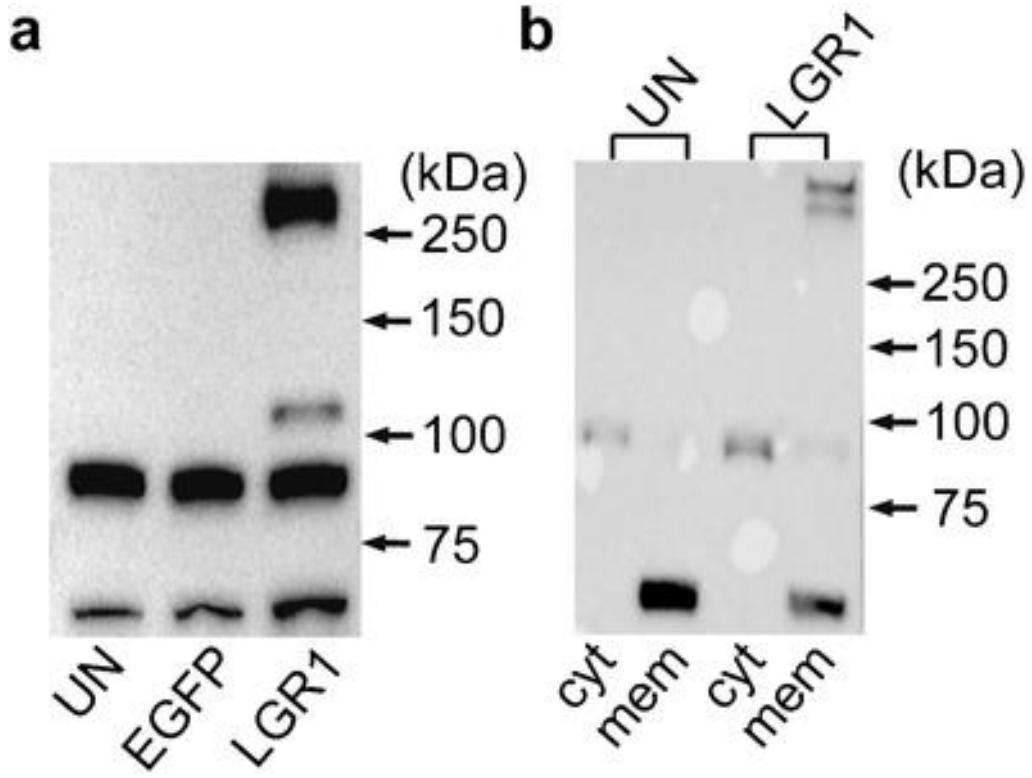

Figure 2-8: Western blot analysis of (**a**) protein isolated from untransfected (*UN*), EGFP-transfected (*EGFP*) and LGR1-transfected (*LGR1*) CHO-K1 cells and (**b**) cytosolic (*cyt*) and membrane-associated (*mem*) protein fractions from untransfected (*UN*) and LGR1-transfected (*LGR1*) CHO-K1 cells. An expected sized band at ~112 kDa, along with high-molecular weight multimers >250 kDa were only observed in lanes containing protein isolations from LGR1-transfected cells (**a**). LGR1-positive bands were likewise observed in isolated membrane protein fractions but not in cytosolic protein fractions (**b**)

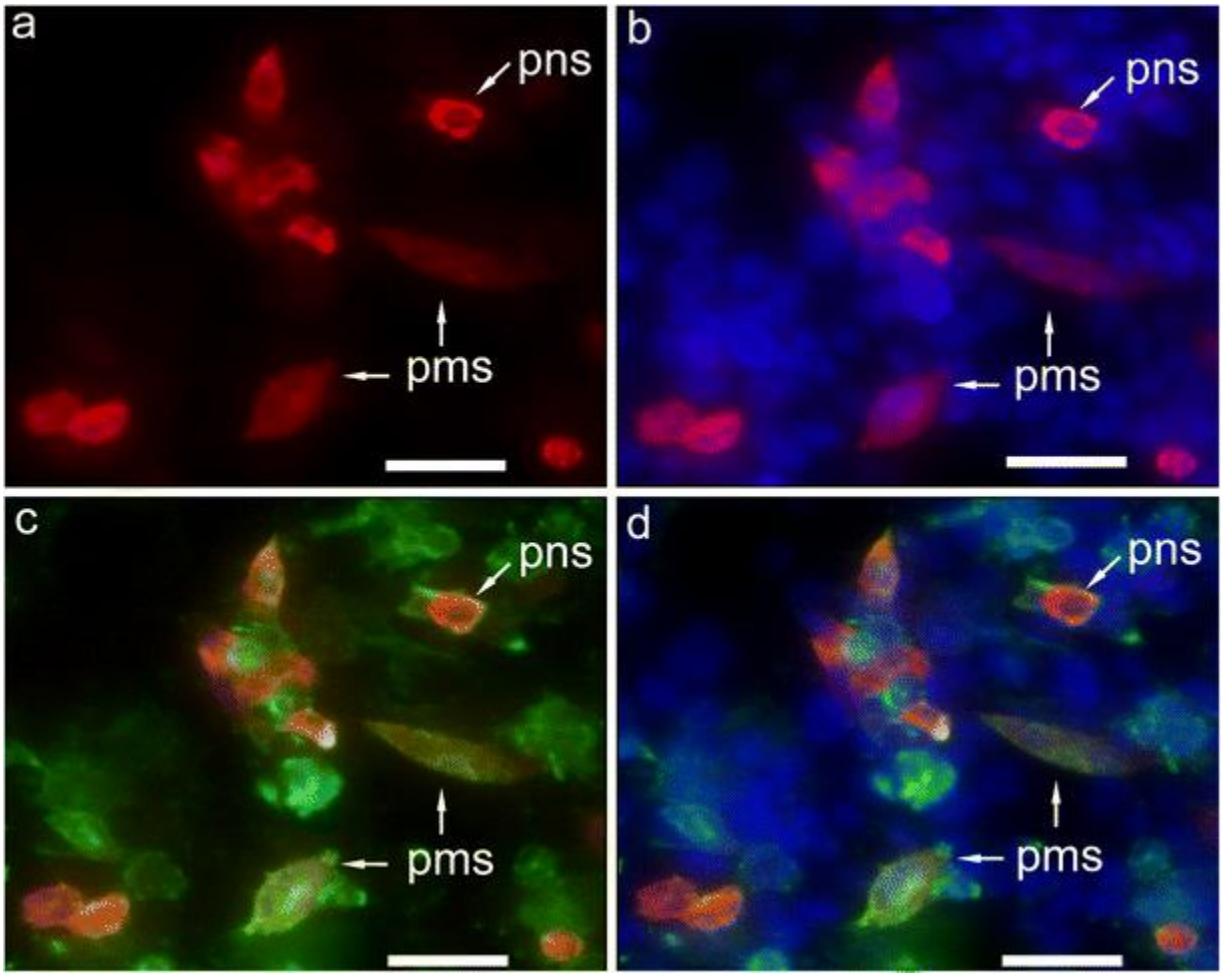

Figure 2-9: Immunocytochemical detection of LGR1-like staining (*red*), DAPI (*blue*) and α-tubulin (*green*) in transiently transfected CHO-K1 cells. Strong LGR1-like perinuclear staining (*pns*) was observed in transiently transfected CHO-K1 cells and was also noted in the plasma membrane (*pms*) of some cells. Co-localization with α-tubulin, used as a cytosolic marker, was not observed (**c**). *Scale bars* 50 μm

2.4.4 Western blot and immunocytochemical analyses of stably transfected HEK 293T cells

Western blot experiments using an ectodomain-targeted custom antibody to detect *A. aegypti* LGR1 expression in transiently transfected CHO-K1 cells indicated the antibody was not ideal for use under denaturing conditions. Although the custom antibody-recognized LGR1 monomeric expression (Fig. 2-8a) and high-molecular-weight multimers associated with the LGR1 protein, other non-specific products were also recognized (Fig. 2-8a, b). Given that this sub-optimal detection in western blots could be due to a combination of variables (i.e., inappopriate binding of the custom antibody, the type of cell line and/or the transient transfection system), to improve detection of LGR1 monomers and minimize non-specific product detection, we created a HEK 293T cell line stably expressing an LGR1–EGFP fusion protein. Moreover, the fusion protein was used to better characterize properties of the LGR1 protein under denaturing conditions using an EGFP-specific antibody and also to confirm if EGFP expression co-localized with LGR1-like staining detected in immunocytochemistry.

Immunocytochemistry was performed on either untransfected (negative control) or HEK 293T cells stably expressing LGR1–EGFP (Fig. 2-10). EGFP fluorescence was then compared to immunoreactive staining revealed with the *A. aegypti* LGR1 custom antibody to verify its specificity. Results indicate EGFP fluorescence (Fig. 2-10a–c) strictly co-localizes with *A. aegypti* LGR1-like immunoreactivity detected in the membrane of cells stably expressing the LGR1–EGFP fusion protein (Fig. 2-10b, c). Importantly, detection of LGR1 immunoreactivity was abolished when the custom antibody was pre-incubated with the LGR1 antigen sequence (Fig. 2-10d), though EGFP expression remained (Fig. 2-10e). No EGFP fluorescence or LGR1-like staining was observed in untransfected HEK 293T control cells (Fig. 2-10f).

To verify that EGFP fluorescence served as a sufficient marker for LGR1 expression in mammalian cell lines, we used a GFP-targeted antibody and sought a predicted band size of 132 kDa in molecular weight (LGR1: 105 kDa+EGFP: 27 kDa). Results showed that, regardless of the transient or stable transfection system used with HEK 293T cells, a band migrating slightly higher (~139 kDa) than the monomeric form of the fusion construct was intensely detected (Fig. 2-11a). Moreover, no detection of any immunopositive protein band was observed in untransfected negative control cells (Fig. 2-11b). Under transient transfection conditions, a second band associated with a multimeric form of the fusion protein (>250 kDa) was observed, which was not present in cells stably expressing the LGR1–EGFP fusion protein (Fig. 2-11a). As a result, since expression in the HEK 293T stable cell line was improved compared to transient expression of LGR1–EGFP, several colonies of stably expressing HEK 293T cells were examined for the greatest LGR1–EGFP expression. Detection of a measured 139 kDa monomeric band was observed in all LGR1–EGFP stably expressing cell lines (Fig. 2-11b) and was absent in protein isolated from untransfected (control) HEK 293T cells. As select stable cell lines appeared to better express the LGR1–EGFP fusion protein (e.g., stable line 3 had the strongest expression) (Fig. 2-11b), this stable line was selected for subsequent experiments. Similar to studies in CHO-K1 cells transiently expressing *A. aegypti* LGR1 (Fig. 2-8b), the LGR1–EGFP fusion protein is membrane associated and is not present in cytosolic fractions of stably expressing cells and is also absent in cytosolic and membrane fractions of untransfected cells (Fig. 2-11c). Moreover, upon deglycosylation treatments with PNGase F, the monomeric 139 kDa band migrated at a lower molecular weight decreasing by 7 kDa where it matches the predicted molecular size of the LGR1–EGFP fusion construct at 132 kDa (Fig. 2-11d).

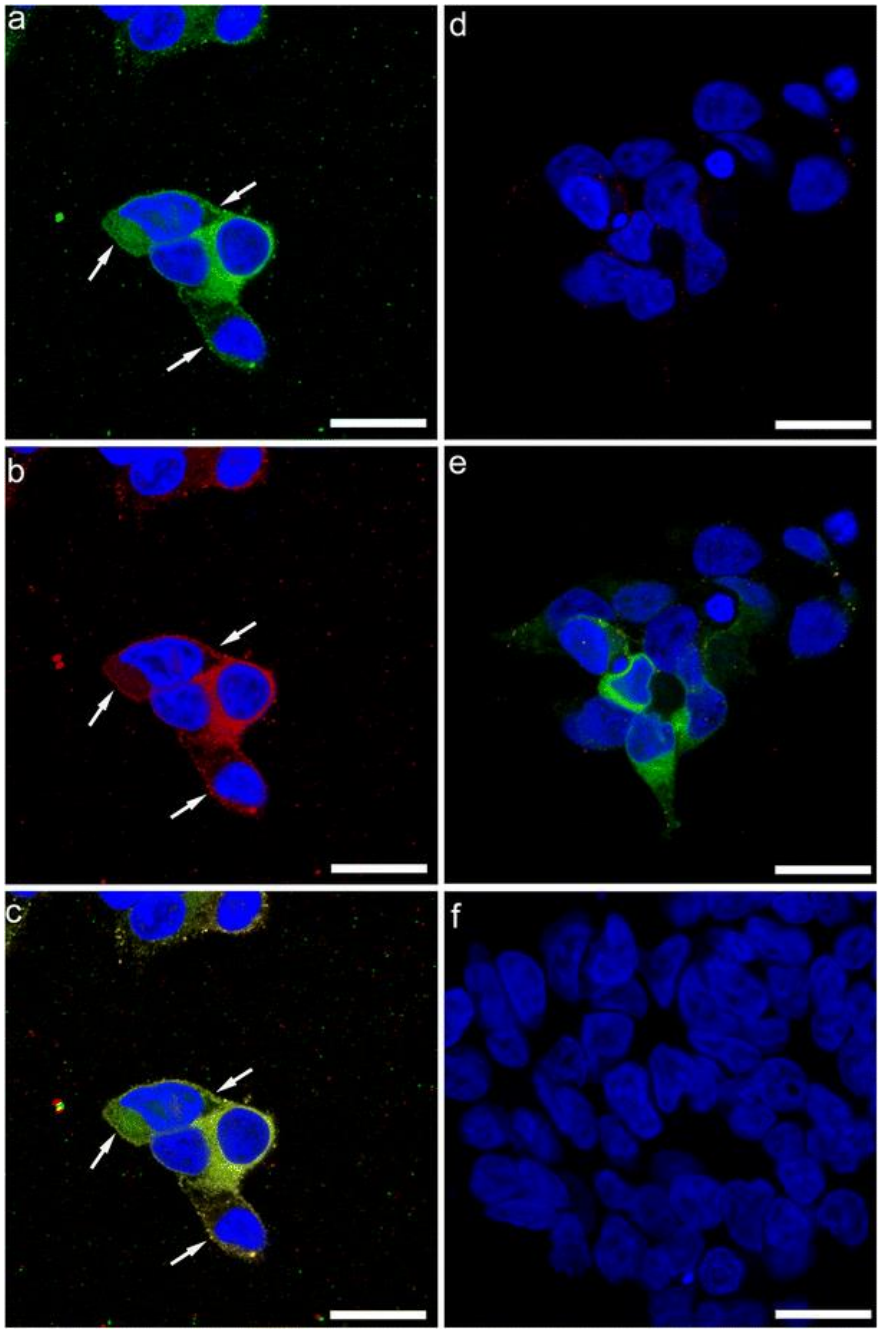

Figure 2-10: Immunocytochemical detection of LGR1-like staining (*red*), DAPI (*blue*) and EGFP (*green*) in LGR1–EGFP stably transfected and untransfected HEK 293T cells. EGFP fluorescence (**a**) and LGR1-like immunoreactivity (**b**) strictly co-localize in plasma membranes (indicated by *arrows*) of stably transfected HEK 293T cells (**c**). LGR1-like staining is abolished when the LGR1-antibody is preabsorbed with LGR1 synthetic antigen (**d**) though EGFP fluorescence remains (**e**) in stably expressing cells. EGFP as well as LGR1-like immunofluorescence is absent in untransfected control cells (**f**). *Scale bars* 50 μm

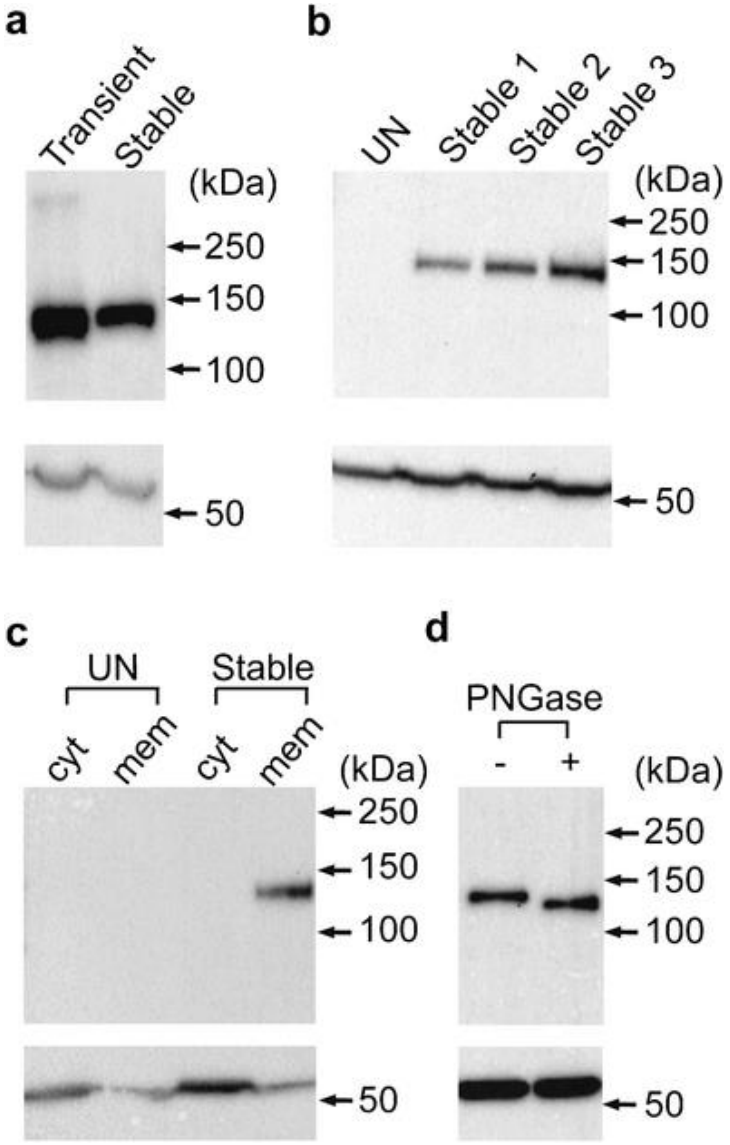

Figure 2-11: Western blot analysis of protein isolated from (**a**) LGR1–EGFP transiently (transient) and stably (stable) transfected HEK 293T cells, (**b**) untransfected (UN) HEK 293T cells or three different stable cell lines (Stable 1–3), (**c**) cytosolic (*cyt*) and membrane-associated (*mem*) protein fractions from untransfected or stably transfected cells and (**d**) stably transfected cells treated with PNGase. A band size slightly higher than the predicted molecular weight of LGR1–EGFP (132 kDa) at 139 kDa was detected in both transiently and stably transfected cells (**a**) but was absent in untransfected cells (**b**). LGR1 multimers (>250 kDa) were observed in transiently transfected HEK 293T cells (**a**) but were absent in all stable lines tested (**a**, **b**). Since stable line 3 exhibited the highest expression of LGR1–EGFP (**b**), it was chosen for cell fractionation (**c**) and de-glycosylation treatments (**d**). LGR1–EGFP monomer band is present in membrane-protein fractions but is not present in cytosolic protein fractions of stably transfected cells, or any fractions from untransfected cells (**c**). Upon treatment with PNGase, the 139-kDa monomer correlating to the LGR1–EGFP monomer migrates as a lower molecular weight band matching the predicted molecular weight at 132 kDa (**d**)

2.5 Discussion

The GPA2/GPB5 glycoprotein hormone signalling system evolved prior to the emergence of bilateral metazoans (Hauser et al. 1998; Vibede et al. 1998), indicating that it may be of physiological relevance to many organisms, including invertebrates such as mosquitoes. However, contrary to the classic glycoprotein hormone relatives that include the gonadotropins and TSH, the physiological role of GPA2/GPB5 in both vertebrates and invertebrates remains unclear. In order to begin elucidating the function of GPA2/GPB5 in the mosquito *A. aegypti*, we characterized the tissue and sex-specific distribution patterns of LGR1 expression. To our knowledge, this is the first study conducted on an invertebrate model to examine protein-level expression and characterization of the GPA2/GPB5 receptor, LGR1. Through the use of RT-qPCR, immunocytochemistry, immunohistochemistry and immunoblot techniques, we found intriguing patterns of LGR1expression with regards to tissue distribution and cellular localization as well as post-translational processing.

2.5.1 Spatial and sex-specific distribution patterns of LGR1

Since previous research had not examined transcript or protein-level expression profiles of LGR1 in the anterior midgut, diverticula and reproductive tissues of *A. aegypti* (Paluzzi et al. 2014), our results provide new insight into possible functions for GPA2/GPB5. Of the reproductive tissues examined, the ovaries and testes of adult *A. aegypti* exhibited LGR1 transcript expression and strong LGR1-like immunoreactive staining. Interestingly, while LGR1-like immunoreactivity was generally dispersed throughout female ovaries in the previtellogenic-staged mosquitoes studied here, discrete regionalization of LGR1-like expression was observed within distal regions of the adult male testes. In adult mosquitoes, the testes are compartmentalized into different zones of spermatogenesis (Pitts et al. 2014). From the hooked apex, located at the most distal

region of the testes, to the base where it meets the vas efferens, germ cells grow, mature and transform into spermatozoa (Hodapp, CJ and Jones 1961; Pitts et al. 2014). LGR1-like protein expression was observed in zones where germ cells undergo mitotic growth (Pitts et al. 2014) (Fig. 2-3b) indicating that LGR1, presumably under activation by GPA2/GPB5, may play a role during the mitotic divisions of germ cells in the adult mosquito testes. Comparatively, LGR1-like expression was not compartmentalized in the ovaries of adult female *A. aegypti*, though LGR1-like staining was clearly evident relative to controls. Most species of mosquitoes like *A. aegypti* are anautogenic, whereby a blood meal from a vertebrate host is required to initiate the process of egg development or oogenesis (Clements 2000). In adult females, previtellogenesis is the first stage of egg development that begins at the onset of adult emergence and ends when a blood meal is acquired. During this reproductive stage, hormones such as juvenile hormone, ovary ecdysteroidogenic hormone and insulin-like peptides help mosquitoes become competent to respond to a blood meal and initiate vitellogenesis, characterized by a mass production of nutrients that are sequestered by developing oocytes to ensure proper egg development (Sappington et al. 1996). Together, these hormones facilitate the production of ecdysone in the ovaries (Brown et al. 1998, 2008; Dhara et al. 2013) and yolk protein production in the fat body (Raikhel and Lea 1983, 1990; Matsumoto et al. 1989) and at the level of the follicles, initiate the onset of oocyte growth and follicular epithelial differentiation (Gwadz RW and Spielman A 1973; Raikhel and Lea 1985). Given that LGR1-like staining was observed in follicular epithelia and nurse cells of non-blood fed mosquitoes, it suggests GPA2/GPB5 acting through its receptor (LGR1) may be involved in physiological processes related to previtellogenesis. Similar patterns of immunohistochemical staining in follicular epithelia and nurse cells were observed with the mosquito insulin receptor, a known regulator of ecdysone production in adult mosquitoes

(Brown et al. 1998; Helbling and Graf 1998). As a result, future experiments will need to examine whether LGR1 expression changes over the course of a blood meal and further delineate whether this glycoprotein hormone signalling system is involved in regulating previtellogenic processes such as ecdysteroidogenesis.

2.5.2 The alimentary canal: Foregut, midgut, hindgut and Malpighian tubules

LGR1 mRNA and protein-like immunoreactivity was observed in most regions of the alimentary canal; one notable exception being the paired dorsolateral diverticula of adult mosquitoes where little to no LGR1-like immunoreactivity was observed. Considering LGR1 transcripts are enriched in adult midguts, hindguts and Malpighian tubules (Paluzzi et al. 2014), our results confirm LGR1 protein expression based on the localization of LGR1-like immunoreactivity identified in these tissues. Moreover, our findings present new information regarding the expression of the LGR1 transcript and protein-level distribution in mosquito epithelia that had not previously been examined, including the anterior midgut and diverticula (Figs. 2-1, 2-5a, b). Consistent with previous studies (Paluzzi et al. 2014; Vandersmissen et al. 2014), our data suggest a sex-specific role for GPA2/GPB5 in invertebrates, given that LGR1 transcript is more abundant in adult females compared to males. However, although transcript data showed sex-specific differences, our immunohistochemical results examining LGR1 protein distribution did not reveal significant differences between sexes.

GPA2/GPB5 may have an ionoregulatory function in adult *A. aegypti* since LGR1 transcript was found to be upregulated in the hindgut. In support of this possibility, recombinant GPA2/GPB5 modulates ion transport across specific regions of the hindgut, including the posterior ileum and rectum (Paluzzi et al. 2014). Studies in the fruit fly *D. melanogaster* have

likewise supported a ionoregulatory function for GPA2/GPB5 as the receptor is expressed in osmoregulatory epithelia (Sellami et al. 2011) and also a developmental role, since LGR1 knockdown led to developmental abnormalities and increased susceptibility to desiccation stress (Vandersmissen et al. 2014). Further, in a behavioral screen to examine genes influencing water consumption, knockdown of the GPA2/GPB5 receptor (LGR1) in *D. melanogaster* led to a substantial increase in water consumption (Jourjine et al. 2016), indicating a central role related to organismal water and ion homeostasis. In accordance with previous findings examining transcript expression profiles, LGR1 transcript and LGR1-like immunoreactivity is widespread across gut epithelia, supporting a role in hydromineral balance for GPA2/GPB5. For example, LGR1 mRNA and immunoreactivity was observed in the ventral diverticulum, which suggests an ionoregulatory function during sucrose-engorgement by adult male and female *A. aegypti* since sucrose is deposited into the diverticula, in contrast to the passage of blood that is directly deposited into the midgut during bloodmeal engorgement by adult females (MacGregor 1930, 1931; Bishop and Gilchrist 1946; Day 1954; Christophers 1960).

2.5.3 Unraveling epithelial versus muscle tissue distribution

The alimentary canal of adult mosquitoes is analogous to a simple epithelial tube surrounded by layers of circular and longitudinal smooth muscle that collectively regulate physiological processes like digestion and excretion (Phillips et al. 1988). Previous studies on the GPA2/GPB5 receptor, LGR1, quantified transcript abundance in tissues of *D. melanogaster* and *A. aegypti* without differentiating whether the receptor is present in epithelial and/or muscle tissues (Sellami et al. 2011; Paluzzi et al. 2014; Vandersmissen et al. 2014). Thus, to determine if LGR1 is expressed in either epithelial tissue, muscle tissue or both, our study utilized immunolocalization

techniques to identify LGR1-like expression patterns in comparison to the distribution of muscle-enriched F-actin that was detected using phalloidin. Our results identified distinct patterns of longitudinal and circular smooth muscle arrays in the hindgut, midgut and diverticula of the adult male and female alimentary canal but that lacked any co-localization with LGR1-like immunoreactivity. Additionally, tissues such as the Malpighian tubules and the male testes, which lack associated muscle tissue in *A. aegypti* (Hodapp, CJ and Jones 1961; Clements 2000), still showed obvious LGR1-like immunoreactivity. In adult female *A. aegypti*, the ovaries comprise two types of membranes; an ovarian membrane that coats the entire ovary and an ovariolar membrane that encloses the ovarioles (Clements 2000). The ovariolar membrane and the central chamber (termed the calyx), which medially penetrate the ovaries, are well-tracheated and covered by a muscle sheath (Nicholson 1921; Clements 2000). Some co-localization of phalloidin-stained muscle with LGR1-like staining was noted in the medial portions of the ovaries; however, this may be attributed to the close association of muscle fibers with the ovariolar membrane and calyx.

2.5.4 Cellular distribution patterns of LGR1

Since hormones are released into circulation prior to acting on their target tissues, we anticipated LGR1-like staining would be localized to basolateral surfaces of gut epithelia. To determine the cellular distribution of LGR1 in different tissues, LGR1-like immunoreactivity was compared to the distribution of NKA, which has strict localization to basolateral membrane surfaces in *A. aegypti* gut epithelia (Patrick et al. 2006). Relative to the distribution of NKA immunoreactivity, optical sections of wholemounts from male and female *A. aegypti* tissues show LGR1-like staining is present on basolateral membranes as well as intracellular sites of gut epithelia.

Interestingly, intracellular LGR1-like immunoreactivity appeared as defined punctate staining that may reflect LGR1-like vesicular trafficking, which is possible given heterologous expression in mammalian cells confirmed LGR1 is membrane-associated.

The predicted molecular weight of *A. aegypti* LGR1 is ~105 kDa (Paluzzi et al. 2014) and bands at 112 kDa, along with high-molecular-weight multimers, were present in immunoblots examining total protein isolated from LGR1-transiently transfected CHO-K1 cells. Though the monomeric 112-kDa band size does not exactly match the predicted molecular weight of LGR1 at ~105 kDa, the higher migration pattern could be due to glycosylation since three glycosylation sites are predicted in the *A. aegypti* protein sequence (Paluzzi et al. 2014), in combination with improper folding, post-translational modifications and self-association of the protein under the transient transfection system (Mizrachi and Segaloff 2004; Latif et al. 2010). In addition, high-molecular-weight multimers >250 kDa corresponding to LGR1 could be due to CHO-K1 cells transiently over expressing the protein at very high levels in a subset of cells, which could lead to mis-localization, improper processing and intracellular retention (Tao et al. 2004).

As previous studies on LGR1-related receptors found that establishment of a stable cell line would improve receptor expression towards more physiologically relevant levels (Mizrachi and Segaloff 2004; Latif et al. 2010), we aimed to address this challenge with the *A. aegypti* LGR1 receptor. The mislocalization of LGR1 protein and its particularly high expression levels in perinuclear regions suggest that the transient expression of LGR1 may have saturated the secretory capacity of the CHO-K1 cells (Hobman et al. 1997). This may be due to accelerated protein synthesis associated with the transient transfection system, which may inundate protein processing machinery, leading to protein misfolding and defects in secretory pathway traffic

(Sadeghi et al. 1997). Therefore, the high level of LGR1-like staining from immunocytochemical results, as well as the receptor multimerization routinely detected in western blot assays could be explained by aggregation of immature LGR1 proteins from either self-association or coupling to other proteins. Furthermore, retention of LGR1 protein in the endoplasmic reticulum may be a result of chaperone malfunction (Hebert and Molinari 2007). Such perinuclear receptor localization and multimerization has been observed in earlier studies, particularly of the human lutropin receptor (hLHR) (Tao et al. 2004). The difference in hLHR localization in transiently versus stably expressing mammalian cells was striking. Cells transiently expressing hLHR yielded mostly aggregated immature forms associated with the endoplasmic reticulum, whereas the majority of stably expressed hLHR was found to be mature and localized exclusively on the plasma membrane in either dimerized or oligomerized forms (Tao et al. 2004). Moreover, western blot analyses of hLHRs revealed the presence of a double band at high-molecular-weight sizes, which was correlated with the aggregated immature form of hLHRs in the endoplasmic reticulum during transient expression. In contrast, the dimeric form of protein at lower molecular weight was observed in a stable hLHR expressing cell line (Tao et al. 2004). This likely explains the western blot results using protein isolated from transiently expressing CHO-K1 cells that detected intense multimer bands >250 kDa as well as the monomer at 112 kDa. The mature form of LGR1 may be more prevalent in a stable cell line and LGR1 multimerization could be lessened while improving detection of monomeric form. Taken together, just as the hLHR belongs to the same GPCR superfamily as LGR′s, *A. aegypti* LGR1 is presumably highly localized to the endoplasmic reticulum in multimeric forms in transiently transfected cells.

With the aim of minimizing receptor multimerization and improving detection of LGR1 monomers, we implemented an alternate heterologous system using HEK 293T cells that were

found to be optimal for stable expression of LGR1. The construct for creating the stable cell line *involved A. aegypti* LGR1 fused to EGFP, which was used as an indirect, fluorescent marker for receptor expression localization. The predicted molecular weight of the LGR1–EGFP fusion construct is ~132 kDa (LGR1: ~105 kDa+EGFP: 27 kDa). Due to the fact that an EGFP-targeted antibody recognized a single band at 139 kDa, which was very close to the calculated mass of the fusion protein, EGFP proved to be a useful marker for further characterizing *A aegypti* LGR1 expression.

Immunocytochemical results confirmed LGR1-like immunoreactivity co-localized with GFP fluorescence in HEK 293T stable cell lines. Moreover, detection was abolished when the LGR1 antibody was pre-incubated with its synthetic antigen, which is consistent with observations in the immunohistochemistry studies on *A. aegypti* gut and reproductive tissues. As a result, it is reasonably concluded that the custom antibody is indeed suitable for detection of *A. aegypti* LGR1 expression in immunocytochemical and immunohistochemical techniques. For western analysis, however, the LGR1 antibody is suboptimal. While a suitable-sized band correlating to the LGR1 protein was recognized (112 kDa) in LGR1-transfected cells, non-specific bands were also detected in all cell isolates.

In accordance with previous studies on LGRs (Mizrachi and Segaloff 2004; Latif et al. 2010), stable expression in a heterologous system improved LGR1 expression compared to transient transfection conditions. A stable cell line expressing LGR1–EGFP demonstrated a dramatic decrease in receptor multimerization (>250 kDa), while detection of monomeric mature forms (~139 kDa) was improved. This could be due to the expression of LGR1 at more physiological levels in stably expressing cell lines (Mizrachi and Segaloff 2004; Latif et al. 2010). LGR1–EGFP monomers were only detected in membrane-associated fractions of stably

expressing HEK 293T cells but absent in all samples collected from untransfected HEK 293T cells as well as cytosolic fractions of stably expressing cells. Therefore, consistent with experiments using the *A. aegypti* LGR1 antibody, our results confirm LGR1 as being membrane-associated.

Lastly, to examine whether the slightly higher than calculated migration pattern of LGR1 monomers could be due to post-translational processing at predicted glycosylation sites on the LGR1 protein sequence (Paluzzi et al. 2014), deglycosylation treatments were performed. Results showed a decrease in band size from 139 kDa to the predicted band size of the fusion protein at 132 kDa. Similarly, small-molecular-weight changes upon deglycosylation techniques have been observed with other glycoprotein hormone receptors, such as the human TSH receptor (Oda et al. 1999), characterized by three absolutely conserved glycosylation sites across a number of vertebrate species (Núñez Miguel et al. 2017). Future studies will examine whether glycosylation of LGR1 is required for normal membrane expression, functional activation and/or required for ligand (GPA2/GPB5) binding.

2.5.5 Concluding remarks

To our knowledge, this study is the first to comprehensively examine the GPA2/GPB5 receptor, LGR1, at the transcript and protein level in a non-vertebrate organism. Observed results support an ionoregulatory and osmoregulatory function for GPA2/GPB5 as suggested previously (Sellami et al. 2011; Paluzzi et al. 2014; Vandersmissen et al. 2014; Jourjine et al. 2016; Rocco and Paluzzi 2016), since LGR1 transcript and LGR1-like immunoreactivity was localized to gut epithelial tissues that function in coordinating ion and water homeostasis. Moreover, our results also indicate that the heterodimeric glycoprotein hormone, GPA2/GPB5, may play a

reproductive function since LGR1 transcript and LGR1-like immunoreactivity was found in regions of the testes of adult male and follicular epithelium and nurse cells in the ovary of previtellogenic-staged adult female mosquitoes. Transcriptional expression analysis examining LGR1 tissue distribution supports a sex-specific role for GPA2/GPB5; however, our immunohistochemical mapping of the receptor at the protein level did not reveal obvious differences in immunoreactive staining between sexes. Finally, future experiments should verify whether receptor transcript abundance correlates with protein abundance in different life stages of *A. aegypti,* in order to confirm whether GPA2/GPB5 holds a developmental role in mosquitoes as suggested in the fruit fly (Vandersmissen et al. 2014). Thyrostimulin, the mammalian homolog of GPA2/GPB5, is believed to play more than one physiological role in vertebrates. Comparatively, it is plausible that GPA2/GPB5 may also be pleiotropic in mosquitoes as our results identified multiple prospective tissue targets that supports the possibility of multiple physiological roles.

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CHAPTER 3:

GLYCOPROTEIN HORMONE RECEPTOR KNOCKDOWN LEADS TO REDUCED REPRODUCTIVE SUCCESS IN MALE *AEDES AEGYPTI*

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3.1 Summary

Glycoprotein hormone receptors mediate a diverse range of physiological functions in vertebrate and invertebrate organisms. The heterodimeric glycoprotein hormone GPA2/GPB5 and its receptor LGR1, constitute a recently discovered invertebrate neuroendocrine signalling system that remains to be functionally characterized. We previously reported that LGR1 is expressed in the testes of adult *Aedes aegypti* mosquitoes, where its immunoreactivity is particularly regionalized. Here, we show that LGR1 immunoreactivity is associated with the centriole adjunct of spermatids and is observed transiently during spermatogenesis in mosquitoes, where it may act to mediate the regulation of flagellar development. RNA interference to downregulate LGR1 expression was accomplished by feeding mosquito larvae with bacteria that produced LGR1-specific dsRNA, which led to defects in spermatozoa, characterized with shortened flagella. LGR1 knockdown mosquitoes also retained ~60% less spermatozoa in reproductive organs and demonstrated reduced fertility compared to controls. To date, the endocrine regulation of spermatogenesis in mosquitoes remains an understudied research area. The distribution of LGR1 and detrimental effects of its knockdown on spermatogenesis in *A. aegypti* indicates that this heterodimeric glycoprotein hormone signalling system contributes significantly to the regulation of male reproductive biology in this important disease-vector.

3.2 Introduction

The mosquito *Aedes aegypti* serves as a vector for a variety of pathogens causing diseases including Zika, yellow fever, chikungunya and dengue virus, the latter of which is the most widespread arbovirus disease in humans infecting nearly 400 million people annually (Bhatt et al. 2013). Currently, promising control strategies that aim to curb viral transmission by mosquitoes target their reproductive physiology. For example, the classical sterile insect technique (SIT), which involves the mass production and release of sterilized lab-reared male insects into field populations, has proven effective in the elimination of the New World screwworm *Cochliomyia hominivorax* from the southern United States, Central America, Mexico and Panama, and has had success for a number of mosquito species (Benedict and Robinson 2003; Wyss 2006; Robinson et al. 2009; Oliva et al. 2014). A second promising control strategy to reduce disease transmission by mosquitoes involves introducing the bacterium *Wolbachia* to mosquito populations, which renders the sperm of infected males incapable of fertilization (LePage and Bordenstein 2013).

Control methods that target reproduction in disease vectors can be improved with a comprehensive knowledge of their reproductive biology, including its regulation. In *A. aegypti*, spermatogenesis is asynchronous and occurs in a pair of testes that are not always the same size nor in the same state of development (Hutcheson 1972). Each testis resembles a follicle organized into different compartments called cysts, characterized with an external wall that envelopes numerous germ cells developing in a synchronous fashion (Wandall 1986; Clements 2000). From the apex to the base of the testis, cysts progressively increase in size and maturity so that smaller compartments with undifferentiated spermatogonia are found on distal ends, whereas larger cysts containing mature spermatozoa are located at the base (Wandall 1986; Clements

2000). As maturation proceeds in the testis, spermatogonia proliferate and differentiate into spermatocytes, which undergo two meiotic divisions to produce spermatids and finally spermatozoa; which are then transported from each testis, via the vas deferens, to a common storage unit called the seminal vesicle. Upon copulation with a female, the seminal vesicle is emptied, and a new wave of spermatogenesis occurs to ensure that a continuous supply of sperm is made available for a subsequent mating (Clements 2000). While much is known regarding the regulation and control of reproductive processes in female insects including oogenesis, research dedicated to understanding the regulation and control of spermatogenesis by factors including neuropeptides and/or neurohormones remains largely unexplored (Raikhel et al. 2005).

In vertebrates, hormonal regulation of spermatogenesis is governed by the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Pierce and Parsons 1981; Kerr and Sharpe 1985; Stockell Hartree and Renwick 1992; Dierich et al. 1998). FSH, LH, thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG) comprise the classic glycoprotein hormone family, each formed by the heterodimerization of a common alpha subunit and a hormone-specific beta subunit (Pierce and Parsons 1981). A fifth glycoprotein hormone comprised of two distinct subunits (GPA2/GPB5) called thyrostimulin was discovered and, unlike the classic heterodimeric glycoprotein hormones that are restricted to vertebrate species, homologous GPA2/GPB5 subunit genes have been identified in most bilaterians (Hsu et al. 2002; Nakabayashi et al. 2002; Santos et al. 2011; Rocco and Paluzzi 2016). Though the exact physiological role of GPA2/GPB5 remains unclear in any given organism, expression studies of the GPA2/GPB5 receptor indicate it may possess a reproductive function in the rat (Sun et al. 2010), mosquito (Rocco et al. 2017), amphioxus (Tando and Kubokawa 2009) and lamprey (Hausken et al. 2018). In invertebrates, GPA2/GPB5 binds to a receptor called the leucine-rich

repeat-containing G protein-coupled receptor 1 (LGR1), which shares ~50% amino acid sequence homology to the membrane-spanning regions of the classic glycoprotein hormone receptors (Hauser et al. 1998; Nishi et al. 2000; Sudo et al. 2005). In the yellow fever mosquito *A. aegypti*, an investigation on the expression profile of LGR1 revealed transcript expression along with strongly regionalized immunoreactivity in the mosquito testes, suggesting GPA2/GPB5 may be linked to regulating mosquito spermatogenesis (Rocco et al. 2017). In the present study we sought to further delineate the role of LGR1 in *A. aegypti* spermatogenesis by (i) characterizing the distribution of LGR1 expression over the course of spermatogenesis and (ii) using LGR1-targeted RNA interference to examine effects on net spermatozoa output, flagellar length and male fertility.

3.3 Materials and methods

3.3.1 A. aegypti colony rearing

Experimental animals were acquired from colonies raised under conditions described previously(Rocco et al. 2017). To ensure virgin adults, pupae were isolated individually in 25 cm^2 cell culture flasks (Corning, NY, USA) containing 3mL of de-ionized water (dH_2O) and covered with a cotton ball wick soaked in a 10% sucrose solution. Upon emergence, adults were sexed and processed for immunohistochemical and fluorescence *in situ* hybridization experiments.

3.3.2 Wholemount immunohistochemistry

Male reproductive organs (testes, vas deferens and seminal vesicle) and female spermathecae of one-day old virgin adult *A. aegypti* were collected and analyzed for LGR1 expression using immunohistochemical techniques described earlier (Rocco et al. 2017). Primary antibodies included a custom affinity-purified rabbit polyclonal antibody raised against the *A. aegypti* LGR1 protein (0.5 µg ml^{-1}, Genscript Inc., Piscataway, NJ) with specificity validated previously (Rocco et al. 2017), and a mouse monoclonal anti-γ-tubulin antibody (1:10, Sigma-Aldrich, Oakville, ON). Secondary antibodies used included Alexa Fluor 594- or Alexa Fluor 488-coupled goat anti-rabbit Ab (1:200, Invitrogen, Carlsbad, CA), and Alexa Fluor 647-coupled goat anti-mouse Ab (1:200, Invitrogen, Carlsbad, CA). Filamentous actin (F-actin) was visualized using Alexa Fluor 488-conjugated phalloidin (0.165M, Invitrogen, Carlsbad, CA). For visualization of the mitochondrial derivatives associated with spermatozoa, samples were incubated in MitoTracker Red CMXRos (100 nM, Invitrogen, Carlsbad, CA) in nuclease-free phosphate-buffered saline (PBS) at room temperature (RT) for 2 h prior to fixation. DNA was

stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (4 µg mL^{-1}, Sigma-Aldrich, Oakville, ON), and samples were optically sectioned using a Yokogowa CSU-X1 Zeiss Cell Observer Spinning Disk confocal microscope. Images were processed using Zeiss Zen and ImageJ software.

3.3.3 Fluorescence in situ hybridization

Target cDNA fragments were amplified from previously prepared constructs that contained the *A. aegypti* LGR1 open reading frame (Paluzzi et al. 2014) using gene-specific primers (Table 3-S1). PCR amplicons were ligated to pGEM-T-Easy vector (Promega, Madison, WI) and transformed into NEB 5-alpha high efficiency competent *E. coli* (New England Biolabs, Whitby, ON). The T7 promoter sequence was incorporated onto the 5' end of the sense and anti-sense strands using modified primers (see Table 3-S1), and amplification was carried out using the T7 promoter oligonucleotide and either anti-sense or sense gene-specific primers to create anti-sense or sense strand template cDNA. Base accuracy was verified by Sanger sequencing (The Centre for Applied Genomics, Sick Kids Hospital, Toronto, ON, Canada), and cDNA templates were used to generate *in vitro* transcribed digoxigenin (DIG)-labelled sense and anti-sense RNA probes using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Whitby, ON) and replacing the provided nucleotides with DIG RNA Labelling Mix (Sigma-Aldrich, Oakville, ON). After overnight synthesis at 37 °C, RNA probes were treated with DNAse I (New England Biolabs, Whitby, ON), electrophoresed on a 1% non-denaturing agarose gel to confirm probe integrity and DNA template removal, and subsequently quantified. The localization of LGR1 transcript in the testes of *A. aegypti* was determined with fluorescence *in situ* hybridization (FISH) following a previously reported protocol (Paluzzi et al. 2008), using 10 ng

Table 3-S1. Primers utilized for RNA probe template generation, bacteria-mediated RNA interference dsRNA target design, and quantitative PCR analysis of LGR1 in *Aedes aegypti* (Genbank accession number: KF711859). Positions of oligonucleotides refer to the LGR1 full open reading frame as reported previously (Paluzzi et al. 2014)

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Oligonucleotide Name:	Oligonucleotide Sequence:	Product size (bp)	Nucleotide position (exons, base number):	Application:
LGR1-F	TGTTAAGTGCTACCCGATGC	610	11-13, 1608-2218	RNA probe generation for fluorescence *in situ* hybridisation
LGR1-R	TGACGATGATGATGAAGGCC			
T7 Promoter	TAATACGACTCACTATAG			
LGR1-F	ATGCGAGGACGTAATGGGAT	735	11-13, 1644-2378	dsRNA Target Sequence for bacteria-mediated RNA interference
LGR1-R	ATGGCTGTCAGTCCGAAGAA			
LGR1-F	GCCGGTTGCGTATCTTTTC	290	9-11, 1319-1608	Quantitative PCR
LGR1-R	ATCAAATGTTGTGGGCGTAAG			
rp49-F	ACAAGCTTGCCCCCAAC	214		
rp49-R	GCGATTTCGGCACAGTAGA			

ul^{-1} sense and anti-sense DIG-labelled RNA probes. Modifications to the protocol reported previously included fluorescent detection with Alexa Fluor 488 tyramide dye diluted (1:100) in amplification buffer (Life Technologies, Eugene, OR) containing 0.0015% H_2O_2 and optical sections were performed using a Yokogowa CSU-X1 Zeiss Cell Observer Spinning Disk confocal microscope. Images were processed using Zeiss Zen and ImageJ software.

3.3.4 Transmission electron microscopy: Upon dissecting *A. aegypti* testes in PBS, tissue samples were processed for conventional methods of transmission electron microscopy as previously described (Scudeler et al. 2017). Briefly, tissues were fixed overnight in a 2.5% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) and post-fixed for 2 h in 1% osmium tetroxide. Tissue samples were contrasted with 0.5% uranyl acetate for 2 h, dehydrated in a graded acetone series (50%, 70%, 90% and 100%), and embedded in Araldite resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and analyzed using a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, Netherlands).

3.3.5 Bacteria-mediated RNA interference

Double-stranded RNA (dsRNA) target cDNA sequences for *A. aegypti* LGR1 were amplified using primers listed (Table 3-S1) using a previous plasmid construct containing the LGR1 open reading frame (Rocco et al. 2017). Primers amplified a select region in the N-terminal ectodomain region (Paluzzi et al. 2014), which did not span the region containing the antigen site for the custom LGR1 antibody used in immunohistochemical experiments nor did it overlap with the primer-binding sites targeted in quantitative RT-PCR (Rocco et al. 2017). The amplified

dsRNA template sequence was then cloned into the pGEM-T-Easy sequencing vector (Promega, Madison, WI) and subcloned into the dsRNA-producing plasmid L4440 (Fire et al. 1998; Montgomery et al. 1998), which was a gift from Andrew Fire (Addgene plasmid # 1654). L4440 plasmids containing the LGR1 target sequence or an empty vector (used as a control) was then transformed into RNAse III-deficient HT115 (DE3) *Escherichia coli* bacterial strain provided by the *Caenorhabditis* Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). HT115 (DE3) *E. coli* containing the L4440 plasmid with or without the LGR1 target sequence were grown in 5 mL LB media supplemented with ampicillin (100 µg ml^{-1}) and tetracycline (10 µg ml^{-1}) (LB-Amp-Tet). Overnight cultures were then used to inoculate 100 mL of pre-warmed LB-Amp-Tet with shaking at 37 °C and bacterial growth was measured hourly using a Synergy 2 Multi-Mode Microplate Reader (BioTek) until an OD_{595} of 0.7-0.8 was reached. Bacterial cultures were then induced with 0.4 mM ispropyl β-D-1-thiogalactopyranoside (IPTG) to produce dsRNA for 4 h, shaking at 37 °C. After IPTG induction, cultures were centrifuged at 5000 rpm for 10 minutes, and bacterial pellets were resuspended in 1 mL LB media (without antibiotics) and stored overnight at −20 °C. The next day, bacteria were thawed, mixed with an equal volume of larval feed (2% liver powder: brewers yeast mixture, 1:1) and used to feed 2nd instar *A. aegypti* larvae which had been starved overnight. Following 48 hours of exposure to control bacteria or LGR1 dsRNA-expressing bacteria, larval baths were changed with clean dH_2O and larvae were fed normal larval feed until pupation. Pupae were kept isolated to ensure emerging adults were virgin for future experiments.

3.3.6 Evaluation of LGR1 knockdown efficiency

RT-qPCR

Total RNA was isolated from 4th instar larvae and one-day old virgin adult *A. aegypti* that were fed on *E. coli* expressing control dsRNA (empty L4440) or LGR1 dsRNA using the Monarch Total RNA Miniprep Kit (New England Biolabs, Whitby, ON) following manufacturer's instructions that included an on-column DNAse I treatment to remove residual genomic DNA. Total RNA (50 ng) was then used as template for cDNA synthesis using the iScript™ Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada) following recommended guidelines and diluted ten-fold prior to quantitative RT-PCR. LGR1 transcript levels were then quantified using PowerUP™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) and measured on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) following conditions described previously (Gondalia et al. 2016). Primers used to quantify LGR1 transcript abundance overlap exon-exon boundaries (Table 3-S1), and data was normalized to the stably expressed reference gene ribosomal protein 49 (GenBank accession: AY539746) according to the ΔΔCt method (Paluzzi et al. 2014). Measurements were taken from a minimum of three independent rounds of bacterial feedings, each consisting of 50 control or dsLGR1-treated larvae, to serve as biological replicates for each developmental stage.

Immunofluorescence

LGR1 immunoreactive staining intensity was compared between testes, particularly in cysts containing late-staged spermatids, of one-day old virgin *A. aegypti* that were either fed on *E. coli* expressing control dsRNA (empty L4440) or LGR1 dsRNA. All microscope settings were identical when acquiring images of control and dsLGR1-treated testes and saturation was

avoided by setting appropriate acquisition parameters as recommended to improve the accuracy and precision of fluorescence microscopy-based quantitative measurements (Waters 2009). Using ImageJ software, immunofluorescent images were converted to 8-bit grayscale images, over an intensity scale of 0-255, and the mean gray value from cysts within the testes containing LGR1 immunoreactivity was recorded and compared between treatments. Measurements of LGR1 staining intensity compared isolated control or dsLGR1-treated testes (n = 11-13) that served as independent biological replicates. Testes of adult mosquitoes collected for analysis were gathered from multiple batches of bacterially-fed larvae.

3.3.7 Quantitation of sperm and flagella length

Sperm quantitation was performed following a protocol that was previously shown to obtain accurate sperm counts (Ponlawat and Harrington 2007). Male reproductive organs (testes and seminal vesicle) were isolated in PBS from adult virgin *A. aegypti* that were fed as larvae with either *E. coli* expressing control dsRNA (empty L4440) or LGR1 dsRNA. For each specimen, testes were separated from the seminal vesicle and placed into designated wells of a 24-well tissue culture plate containing 100 µl PBS. Organs were then carefully torn using 0.1 mm insect pins to release sperm. Pins were washed with an excess of 100 µl PBS for a final volume of 200 µl $well^{-1}$ $tissue^{-1}$. Upon aspirating each well with a P10 pipette to obtain a homogenous distribution of sperm, 5 µl of PBS from each well was spotted 10 times onto microscope slides, air-dried and fixed with 70% ethanol. After fixation, slides were stained with NucBlue® Cell Stain Ready Probes™ (Life Technologies, Eugene, OR) and elongated nuclei, characteristic of mature spermatozoa, were counted in each 5 µl aliquot. The average number of spermatozoa in a 5 µl droplet was calculated and multiplied by the dilution factor to obtain a measure of the total

number of spermatozoa per tissue sample. Total number of spermatozoa from the testes or seminal vesicle were compared between control and LGR1 knockdown treatments (n = 17 mosquitoes, collected from multiple batches of bacterially-fed larvae). Counts were performed blindly two times to maintain accuracy in sperm quantitation. Flagella associated with mature spermatozoa were visualized by phase-contrast microscopy and lengths were measured using an EVOS FL Auto Imaging System (Life Technologies, Burlington, ON). The average flagellar length of 60 testis-derived spermatozoa was compared between mosquitoes treated with control or dsLGR1 bacteria (n = 14-15 mosquitoes, collected from multiple batches of bacterially-fed larvae).

3.3.8 Mating assays

1-day old virgin male *A. aegypti*, fed as larvae on either control or LGR1-dsRNA producing bacteria, were individually transferred into flasks containing a 6-day old untreated virgin female (each flask contained one male per female), and provided a cotton ball wet with dH_20 to entice a bloodmeal the following day. Individual females were blood fed on the arm of a consenting adult over the mating flask that contained a small opening lined with black aluminum screening. Three days post bloodmeal, filter paper was provided for eggs to be oviposited, and the eggs from each female were collected and counted 72 hours later. Upon semi-desiccating eggs for 48 hours, eggs were placed into 100 mL dH_20 containing 500 ul of larval feed for 3 days, and percent larval hatching was calculated. Each egg batch laid by a given female mosquito served as an independent biological replicate (n = 13).

3.4 Results

3.4.1 A. aegypti LGR1 expression localizes to the centriole adjunct of spermatids

To identify the role of LGR1 in *A. aegypti* spermatogenesis, we first analysed the subcellular distribution of LGR1 in each developmental stage of spermatogenesis using immunofluorescence microscopy and *in situ* hybridization. Receptor immunoreactivity and transcript were detected in early spermatogenic cells including spermatogonia, spermatocytes and spermatids, but not in mature spermatozoa (Fig. 3-1). In spermatogonia, LGR1 immunoreactivity was detected as dispersed foci that encircled the cell perimeter and became more abundant in the spermatocyte stages (Fig. 3-1A, B). Fusomes are regions of actin-rich vesiculated cytoplasm that extend through intercellular bridges called ring canals, proposed to function in coordinating male germline cell division in early spermatogenesis (Hime et al. 1996; Wilson 2005). Phalloidin-labelled fusomes were found to be abundant and highly branched in cysts containing spermatogonia, and less abundant and more globular-shaped in spermatocyte cysts; LGR1 immunoreactivity did not colocalize with fusomes in spermatogonia and spermatocyte stages (Fig. 3-1A, B). In early spermatids, which are characterized with condensed, round-shaped nuclei, LGR1 immunoreactivity concentrates to single spots that are polarized to either end of the nuclei and, as nuclear elongation proceeds in late spermatids, receptor immunoreactivity is prominently detected as an elongated structure distinct from but remaining closely associated to the nuclei (Fig. 3-1B, C). Upon completion of spermiogenesis, LGR1 immunoreactivity was not associated with spermatozoa, but rather detected in the cyst cytoplasm as punctate foci that are encapsulated within F-actin stained waste bags (Fig. 3-1D) (Texada et al. 2008), which are organelles responsible for disposing of excess contents after sperm individualization (Metzendorf and Lind 2010). LGR1 protein was found to stain basolateral surfaces of epithelia of the vas

deferens and associated reproductive tissues in females (Fig. 3-1E, F), but again, was not associated with spermatozoa in the vas deferens or the spermathecae of female mosquitoes (Fig. 3-1E, F). Taken together, LGR1 immunolocalization (Fig. 3-1A-F) along with the distribution of LGR1 transcript (Fig. 3-1G-L), revealed expression associated with immature stages of sperm, but not in spermatozoa, suggesting that LGR1 may contribute towards coordinating the development of sperm.

In late spermatids, LGR1 immunoreactivity most prominently associated with an elongated region that attached to nuclei (Fig. 3-1C). To structurally identify this elongated region, co-localization experiments were performed with markers that stain in the spermatid collar and tail regions. Results indicated LGR1 immunoreactivity was associated with the centriole adjunct in the collar region of developing spermatids, that was stained using an antibody targeting gamma tubulin (Dallai et al. 2016), rather than mitochondrial derivatives in flagella (Fig. 3-2). The centriole adjunct is a region found between the nucleus and flagella of sperm (Fig. 3-2D), and contains more than 100 proteins responsible for nucleating cytoplasmic microtubules, ultimately required for proper flagellar development during spermatid stages (Nigg and Raff 2009; Dallai et al. 2016). Since LGR1 immunoreactivity was associated with this region in developing spermatids, we next sought to identify whether knockdown of LGR1 expression influenced (i) flagellar development, (ii) the quantity of spermatozoa and (iii) male fertility.

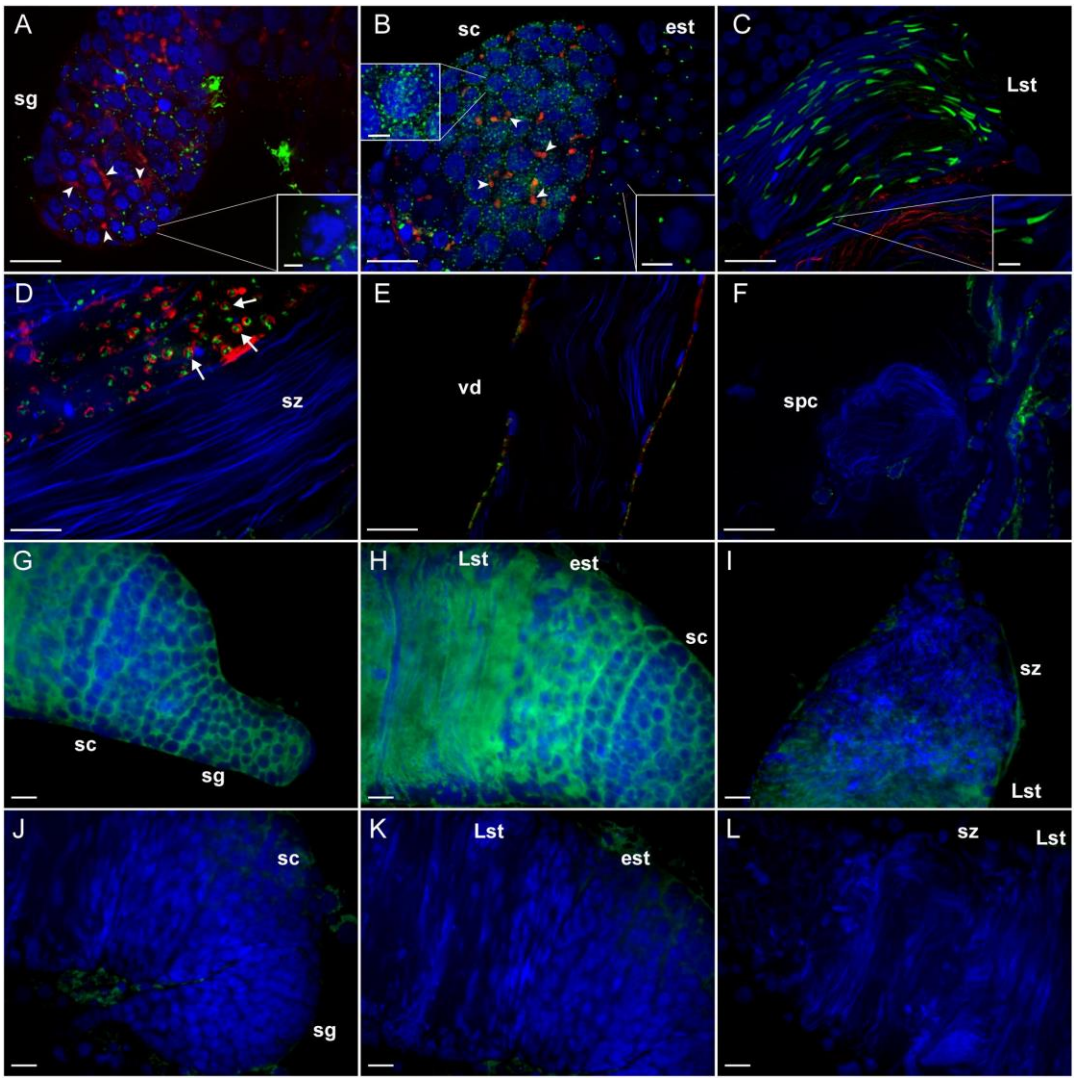

Figure 3-1: Immunohistochemical localization and transcript detection by fluorescent *in situ* hybridization of LGR1 during spermatogenesis in adult *A. aegypti* testes. (**A** and **B**) LGR1-immunoreactivity (green), which is distinct from phalloidin-labelled filamentous-actin-rich fusomes (red) (arrowheads) and nuclei (blue), is present as dispersed foci within the cytoplasm of cysts containing spermatogonia (sg) that increase in quantity in cysts containing spermatocytes (sc). (**B**) In early-staged spermatids (est), LGR1 immunoreactivity is localized to a single spot that is found on one end of each nucleus and (**C**) elongates as nuclear elongation occurs in late-staged spermatids (Lst). (**D**) LGR1 immunoreactivity is encapsulated by phalloidin-labelled filamentous-actin rings (arrows) known as waste bags (Texada et al. 2008) and (**D-F**) is not associated with the nuclei of mature spermatozoa (sz) within the testis base, vas deferens (vd) or female spermatheca (spc). (**G-I**) Digoxigenin-labelled anti-sense probes used to localize LGR1 mRNA-expressing (green) cells detected receptor transcript in all developmental stages except spermatozoa (nuclei, blue). (**J-L**) Though non-specific staining was associated with fat body surrounding testes using sense probes, LGR1 transcript staining was absent in all spermatogenic cells. Scale bars: 20 µm in **A-C**, **E-L**, 10 µm in **D** and 5 µm in **A-C** insets.

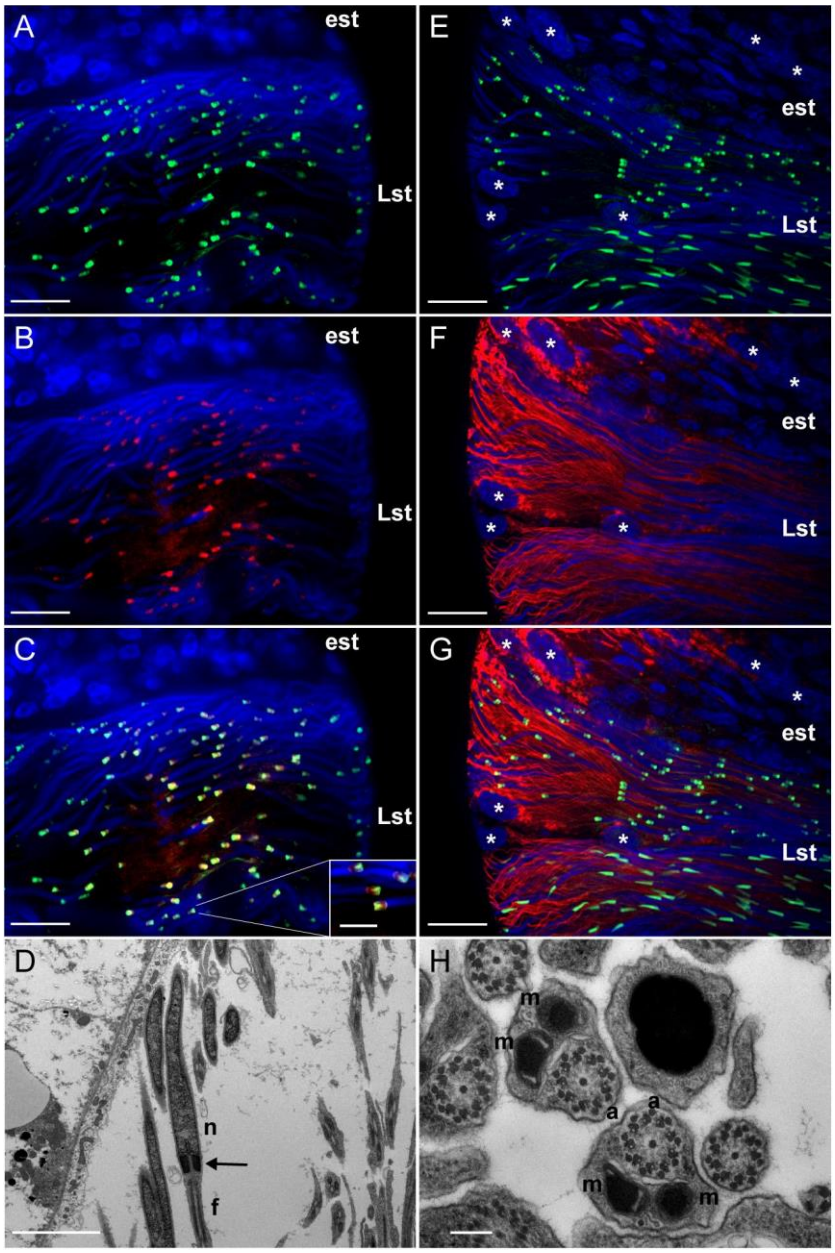

Figure 3-2: Distinct localization of LGR1 immunoreactivity in late-staged spermatids of adult *A. aegypti* testes. **(A, C, E, G)** Testes were stained with anti-LGR1 (green), (**B**-**C**) anti-gamma tubulin (red) as a marker for the centriole adjunct (Dallai et al. 2016), (**F**-**G**) MitoTracker (red) to detect mitochondrial derivatives in flagella, and nuclei (blue). (**C**) LGR1 immunoreactivity co-localizes with gamma-tubulin at the centriole adjunct. (**D**) Transmission electron microscopy (TEM) image showing the centriole adjunct (arrow) is positioned between the nucleus (n) and flagella (f) of spermatids. (**G**) LGR1 immunoreactivity is not found associated with the mitochondrial derivatives in the flagella of late-staged spermatids, comprised of (**H**) two mitochondrial derivatives (m) and an axoneme (a). Early-staged spermatids (est), late-staged spermatids (Lst), cyst cell nuclei (*). Scale bars: 10µm in **A**-**C**, 5 µm in **D**, 20 µm in **E**-**G** and 200 nm in **H**.

3.4.2 Bacteria-mediated LGR1 knockdown

RNA interference of the *A. aegypti* LGR1 gene was accomplished using a bacteria-mediated gene-silencing mechanism established previously (Whyard et al. 2015). Larvae were fed RNAse III-deficient HT115 *E. coli* (Timmons et al. 2001) producing LGR1-targeted or control (no insert) dsRNA. Relative to control mosquitoes fed with bacteria containing dsRNA derived from non-recombinant pL4440 plasmid, LGR1 transcript was significantly reduced by ~57% in whole fourth-instar larvae and ~73% in newly emerged (< 1 day old) adults (Fig. 3-3A). Moreover, LGR1 protein expression in the testes of newly emerged adults, assessed by comparing the intensity of LGR1 immunoreactivity in cysts containing spermatids, was significantly decreased in dsLGR1-fed mosquitoes compared to controls (Fig. 3-3B-F).

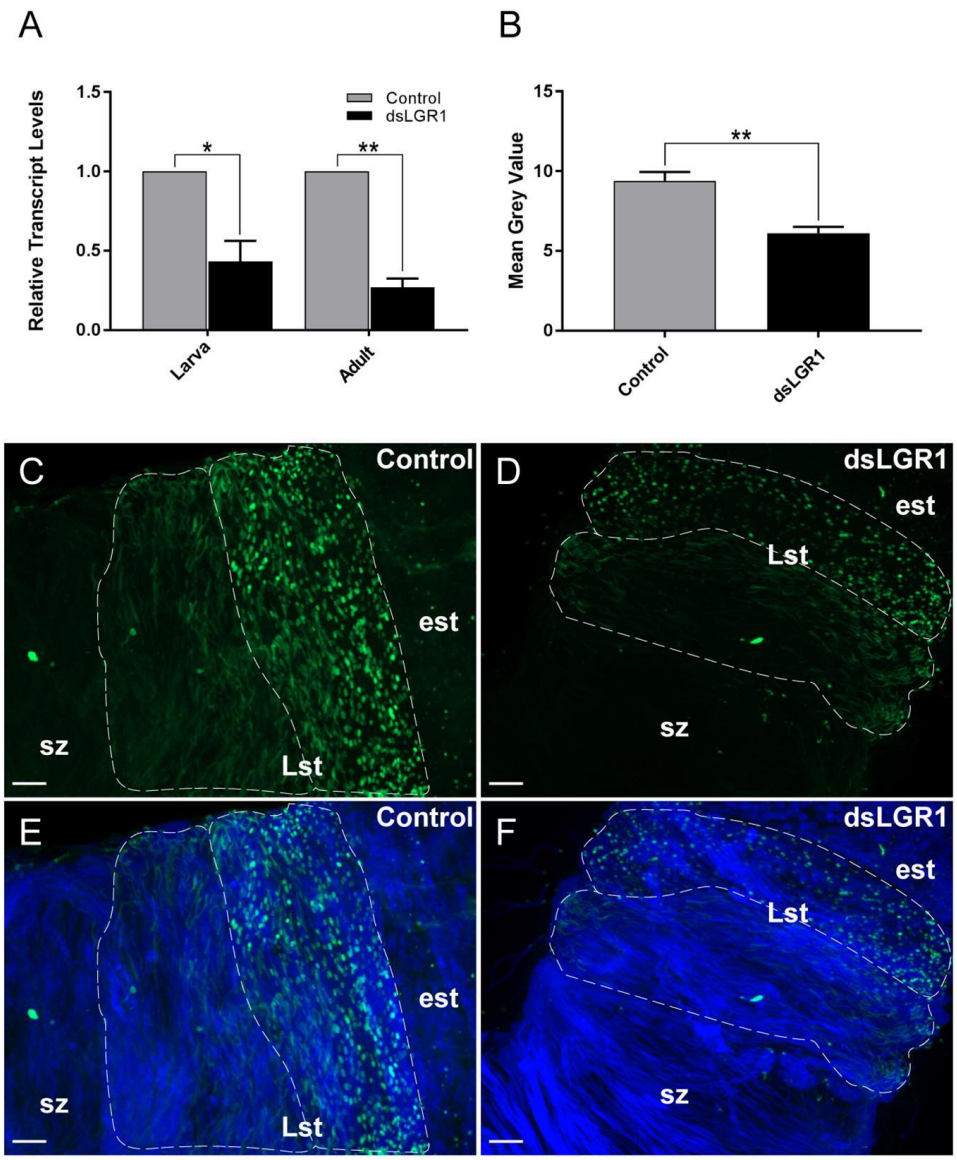

Figure 3-3: LGR1 knockdown efficiency in adult *A. aegypti*. 2nd instar larvae were fed with *E. coli* expressing LGR1 dsRNA or control dsRNA (empty L4440 vector). After feeding, LGR1 transcript knockdown efficiency was determined in (**A**) whole 4th-instar larvae and one-day old adults by quantitative RT-PCR. LGR1 immunoreactivity was examined in control and LGR1 knockdown treatments within (**B**-**F**) the testes of virgin one-day adult males (LGR1, green; nuclei, blue). (**A**) LGR1 transcript levels of 4th instar larvae and newly emerged adults fed with LGR1 dsRNA were significantly reduced compared to control dsRNA-fed mosquitoes. (**B**) Quantification of LGR1 immunoreactivity from control and dsLGR1-treated larvae confirms downregulation. Representative confocal images demonstrate reduced LGR1 immunofluorescence in cysts (dotted outline) containing late-staged spermatids (Lst) when comparing (**C**, **E**) control and (**D**, **F**) dsLGR1-treated mosquitoes. All data are presented as mean ± SEM (n = 3 in **A**, n = 11-13 in **B**) with asterisks representing significant differences between control and dsLGR1-treated mosquitoes as determined using an unpaired t-test: **P* < 0.05, ***P* < 0.01. Early-staged spermatids (est), spermatozoa (sz). Scale bars: 20 µm.

3.4.3 LGR1 knockdown influences spermatozoa count, flagellar morphology and male fertility

Sperm was collected separately from the testes and seminal vesicles of newly emerged adult virgin *A. aegypti* males and the quantity of mature spermatozoa was compared between mosquitoes fed with *E. coli*-expressing LGR1-targeted dsRNA or *E. coli* expressing dsRNA derived from a non-recombinant pL4440 plasmid. For spermatozoa collected from testes samples, LGR1 knockdown resulted in a significant reduction in the number of spermatozoa compared to controls (Fig. 3-4A) and a similar trend was observed for spermatozoa isolated from seminal vesicles (Fig. 3-4B), although this difference was not significant. Overall, when examining the total amount of mature sperm present in the male reproductive system, LGR1 dsRNA-treated mosquitoes possessed an average of 60% less mature spermatozoa than controls (Fig. 3-4C). Testes-derived spermatozoa were then further analyzed for differences in flagellar morphology to identify if LGR1 knockdown had any other effect beyond the significant reductions in quantity of mature spermatozoa. The average length of flagella from control mosquitoes was ~220µm, whereas in LGR1 knockdown mosquitoes, the average flagellar length was reduced by ~28% averaging ~159µm (Fig. 3-4D-F). To examine whether these effects on spermatogenesis led to any appreciable changes to male reproductive success, males fed on control or dsLGR1-producing bacteria were individually mated with untreated 6-day old virgin females. After the engorgement of a blood meal, egg batches from each female were counted, and the percentage of larvae that emerged was recorded. There was no significant difference in the number of eggs oviposited by females mated with either control or dsLGR1-treated males; notably, however, there was a significant reduction (~30%) in the number of larvae that emerged from eggs laid by females mated with dsLGR1 males compared to control-treated males (Fig. 3-4G-H).

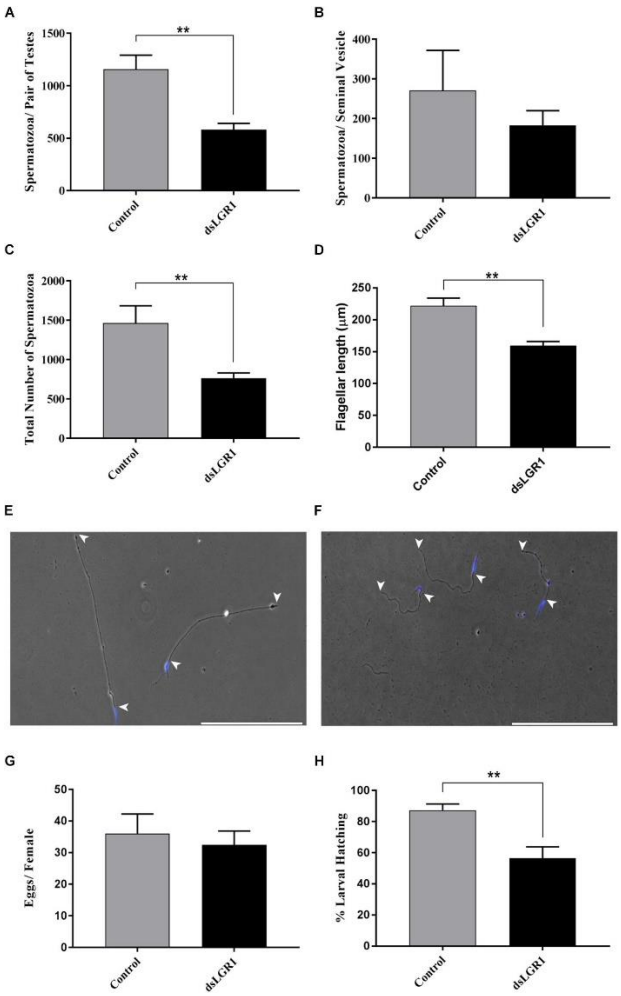

Figure 3-4: Effects of LGR1 knockdown on spermatozoa yield, flagellar length and reproductive success of adult male *A. aegypti*. 2nd instar larvae were fed with *E. coli* expressing LGR1 dsRNA or control dsRNA (empty L4440 vector) and the effects of knockdown on sperm yield, flagellar length and male fertility were analyzed in adult stages. Relative to controls, (**A**-**C**) dsLGR1-fed mosquitoes had significantly less spermatozoa in testes and in the overall reproductive tract (testes + seminal vesicle), while the number of spermatozoa in the seminal vesicle showed a similar trend but was not significant. (**D**) Flagellar lengths from spermatozoa of dsLGR1-treated mosquitoes were significantly shorter than flagella from control mosquitoes. Representative images showing flagellar lengths (arrowheads indicate beginning and end of flagellum) of spermatozoa collected from (**E**) control- and (**F**) dsLGR1-treated mosquitoes (nuclei, blue). (**G**-**H**) No differences in egg yield were observed between females mated with control or dsLGRl-treated males; however, the percentage of larvae hatched from eggs was significantly reduced in matings that involved male LGR1 knockdown treatments. All data are presented as mean ± SEM (n = 17 in **A**-**C**, n = 14-15 in **D**, n = 13 in **G**-**H**) with asterisks representing significant differences between control and dsLGR1-treated mosquitoes as determined using an unpaired t-test: ***P* < 0.01. Scale bars: 200 µm.

3.5 Discussion

Reproduction is tightly regulated by hormones in both vertebrate and invertebrate organisms. In female mosquitoes, oogenesis (the process of egg development) is coordinated by several well-characterized hormonal signalling pathways such as juvenile hormone, ecdysteroids, ovary ecdysteroidogenic hormone, and insulin-like peptides (Raikhel and Lea 1990; Brown and Cao 2001; Raikhel et al. 2005; Gulia-Nuss et al. 2012, 2015; Dhara et al. 2013; Hansen et al. 2014). In males, some evidence for endocrine regulators of spermatogenesis is present in the fruit fly *Drosophila melanogaster* (Kiger et al. 2000; Ueishi et al. 2009; Schwedes et al. 2011). However, research directed towards understanding how spermatogenesis is hormonally regulated in mosquitoes remains unexplored. A previous study examining the expression profile of the glycoprotein hormone receptor LGR1 in *A. aegypti* showed transcript enrichment and LGR1 immunoreactivity in male and female reproductive organs, indicating that its ligand GPA2/GPB5 may encompass a reproductive function (Rocco et al. 2017). In the female ovaries, LGR1 was found expressed in follicular epithelia that house oocytes and nurse cells that aid in egg development (Rocco et al. 2017), whereas in the testes of male mosquitoes, LGR1 immunoreactivity localized to distinct areas of developing sperm (Rocco et al. 2017). Given its confirmed expression at the transcript level and regionalized immunoreactivity in the testes, the current study aimed to elucidate the physiological role of LGR1 in *A. aegypti* spermatogenesis.

3.5.1 LGR1 as a regulator of microtubule nucleation in the spermatid centriole adjunct

LGR1 immunoreactivity was most apparently associated with an elongated structure positioned at the base of spermatid nuclei, which co-localization experiments identified as the centriole adjunct (Fig. 3-1, 3-2). In developing spermatids, the centriole adjunct is a cylindrical sheath that forms around the centriole, that functions to anchor the flagellar axoneme (Dallai et al. 2016).

Associated with the centriole adjunct is electron-dense pericentriolar material which consists of cytoplasmic proteins, such as gamma-tubulin and CP190, that are responsible for nucleating microtubules to form the flagellar axoneme (Riparbelli et al. 1997; Dallai et al. 2016). Experiments that aimed to elucidate the subcellular distribution of LGR1 immunoreactivity during spermatogenesis revealed LGR1 immunoreactivity associates with the cell perimeter (i.e. plasma membrane) of spermatogonia and spermatocytes (Fig. 3-1A, 3-1B), which parallels previous findings that *A. aegypti* LGR1 is a membrane-associated receptor (Rocco et al. 2017). In late-staged spermatids, LGR1 staining is associated with the plasma membrane and regionalized to the centriole adjunct given that receptor staining encapsulates gamma-tubulin staining, which is a cytoplasmic protein. Taken together, it is hypothesized that LGR1 is localized to the membrane of the centriole adjunct where it is exposed and can be activated by its known ligand GPA2/GPB5 to coordinate microtubule nucleating activity in this region in spermatids. Interestingly, strong GPB5 immunoreactivity is associated with rat oocytes (Sun et al. 2010) and GPB5 transcript in previtellogenic-staged oocytes in *Amphioxus* (Tando and Kubokawa 2009), which supports the hypothesis that GPA2/GPB5-LGR1 signalling may be involved in gamete development in mosquitoes.

Microtubule nucleating activity in the centriole adjunct is transient, given that staining for gamma tubulin and other pericentriolar proteins disappear when mature spermatozoa are formed (Riparbelli et al. 1997; Dallai et al. 2016). LGR1 transcript and immunoreactivity was detected throughout early stages of spermatogenesis and late stages of spermiogenesis, but not in mature spermatozoa. The receptor immunoreactivity was localized to waste bags, which are involved in removing excess cellular material following spermiogenesis (Arama et al. 2003; Fabian and Brill 2012; Ben-David et al. 2015) (Fig. 3-1). Given its close localization with gamma-tubulin in

spermatids and transient expression pattern during spermatogenesis, we tested whether LGR1 is involved in microtubule nucleation and, consequently, flagellar development.

3.5.2 LGR1 as a regulator of spermatogenesis

Depending on the mosquito species, spermatogenesis predominantly occurs in late larval to pupal developmental life stages (Lum 1961; Barr 1974). As a result, to assess the influence of LGR1 knockdown on spermatogenesis in *A. aegypti*, *E. coli* bacteria expressing LGR1-targeted dsRNA were fed to 2nd instar larvae. The effectiveness of LGR1 knockdown was then measured throughout mosquito developmental stages, as well as in the testes of adult males, and measured relative to control mosquitoes fed with *E. coli* expressing dsRNA from a non-recombinant pL4440 plasmid (Fig. 3-3). Results showed that LGR1 gene knockdown was maintained to adulthood, where LGR1 transcript was significantly downregulated by ~73%. Moreover, in the testes of adults, LGR1 immunoreactive staining intensity in spermatid-containing cysts was decreased by ~35% relative to the cysts of control testes (Fig. 3-3).

LGR1 knockdown mosquitoes were examined for abnormal phenotypes associated with spermatogenesis including morphological defects as well as differences in the quantity of spermatozoa from reproductive organs since defective sperm likely undergo apoptosis and/or may be targeted for degradation (57, 58). Relative to control mosquitoes, LGR1 knockdown led to a significant reduction in the quantity of mature spermatozoa, and the remaining spermatozoa were characterized with significantly shorter flagellar lengths (Fig. 3-4). Given the impact of LGR1 knockdown on *A. aegypti* spermatogenesis, we next sought to determine whether this had any consequences on the reproductive success of male mosquitoes. Interestingly, a significantly reduced proportion of eggs hatched when females were mated with LGR1 knockdown males

compared to control males (Fig. 3-4). This could be interpreted as a result of abnormalities in sperm quality and reduced quantity from LGR1 knockdown males, which displayed a lower number of mature spermatozoa, and of the remaining spermatozoa, flagellar morphological defects were evident.

3.5.3 Concluding remarks

In summary, our data provide evidence for the involvement of LGR1 in mosquito spermatogenesis, given that knockdown of this receptor decreases sperm yield, impairs flagellar morphology and renders males less fertile. Specifically, our data indicates that LGR1 functions to regulate flagellar development during spermatogenesis since LGR1 immunoreactivity was regionalized to the centriole adjunct of spermatids, a region that houses proteins important for producing the axoneme. Given that LGR1 is the receptor target of the glycoprotein hormone GPA2/GPB5 (Sudo et al. 2005), our results support a reproductive function for GPA2/GPB5 in the mosquito. To our knowledge, this is the first study that examines endocrine signalling in the regulation of reproductive processes in male *A. aegypti*. In past studies, expression profiling of the allatotropin and allatostatin neuropeptide receptors identified transcript enrichment in the testis (Mayoral et al. 2010; Nouzova et al. 2013), suggesting that alternate endocrine signalling pathways exist and may also control sperm development in mosquitoes. Though most research allocated to understanding mosquito biology has been directed towards female reproduction, male reproductive biology is understudied and should be considered in future research. Research focused on better understanding the reproductive biology and its endocrine of male mosquitoes will be vital for the development of novel approaches and the improvement of existing pest control strategies aimed at lessening the burden of these medically-important vectors of disease.

3.6 References

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CHAPTER 4:

EXPRESSION PROFILING, DOWNSTREAM SIGNALLING AND INTER-SUBUNIT INTERACTIONS OF GPA2/GPB5 IN THE ADULT MOSQUITO, *AEDES AEGYPTI*

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4.1 Summary

GPA2/GPB5 and its receptor constitute a glycoprotein hormone-signalling system native to the genomes of most vertebrate and invertebrate organisms, including humans and mosquitoes. Unlike the well-studied gonadotropins and thyrotropin, the exact function of GPA2/GPB5 is unclear, and whether it elicits its functions as heterodimers or as independent monomers or homodimers remains unknown. To better characterize GPA2/GPB5 signalling in the mosquito *Aedes aegypti*, the current study aimed to describe the spatial expression patterns of each glycoprotein hormone subunit in detail in the adult stage. Moreover, using heterologous systems involving mammalian cell lines to produce recombinant *A. aegypti* and *Homo sapiens* GPA2 and GPB5, subunit interactions were studied and the functionality of individual or both subunits in receptor activation were examined. In adult mosquitoes, GPA2 and GPB5 transcripts and immunoreactivity of each subunit was co-localized to the same bilateral pairs of neuroendocrine cells within the first five abdominal ganglia of the central nervous system. Unlike human GPA2/GPB5 that demonstrated strong heterodimerization between subunits, *A. aegypti* GPA2/GPB5 subunit heterodimers were not detected in our experiments. Rather, cross-linking analysis to determine subunit interactions revealed *A. aeygpti* and *H. sapiens* GPA2 and GPB5 subunits form homodimers, and treatments with independent subunits did not activate *A. aegypti* LGR1 or *H. sapiens* TSH receptor, respectively. Given that recombinant heterodimers were not formed by expression in HEK 293T cells, a tethered *A. aegypti* GPA2/GPB5 fusion construct was generated for expression of GPA2/GPB5 as a single polypeptide chain. In control unstimulated conditions, *A. aegypti* LGR1 elicited constitutive activity that elevated levels of cAMP as determined by increased cAMP-dependent luminescence. However, upon incubation with recombinant tethered GPA2/GPB5, an inhibitory G protein (Gi) signalling cascade is

initiated and forskolin-induced cAMP production is inhibited. These data are the first to demonstrate *A. aegypti* and *H. sapiens* GPA2 and GPB5 homodimerization *in vitro.* Moreover, these data further support that both subunits (i.e. heterodimerization) are required for glycoprotein hormone receptor activation.

4.2 Introduction

Members of the cystine knot growth factor (CKGF) superfamily, characterized with a CKGF domain as their primary structural feature, include (i) the glycoprotein hormones, (ii) the invertebrate bursicon hormone, (iii) the transforming growth factor beta (TGFβ) family, (iv) the bone morphogenetic protein (BMP) antagonist family, (v) the platelet-derived growth factor (PDGF) family and (vi) the nerve growth factor (NGF) family. Of the members of the CKGF superfamily, the glycoprotein hormones are of fundamental importance in the regulation of both vertebrate and invertebrate physiology.

In vertebrates, the glycoprotein hormones include follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH) as well as chorionic gonadotropin (CG) and are implicated in governing several aspects of physiology like reproduction, energy metabolism along with growth and development. Structurally, these hormones are formed by the heterodimerization of two cystine-knot glycoprotein subunits, an α subunit that is structurally identical for each hormone (GPA1), and a β subunit that is hormone-dependent (GPB1-4) (Pierce and Parsons 1981; Stockell Hartree and Renwick 1992).

Not long ago, two novel glycoprotein hormone subunits were identified in the human genome, glycoprotein α2 (GPA2) and glycoprotein β5 (GPB5), and were found to heterodimerize (GPA2/GPB5) and act on the same receptor as TSH. As a result, GPA2/GPB5 was coined the name thyrostimulin to differentiate it from TSH in vertebrates (Nakabayashi et al. 2002). Unlike other glycoprotein hormones which are vertebrate-specific, homologous genes encoding GPA2/GPB5 subunits exist in both vertebrates and invertebrates, where its function appears to be pleiotropic (Hsu et al. 2002; Nakabayashi et al. 2002; Dos Santos et al. 2011). In vertebrates, GPA2/GPB5 function has been implicated, or at least suggested to be involved in

reproduction (Sun et al. 2010), thyroxine production (Nakabayashi et al. 2002; Okada et al. 2006), skeletal development (Duncan Bassett et al. 2015), immunoregulation (Suzuki et al. 2009; van Zeijl et al. 2014) and the proliferation of ovarian cancer cell lines (Huang et al. 2016). For invertebrates, GPA2/GPB5 is suggested to function in development (Heyland et al. 2012; Paluzzi et al. 2014; Vandersmissen et al. 2014; Rocco and Paluzzi 2016), ion and water balance (Sellami et al. 2011; Paluzzi et al. 2014; Vandersmissen et al. 2014; Jourjine et al. 2016; Rocco and Paluzzi 2016; Rocco et al. 2017) as well as reproduction (Rocco et al. 2017, 2019).

Non-covalent interactions and heterodimerization between the subunits encompassing FSH, LH, TSH and CG is required for their respective biological functions (Pierce and Parsons 1981; Combarnous 1992). However, whether heterodimerization is required for GPA2/GPB5 to activate its receptor and exert its physiological role in vertebrates and invertebrates is debated. For FSH, LH, TSH and CG, the subunits are co-expressed in the same cells and each hormone is released into circulation as heterodimers (Galet et al. 2004; Xing et al. 2004). On the other hand, for vertebrates and invertebrates, GPA2 and GPB5 subunit expression profiles do not always occur in the same cells, and GPA2 is often expressed more abundantly than GPB5 in some tissues (Nagasaki et al. 2006; Okada et al. 2006; Dos Santos et al. 2009; Tando and Kubokawa 2009a; Heyland et al. 2012). Additionally, unlike the beta subunits of the classic glycoprotein hormones, the structure of vertebrate and invertebrate GPB5 lacks an extra pair of cysteine residues that form an additional disulfide linkage, called the ‘seatbelt’, which strengthens and stabilizes its heterodimeric association with GPA2 (Alvarez et al. 2009).

Relative to other G protein-coupled receptors (GPCRs), members of the leucine-rich repeat-containing G protein-coupled receptor (LGR) family are characterized with a large extracellular domain responsible for the selective binding of ligands on the amino terminus

(Krause et al. 2012). All glycoprotein hormone receptors, which includes the FSH, LH/CG, TSH and GPA2/GPB5 receptors, belong to the Type A LGRs that are characterized with a long hinge region and 7-9 leucine-rich repeats (Van Hiel et al. 2012). Bursicon, an invertebrate neuropeptide formed by the heterodimerization of burs α and burs β protein subunits, activates LGR2 which belongs to the Type B LGRs, with 16-18 leucine-rich repeats and a short hinge region (Luo et al. 2005; Mendive et al. 2005; Van Hiel et al. 2012).

Not long after its initial discovery, the invertebrate receptor for GPA2/GPB5, called LGR1, was deorphanized (Sudo et al. 2005). In the fruit fly *Drosophila melanogaster*, GPA2/GPB5 heterodimers were found to activate LGR1 and shown to increase cellular levels of cyclic AMP (cAMP) (Sudo et al. 2005). Interestingly, stimulatory G protein (Gs) coupling and signalling to elevate cAMP was also shown with GPA2/GPB5-TSH receptor activation in humans (Nakabayashi et al. 2002; Sun et al. 2010).

In the mosquito *Aedes aegypti*, genes encoding for GPA2, GPB5 and LGR1 were identified and shown to be expressed in all developmental life stages, with greater expression in adults compared to juvenile stages (Paluzzi et al. 2014). In adults, LGR1 was found localized to epithelia throughout the gut where GPA2/GPB5 could regulate feeding-related processes and hydromineral balance (Rocco et al. 2017). Interestingly, LGR1 expression was also observed in the reproductive tissues of males and females, and knockdown of LGR1 expression led to a significant impedance of normal spermatogenesis in the male testes, with spermatozoa eliciting abnormalities such as shortened flagella. Moreover, LGR1 knockdown in males resulted in 60% less spermatozoa and reduced male fertility relative to control mosquitoes.

With an interest in better understanding GPA2/GPB5 signalling in *A. aegypti* mosquitoes, our work herein set out to characterize the tissue-specific and cellular distribution expression

profile of GPA2/GPB5 in mosquitoes. As well, we sought to determine GPA2/GPB5 interactions and demonstrate GPA2/GPB5-LGR1 interaction and activation using a mammalian cell line heterologous system. Using a combination of molecular techniques, we have demonstrated GPA2/GPB5 cellular co-expression in the central nervous system of adult mosquitoes, and that both subunits are required to activate LGR1, which exhibits ligand-dependent G protein-coupling activity. Moreover, these results provide evidence for human and mosquito GPA2 and GPB5 homodimers, which were not capable of activating TSH receptor or LGR1, respectively. Overall, these findings significantly advance our understanding GPA2/GPB5 signalling in mosquitoes and provide direction to uncover possible functions for homologs in other organisms.

4.3 Materials and methods

4.3.1 Animals

Adult *Aedes aegypti* (Liverpool) were derived from an established laboratory-bred colony in the Department of Biology, York University. Eggs were oviposited onto Whatmann Filter Papers (GE Bioscience) and semi-desiccated before being hatched in 2 L plastic containers half-filled with deionized water at room temperature (RT). Larvae were fed daily with 2% brewers yeast, 2% liver powder solution, whereas adults were maintained on cotton ball wicks that were soaked with a 10% sucrose solution. Upon larval-pupal ecdysis, groups of pupae were separated into glass microchambers and adults that emerged were used for experimentation and were fed *ad libitum* on sucrose. Larvae, pupae and adults to be used for experimentation were maintained on a 12:12 h light:dark cycle at 26 °C, whereas colony-bred adults were maintained at RT and provided access to an artificial blood feeding system for colony-upkeep, as previously described (Rocco et al. 2017).

4.3.2 GPA2/ GPB5 transcript analysis by RT-qPCR

Male and female adult mosquitoes four-days post-eclosion were CO_2 anesthetized and dissected in nuclease-free Dulbecco's phosphate-buffered saline (PBS; pH 7.3) (Wisent, St. Bruno, QC, Canada) at RT. Dissected tissues and organs included the brain, thoracic ganglia, abdominal ganglia, alimentary canal (including the midgut, hindgut and Malpighian tubules), reproductive tissues (including accessory reproductive tissues and ovaries/testes) and carcass (containing peripheral fat body and musculature) of each sex. After dissection, tissues were immediately placed into RNA lysis buffer (Bio Basic, Markham, ON, Canada) and stored overnight at -20°C. Upon thawing samples at RT, total RNA was purified using the EZ-10 RNA Mini-Preps Kit (Bio

Basic, Markham, ON, Canada) that included an on-column DNase I treatment to remove potential contaminating genomic DNA (Life Technologies, Burlington, ON, Canada), and quantified using a Synergy 2 Multimode Microplate Reader (BioTek, Winooski, VT, USA). Total RNA was reverse transcribed using iScript™ Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada), and diluted five-fold with molecular-grade nuclease-free water (dH_20) (Wisent Corporation, St. Bruno, QC, Canada). Using PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA), GPA2 and GPB5 transcript abundance was quantified using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) as described previously (Gondalia et al. 2016). RT-qPCR conditions were described previously (Rocco et al. 2017) and GPA2- and GPB5-specific primer sequences were designed in an earlier study (Paluzzi et al. 2014). GPA2 and GPB5 transcript abundance was normalized to the expression of ribosomal protein 49 (GenBank accession: AY539746) and 60S ribosomal protein S18 (GenBank accession: XM_001660270) following the ΔΔCt method as described previously (Paluzzi et al. 2014). Measurements were performed using three technical replicates per plate and three biological replicates for each tissue/organ sample.

4.3.3 Fluorescence in situ hybridization

Using gene-specific primers (Table 4-S1), *A. aegypti* GPA2 and GPB5 sequences were amplified from previously prepared constructs (Paluzzi et al. 2014) that contained the GPA2 and GPB5 complete open reading frames (ORFs). Sense and antisense probes were then generated following a similar protocol as recently reported (Rocco et al. 2019). Briefly, cDNA fragments were ligated to pGEM T Easy vector (Promega, Madison, WI, USA) and used to transform NEB 5-α competent *Escherichia coli* cells (New England Biolabs, Whitby, ON, Canada). After

Table 4-S1: Primers utilized for RNA probe template generation. GenBank accession numbers: BN001241 (*A. aegypti* GPA2), BN001259 (*A. aegypti* GPB5).

Oligonucleotide name	Oligonucleotide sequence (5' – 3')	Product size (bp)	Target and Usage
T7 promoter	TAATACGACTCACTATAG		GPA2 and GPB5 sense and anti-sense RNA probe generation for fluorescence *in situ* hybridisation
Aedes GPA2 F	ATGGAGTGGCTACGATTTGC	363	
Aedes GPA2 R	GTGTTACCACTGTAAAAAGGACTGA		
Aedes GPB5 F	ATGATCCTAATCTCGGTATGGA	486	
Aedes GPB5 R	AAATCGAATCCGAATACGCTTAG		

screening, bacterial colonies for directionality using T7 promoter oligonucleotide and gene-specific primers (Table 4-S1), template sense or anti-sense cDNA strands for GPA2 and GPB5 probe synthesis were created by PCR amplification and verified by Sanger sequencing for base accuracy (The Centre for Applied Genomics, Sick Kids Hospital, Toronto, ON, Canada). Digoxigenin (DIG)-labelled anti-sense and sense RNA probes corresponding to GPA2 and GPB5 subunits were synthesized using the HiScribe T7 High Yield RNA Synthesis kit (New England Biolabs, Whitby, ON, Canada). Fluorescence *in situ* hybridization (FISH) was then used to detect GPA2 and/or GPB5 transcript in the mosquito central nervous system using 4 ng μl^{-1} (GPB5) and/or 6 ng μl^{-1} (GPA2) RNA sense/ antisense probes, following a previously established protocol (Paluzzi et al. 2008). Preparations were analyzed with a Lumen Dynamics X-CiteTM 120Q Nikon fluorescence microscope (Nikon, Mississauga, ON, Canada), or a Yokogowa CSU-XI Zeiss Cell Observer Spinning Disk confocal microscope, and images were processed using Zeiss Zen and ImageJ software. All microscope settings were kept identical when acquiring images of control and experimental wholemount preparations.

4.3.4 Wholemount immunohistochemistry

GPB5 immunoreactivity in the abdominal ganglia of adult mosquitoes was examined in newly emerged and four-day old *A. aegypti* that were CO_2 anesthetized, and dissected in PBS at RT. Tissues were fixed, permeabilized and incubated in a custom rabbit polyclonal GPB5 antibody (1 $\mu g\ ml^{-1}$) designed against an antigen sequence (CDSNEISDWRFP) positioned at residues 204-240 (Paluzzi et al. 2014) for 48 h at 4°C rocking. Control treatments involved pre-incubated *A. aegypti* GPB5 primary antibody solution containing 100:1 peptide antigen:antibody (mol:mol). After several washes, tissues were incubated overnight at 4°C in Alexa Fluor 488-conjugated

goat anti-rabbit Ab (1:200) secondary antibody (Life Technologies) in PBS containing 10% normal sheep serum. The next day, samples were washed, mounted on coverslips containing Diamidino-2-phenylindole dihydrochloride (DAPI), and analyzed using a Lumen Dynamics X-CiteTM 120Q Nikon fluorescence microscope (Nikon, Mississauga, ON, Canada), or optically sectioned using a Yokogowa CSU-XI Zeiss Cell Observer Spinning Disk confocal microscope. All images were processed using Zeiss Zen and ImageJ software. Further details concerning the wholemount immunohistochemical protocol were reported in an earlier study (Rocco et al. 2017).

4.3.5 Plasmid expression constructs

Plasmid expression constructs were designed to study *A. aegypti* and *H. sapiens* GPA2/GPB5 subunit dimerization patterns and receptor signalling. Using previously available hexa-histidine-tagged *A. aegypti* GPA2-His (Paluzzi et al. 2014), as well as FLAG (DYKDDDDK)-tagged (-FLAG) *H. sapiens* GPB5-FLAG (Genscript, Clone OHu31847D) plasmid vectors as template, the full ORF of each (*A. aegypti* and *H. sapiens*) GPA2 and GPB5 subunit coding sequence, including a consensus Kozak translation initiation sequence, was amplified and a hexa-histidine or FLAG tag sequence was incorporated on the carboxyl-terminus of subunits to produce the following fusion proteins; *A. aegypti* GPA2-FLAG, *H. sapiens* GPB5-His (Table 4-S2). Tags were incorporated onto the carboxyl termini with two consecutive PCR amplifications using plasmid miniprep that contained each ORF as the initial template, and PCR amplification was performed using OneTaq® DNA polymerase (New England Biolabs). Conditions for the first PCR amplification were as follows: initial denaturation cycle (94 °C, 30 s) and 35 cycles of denaturation (94 °C, 20 s), annealing for *A. aegypti* GPA2 (involving GPA2 Kozak F1/GPA2

FLAG R1 primers) 10 cycles at 45 °C followed by 25 cycles at 57 °C and *H. sapiens* GPB5 (involving GPB5 Kozak F1/GPB5 FLAG R1 primers) at 64 °C, with each cycle lasting 20 s) and extension (68 °C, 30 s), followed by a final extension (68 °C, 5 min). The subsequent PCR amplification to complete the incorporation of each tag consisted of an initial denaturation (94 °C, 30 s) and 35 cycles of denaturation (94 °C, 20 s), annealing for *A. aegypti* GPA2 (involving GPA2 Kozak F1/GPA2 FLAG R2 primers) 10 cycles at 49 °C and 25 cycles at 58 °C, *H. sapiens* GPB5 (involving GPB5 Kozak F1/GPB5 FLAG R2 primers) 10 cycles at 54 °C and 25 cycles at 60 °C, 20 s) and extension (68 °C, 30 s), followed by a final extension (68 °C, 5 min). Tagged PCR amplicons were cloned into pGEM T Easy vector (Promega, Madison, WI, USA) and confirmed for base accuracy by Sanger sequencing (The Centre for Applied Genomics, Sick Kids Hospital, Toronto, ON, Canada). After sequence verification, plasmid clones were *Not*1 (Anza™ cloning system; ThermoFisher Scientific, Whitby, ON, Canada) digested for 1 h at 37°C, and His- or FLAG-tagged GPA2 and GPB5 sequences were separated by electrophoresis on a 1% agarose-TAE ethidium bromide-stained gel. Samples were then gel-purified and ligated to the mammalian expression vector pcDNA3.1$^+$ (Life Technologies, Burlington, ON) following a previously described protocol (Rocco et al. 2017). A pcDNA3.1$^+$ mammalian expression construct containing mCherry, which was a gift from Scott Gradia (Addgene plasmid # 30125), was utilized to verify cell transfection efficiency. Experiments also utilized previously prepared pcDNA3.1$^+$ constructs with *A. aegypti* GPB5-His and *A. aegypti* LGR1 coding sequences and dual promoter vector pBudCE4.1 containing both *A. aegypti* GPA2-His and GPB5-His (Paluzzi et al. 2014). Additionally, pcDNA 3.1$^+$ mammalian expression vector construct containing FLAG tagged *H. sapiens* thyrotropin receptor (TSHR-FLAG) (Genscript USA Inc., Clone OHu18318D), *H. sapiens* GPA2-FLAG (Genscript USA Inc., Clone

Table 4-S2: Primers utilized for synthesis of *A. aegypti* GPA2-FLAG, *H. sapiens* GPB5-His and tethered *A. aegypti* GPA2/GPB5 sequences for heterologous expression in mammalian cell lines. GenBank accession numbers: BN001241 (*A. aegypti* GPA2), BN001259 (*A. aegypti* GPB5) and HF564672.1 (*H. sapiens* GPA2).

Oligonucleotide name	Oligonucleotide sequence (5' – 3')	Product size (bp)	Target and Usage
Aedes GPA2 kozak F1	GCCACCATGGAGTGGCTA CGATTTGCAACA		Sense and antisense primer sets for the incorporation of a FLAG tag on the carboxyl terminus of *A. aegypti* GPA2
Aedes GPA2 FLAG R1	GTTACCACTGTAAAAAGG ACGATTACAAGGATGAC	381	
Aedes GPA2 FLAG R2	AAGGACGATTACAAGGAT GACGACGATAAGTAG	393	
Homo GPB5 kozak F1	GCCACCATGAAGCTGGCA TTCCTCTTCCTT		Sense and antisense primer sets for the incorporation of a hexa-histidine tag on the carboxyl terminus of *H. sapiens* GPB5
Homo GPB5 His R1	GGCCACCACGGAGTGTGA GACC°ATCCATCATCACC	406	
Homo GPB5-His R2	TGTGAGACCATCCATCAT CACCATCACCATTAG	417	
Tethered GPA2 F1	CGCGAAACGTGGCAGAAA CCAG	309	Sense and antisense primer sets for the incorporation of (i) a portion of a hexa-histidine tag and glycine-serine repeat linker sequence on the amino terminus and (ii) a *Not*1 restriction enzyme and genomic pcDNA3.1 plasmid sequence overhang on the carboxyl terminus of *A. aegypti* GPA2
Tethered GPA2 R1	GCTCGTGTTACCACTGTA AAAAGGACTAG		
Tethered GPA2 F2	CATCATCACCATCACCAT CGCGAAACGTGGCAGAAA CCA	341	
Tethered GPA2 R2	GTTACCACTGTAAAAAGG ACTAGGCGGCCGCTCGAG T		
Tethered GPA2 F3	TCGGGGTCGGGGTCGCAT CATCACCATCACCATCGC G	366	
Tethered GPA2 R3	GACTAGGCGGCCGCTCGA GTCTAGAGGGCC		
Tethered GPB5 F1	GCCACCATGATCAATCTA ATCTCG	492	Sense and antisense primer sets for the incorporation of (i) an *Eco*R1 sequence and pcDNA3.1 plasmid sequence overhang on
Tethered GPB5 R1	GGAAATCGAATCCGAATA CGCT		
Tethered GPB5 F2	GTGGAATTCGCCACCATG ATCAATCTAATC	519	

Tethered GPB5 R2	GGAAATCGAATCCGAATACGCTGGGTCGGGGTCGGGGTCG	548	the N terminus and (ii) a portion of a hexa-histidine tag and glycine-serine repeat linker sequence on the carboxyl terminus of *A. aegypti* GPB5
Tethered GPB5 F3	ACTAGTCCAGTGTGGTGGAATTCGCCACCATG		
Tethered GPB5 R3	CGCTGGGTCGGGGTCGGGGTCGCATCATCACCATCAC		

OHu31847D), *H. sapiens* GPB5-FLAG (Genscript USA Inc., Clone OHu55827D) and pGlosensor™ -22F cyclic adenosine monophosphate (cAMP) plasmid (Promega Corp., Madison, WI) which were commercially purchased for receptor signalling activity assays.

4.3.6 Generation of tethered A. aegypti GPA2/GPB5 construct

The ORFs of *A. aegypti* GPA2 and GPB5 sequences were tethered together in order to promote heterodimer interactions for testing in receptor activity assays with mammalian cell lines. A hexa-histidine tagged artificial linker sequence involving three glycine-serine repeats was employed to fuse the amino-terminus of *A. aegypti* GPA2 propeptide sequence to the carboxyl-terminus of *A. aegypti* GPB5 prepropeptide sequence, using multiple PCR amplifications with several primer sets (Table 4-S2) as performed previously using lamprey GPA2 and GPB5 sequences (Sower et al. 2015). All PCR conditions involved miniprep samples of the corresponding templates and Q5 DNA High Fidelity DNA Polymerase (New England Biolabs, Whitby, ON) for amplification, following manufacturer recommended conditions. Overall, a portion of the hexa-histidine tagged glycine-serine repeat linker sequence was incorporated onto the amino terminus of *A. aegypti* GPA2 propeptide sequence, and a *Not*1 restriction enzyme and pcDNA3.1$^+$ plasmid DNA sequence overhang was made on the carboxyl terminus. For the *A. aegypti* GPB5 prepropeptide sequence, a portion of the linker sequence was incorporated onto the carboxyl terminus and an *Eco*R1 restriction enzyme and pcDNA3.1$^+$ plasmid DNA sequence overhang was made on the amino terminus. Modified *A. aegypti* GPA2 and GPB5 sequences were amplified in three consecutive PCR reactions (i – iii). PCR reaction conditions included i) 1 cycle of denaturation (98 °C, 30 s) and 35 cycles of denaturation (98 °C, 10 s), annealing (GPA2: tethered GPA2 F1/R1 primers and 70 °C, GPB5: tethered GPB5 F1/R1 primers and 66 °C, 30 s),

and an extension (72 °C, 35 s), followed by a final extension (72 °C, 2 min), ii) 1 cycle of denaturation (98 °C, 30 s) and 35 cycles of denaturation (98 °C, 10 s), annealing (GPA2: tethered GPA2 F2/R2 primers and 61 °C, GPB5: tethered GPB5 F2/R2 primers and 62 °C, 30 s) and extension (72 °C, 35 s), followed by a final extension (72 °C, 2 min), iii) 1 cycle of denaturation (98 °C, 30 s) and 35 cycles of denaturation (98 °C, 10 s), annealing (GPA2: tethered GPA2 F3/R3 primers and 70 °C, GPB5: tethered GPB5 F3/R3 primers and 65 °C, 30 s) and extension (72 °C, 35 s), followed by a final extension (72 °C, 2 min) (Table 4-S2). To generate the expression cassette template, empty pcDNA3.1^{+} expression vector was double digested with Anza^{TM} *Eco*R1 and Anza^{TM} *Not*1 (Invitrogen) restriction enzymes for 1 h at 37°C, migrated on a 1% agarose-TAE ethidium-bromide stained gel and gel-purified. Finally, the three products encompassing modified *A. aegypti* GPA2 propeptide, *A. aegypti* GPB5 prepropeptide and digested pcDNA3.1^{+} plasmid was assembled together using HiFi DNA Assembly Master Mix (New England Biolabs, Whitby, ON) using a DNA molar ratio of 1:2 vector:insert. After transforming NEB 5-α competent *E. coli* cells with this glycoprotein fusion construct assembly, bacterial colonies were screened with T7 promoter oligonucleotide and gene-specific primers and verified for base accuracy with Sanger sequencing as described earlier.

4.3.7 Transient transfection of HEK 293T cells

Human embryonic kidney (HEK 293T) cells were grown in complete growth media (Dulbecco's modified eagles medium: nutrient F12 (DMEM) media, 10% heat inactivated fetal bovine serum (Wisent, St. Bruno, QC) and 1X antimycotic-antibiotic) and maintained in a water-jacketed incubator at 37°C, 5% CO_2. Cells were transfected at ~80-90% confluency with plasmid expression constructs in 6-well tissue culture plates (Thermo Fisher Scientific, Denmark) using

Lipofectamine 3000 transfection reagent (Life Technologies, Carlsbad, CA) with 3:1 transfection reagent to DNA (µL:µg). Before transfection, culture media was replaced with either serum-free medium (DMEM and 1X antimycotic-antibiotic) for experiments that collected secreted proteins, or fresh complete growth medium for experiments that dually-transfected cells with pGlosensor^{TM} -22F cAMP plasmid and either *H. sapiens* TSHR, *A. aegypti* LGR1, or mCherry.

4.3.8 Preparation of protein samples

At 48 hours post-transfection, serum-free culture media containing secreted proteins were collected and concentrated in 0.5 mL 3-kDa molecular weight cut-off centrifugal filters (VWR North America). In some experiments, cells were dislodged with PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA; Life Technologies) (PBS-EDTA) pH 8.0, pelleted at 400x*g* for 5 min, resuspended in PBS and transferred to 1.5 mL centrifuge tubes for a subsequent centrifugation. Cell lysates were then prepared by resuspending and sonicating cells for 5 s in cell lysis buffer (37.5 mM Tris, pH = 7.5, 1.5 mM EDTA, pH 8.0, 3% sodium dodecyl sulfate, 1.5% protease inhibitor cocktail (v/v), and 1.5 mM dithiothreitol (DTT)). For receptor activity assays, to minimize carry-over of lysis buffer, cell lysates were concentrated in 3-kDa molecular weight cut-off centrifugal filters and re-constituted back to initial volumes with serum-free media for a total of three repetitions. Proteins were then used for cross-linking analysis, deglycosylation and immunoblotting or used as ligands for bioluminescent receptor activation and signalling assays.

Dissuccinimidyl suberate (DSS), a chemical cross-linker that strengthens protein intermolecular interactions, was employed to study GPA2 and GPB5 protein-protein interactions. *A. aegypti* and *H. sapiens* GPA2-FLAG and GPB5-His proteins were separately, or combined

together (GPA2-FLAG/GPB5-His) and then treated with DSS (Sigma Aldrich, Oakville, ON). In some experiments, *A. aegypti* GPA2-His/GPB5-His were co-expressed using a dual promoter plasmid and as such, media containing both His-tagged subunits were directly treated with DSS. To treat secreted concentrates of culture media containing *H.* sapiens GPA2 and GPB5 proteins, 0.68 mM DSS was used whereas 2.04 mM DSS was used to test the dimerization characteristics of *A. aegypti* GPA2-His/GPB5-FLAG proteins. Cross-linking was performed for 30 min at RT and reactions were quenched with 50 mM Tris, pH 7.4 for 10 min under constant mixing. To remove N-linked sugars, protein samples were treated with peptide-N-Glycosidase F (PNGase) (New England Biolabs, Whitby, ON) following manufacturers guidelines, with the only modification being that protein samples were not heated to 100°C before enzymatic deglycosylation. Experiments that aimed to determine the effects of protein deglycosylation on cross-linking ability first treated *A. aegypti* GPA2-His/ GPB5-His subunits with PNGase and subsequently cross-linked samples after deglycosylation with DSS.

4.3.9 Western blot analysis

Samples were prepared in 2x Laemmli buffer (Sigma Aldrich, Oakville, ON) containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH ~6.8, and resolved on 10% or 15% SDS-polyacrylamide gels under reducing conditions at 120 V for 90-110 min. Using a wet transfer system, proteins were then transferred to polyvinylidene difluoride (PVDF) membranes at 100 V for 75 min. For GPA2-FLAG/GPB5-His heterodimerization experiments, samples were run in duplicate on the same gel and following transfer, membranes were cut in half for separate primary antibody incubations. Membrane blots containing protein samples were blocked for 1 h in PBS containing Tween-20 (Bioshop,

Burlington, ON, Canada) (PBST) and 5% skim milk powder (PBSTB) rocking at RT. After blocking, membranes were incubated overnight at 4°C on a rocking platform in PBSTB containing mouse monoclonal anti-His (1:500 dilution) or mouse monoclonal anti-FLAG (1:500) primary antibody solutions. The next day, membranes were washed with PBST (3 x 15 min washes) and then were incubated in PBSTB containing anti-mouse HRP conjugated secondary antibody (1:2000 – 1:3000 dilution) for 1 h rocking at RT before washing again with PBST (3 x 15 min washes). Finally, blots were incubated with Clarity Western ECL substrate and images were developed using a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Mississauga, ON) and molecular weight measurements and analysis were performed using Image Lab 5.0 software (Bio-Rad Laboratories, Mississauga, ON). Western blots used to study recombinant GPA2 and GPB5 dimerization were repeated at minimum three times.

4.3.10 Bioluminescent assays

HEK 293T cells were co-transfected to express (i) *H. sapiens* TSHR, *A. aegypti* LGR1, or mCherry and (ii) pGlosensor^{TM} -22F cAMP plasmid (Promega Corp., Madison, WI), which encodes for a modified form of firefly luciferase with a fused cAMP binding moiety providing a biosensor for the direct detection of cAMP signalling in live cells. At 48 hours post transfection, recombinant cells were dislodged with PBS-EDTA, pelleted at 400x*g* for 5 min, resuspended in assay media (DMEM:F12 media with 10% fetal bovine serum (v/v)) containing cAMP GloSensor reagent (2% v/v), and incubated for 3 h rocking at RT shielded from light. 96-well luminescence plates (Greiner Bio-One, Germany) were loaded with previously prepared secreted or cell lysate protein concentrates (described above), forskolin or assay media alone, and

incubated at 37°C for 30 min prior to performing receptor activity assays. For stimulatory G-protein (Gs) pathway detection, recombinant cells were pre-treated with 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min at RT with rocking and shielded from light. Using an automatic injector unit, recombinant cells were then loaded into wells containing various treatments, including 250 nM forskolin as a positive control. For negative controls, concentrates of protein fractions from mCherry-transfected cells was used. For inhibitory G-protein (Gi/o) pathway testing, *A. aegypti* GPA2 and/or GPB5 was tested for the ability to inhibit a forskolin-induced cAMP-mediated bioluminescent response. Using an automatic injector, cells expressing or not expressing LGR1 were loaded into wells that contained various ligand treatments. Subsequently, 250 nM or 1 μM forskolin was added to wells using a second automatic injector unit. For bioluminescent assays with tethered GPA2/GPB5 proteins, after the 3 h incubation, recombinant cells expressing or not expressing LGR1 were equally divided to test Gs and Gi signalling pathways simultaneously using the same batch of cells per biological replicate. In all assays, luminescence was measured every 2 min for 20 min at 37°C using a Synergy 2 Multimode Microplate Reader (BioTek, Winooski, VT, USA) and averaged over 4-8 technical replicates for each treatment. To calculate the relative luminescent response, data was normalized to luminescent values recorded from treatments with 250 nM or 1 μM forskolin alone. Assays were performed repeatedly and involved 3-6 independent biological replicates. Relative luminescent values were compiled on Excel and transferred into GraphPad Prism 5.0 for statistical analysis. Data were analyzed using one-way or two-way ANOVA with Tukey's multiple comparison post-test ($p<0.05$).

4.4 Results

4.4.1 A. aegypti GPA2 and GPB5 subunit expression localization

To determine the distribution of GPA2 and GPB5 subunit expression in the central nervous system and peripheral tissues, adult mosquitoes were chosen for RT-qPCR analysis. GPA2 and GPB5 subunit transcript was enriched in the central nervous system of adult male and female mosquitoes, with significantly elevated expression in the abdominal ganglia relative to other nervous and peripheral tissues (Fig. 4-1A, B). Fluorescence *in situ* hybridization techniques were employed to localize GPA2 and GPB5 transcript expression in the abdominal ganglia. Control experiments involving sense probes did not detect cells in the nervous system (Fig 4-1C, D); however, GPA2 and GPB5 anti-sense RNA probes identified two bilateral pairs of neuroendocrine cells (Fig. 4-1E, F) within each of the first five abdominal ganglia in male and female mosquitoes. For a given ganglion, cells expressing GPA2 transcript localized to similar laterally-positioned cells as GPB5 transcript (Fig. 4-1G, H). To determine if cells expressing GPA2 transcript were the same cells expressing GPB5 transcript, abdominal ganglia were simultaneously treated with both GPA2 and GPB5 anti-sense RNA probes. Using this dual probe approach, results confirmed the detection of only two bilateral pairs of cells (Fig. 4-1I, J) that were greater in staining intensity compared to the intensity of cells detected by using either the GPA2 or GPB5 anti-sense probe alone (Fig. 4-1E, F).

Given the availability of an antibody to target the *A. aegypti* GPB5, we next sought to immunolocalize GPB5 protein in the abdominal ganglia. Control treatments with preabsorbed GPB5 antibody failed to detect any cells in ganglia (Fig. 4-2A). However, experimental treatments localized GPB5 immunoreactivity within two bilateral pairs of cells (Fig. 4-2B)

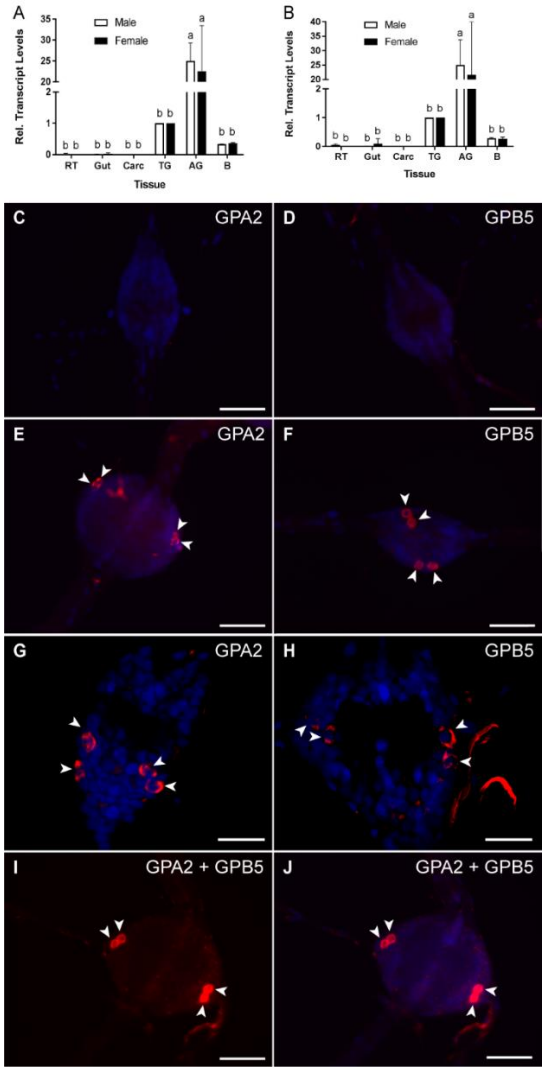

Figure 4-1: GPA2/GPB5 subunit transcript expression and localization in adult *A. aegypti*. (A, B) RT-qPCR to examine GPA2 (A) and GPB5 (B) transcript showed detection in the central nervous system of adult mosquitoes, with significant enrichment in the abdominal ganglia (AG). Subunit transcript abundance is shown relative to their expression in the thoracic ganglia (TG). Mean ± SEM of three biological replicates. Columns denoted with different letters are significantly different from one another. Multiple comparisons two-way ANOVA test with Tukey's multiple comparisons ($P<0.05$) to determine sex- and tissue-specific differences. Fluorescence *in situ* hybridization sense (C, D) and anti-sense (E-J) probes to determine GPA2 and GPB5 transcript localization (GPA2 and/or GPB5 transcript, red; nuclei, blue). Unlike sense probe controls (C, D), two bilateral pairs of cells (arrowheads) were detected with GPA2 (E, G) and GPB5 (F, H) anti-sense probes in the first five abdominal ganglia. Optical sections of ganglia demonstrate cells expressing GPA2 (G) transcript are localized to the same cells expressing GPB5 (H) transcript, which was verified by treating abdominal ganglia with a combination of GPA2 and GPB5 anti-sense probe (I, J) that revealed two, intensely-stained bilateral pairs of cells. In C-F and I-J, microscope settings were kept identical when acquiring images of control and experimental ganglia. Scale bars are 50 µm in C-F, I-J and 40 µm in G and H.

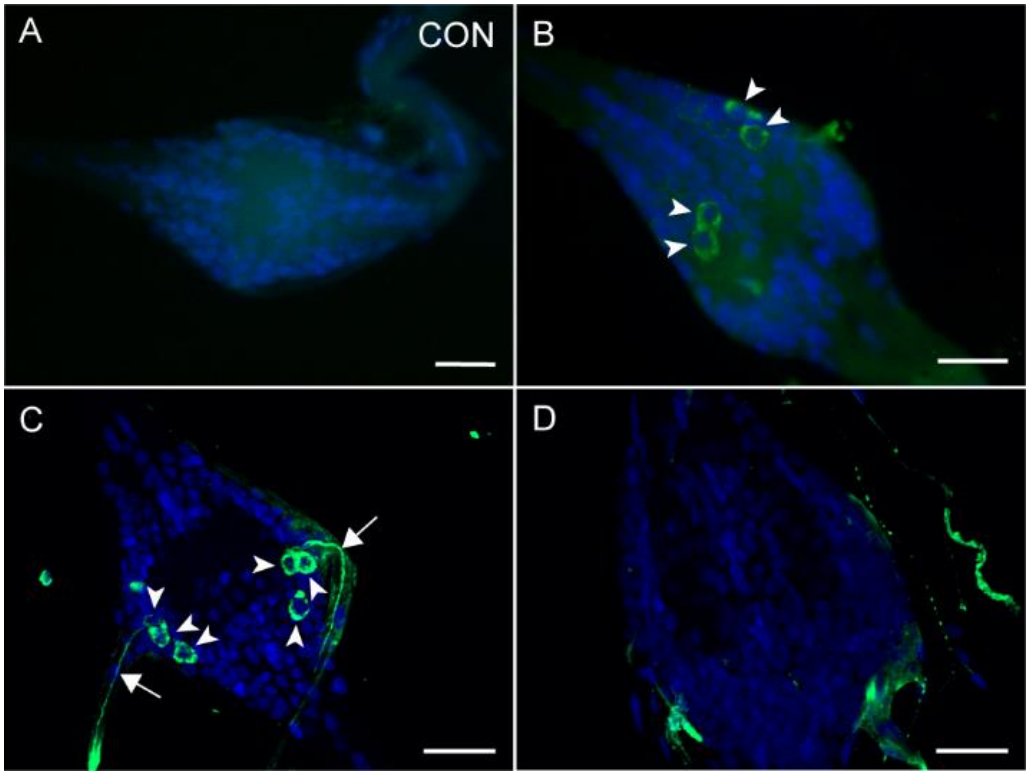

Figure 4-2: Immunolocalization of GPB5 subunit expression in the abdominal ganglia of adult *A. aegypti*. Unlike control treatments (CON) where GPB5 antibody was preabsorbed with GPB5 synthetic antigen (A), experimental treatments demonstrated GPB5 immunoreactivity (green) in two (B), and in some preparations, three (C) bilateral pairs of neuroendocrine cells (arrowheads) within the first five abdominal ganglia of the ventral nerve cord (DAPI, blue). Optical sections of ganglia revealed axons emanate from GPB5-immunoreactive cells through the lateral nerves (arrows). No cells were detected in the sixth, terminal ganglion (D). In A-B or C-D, microscope settings were kept identical when acquiring images of control and experimental ganglia. Scale bars are 25 µm in A-B and 20 µm in C-D.

within the first five ganglia, that were in similar positions to cells expressing GPA2 and GPB5 transcript (Fig. 4-1E-J). At times, three bilateral pairs of cells immunolocalized to the first five abdominal ganglia of the ventral nerve cord (Fig. 4-2C); however, no cells were ever detected in the sixth terminal ganglion (Fig. 4-2D). Along the lateral sides of a given abdominal ganglion, the axons of GPB5 immunoreactive cells were observed to closely associate into a tract of axons that emanated through the lateral nerve (Fig. 4-2C).

4.4.2 Cross-linking analyses to determine A. aegypti GPA2/GPB5 dimerization patterns

A. aegypti GPA2 and GPB5 subunit protein interactions were studied using western blots of recombinant proteins from HEK 293T cells expressing each subunit independently, or co-expressing both subunits in the same cells using a dual promoter plasmid. Expression constructs were designed to incorporate a hexa-histidine tag (His-tag) on the C-terminus of GPA2 and GPB5 for detection using a monoclonal His-tag antibody. Culture media containing secreted His-tag proteins were then collected, concentrated, and treated with DSS cross-linker to study subunit interactions. DSS-treated samples were also digested with PNGase to observe whether deglycosylation affected cross-linked subunit interactions. Under control untreated conditions, GPA2 protein is represented as two bands at 16 kDa and 13 kDa, which correspond to the glycosylated and non-glycosylated forms of *A. aegypti* GPA2, respectively (Fig. 4-3A). After deglycosylation with PNGase, the higher molecular weight band of GPA2 is eliminated, and the non-glycosylated lower molecular weight band intensifies (Fig. 4-3A). Interestingly, when GPA2 protein is tested for cross-linking activity using DSS, an additional strong band at ~32 kDa is detected and migrates to ~30 kDa when cross-linked protein samples are subjected to deglycosylation treatment (Fig. 4-3A). Under control untreated conditions, GPB5 protein is

represented as a band size at 24 kDa and is not affected by PNGase (Fig. 4-3B). Upon treatment with DSS, a faint, second band appears at 48 kDa, that does not change in molecular weight upon an additional treatment with PNGase (Fig. 4-3B). Three independent band sizes at 24 kDa (GPB5), 16 kDa and 13 kDa (glycosylated and non-glycosylated GPA2) were detected in lanes loaded with protein isolated from HEK 293T cells co-expressing GPA2 and GPB5 subunits (Fig. 4-3C). After removing N-linked sugars, the 24 kDa band is not affected but the 16 kDa band disappears and 13 kDa band intensifies (Fig. 4-3C; lane 2), as observed previously (Fig. 4-3A). Cross-linked samples show the addition of two higher molecular weight bands at ~48 kDa and ~32 kDa; which migrate lower to ~30 kDa when subjected to PNGase treatment (Fig. 4-3C).

Given the resolution and thickness of the detected bands, molecular weight bands at ~48 kDa and ~32 kDa could indicate GPA2/ GPB5 heterodimeric interactions (13 or 16 kDa + 24 kDa = 37-40 kDa). Alternatively, these bands could reflect GPA2 (13 or 16 kDa + 13 or 16 kDa = 26-32 kDa) and GPB5 (24 + 24kDa = 48 kDa) homodimers. As a result, to clarify whether *A. aegypti* GPA2 and GPB5 subunits are heterodimeric partners, additional experiments were performed.

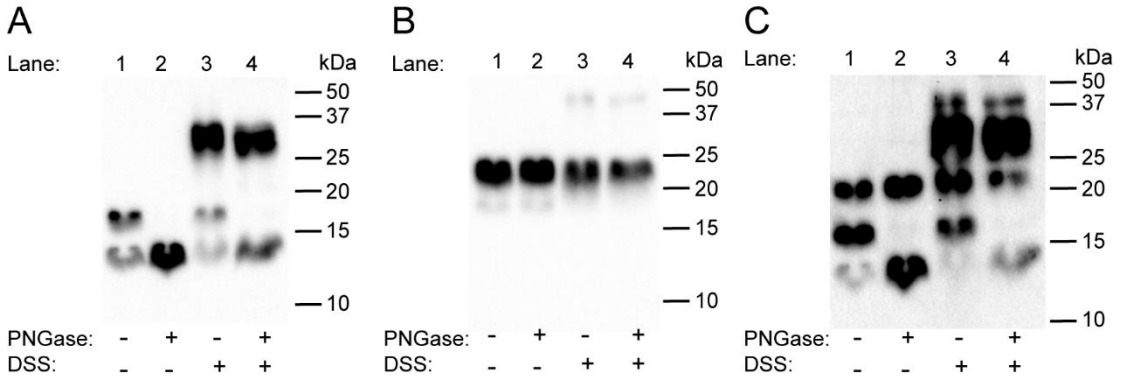

Figure 4-3: Western blot analyses to determine the effects of glycosylation on homo- and heterodimer formation on the glycoprotein hormone (GPA2/GPB5) subunits in the mosquito, *A. aegypti*. (A) Under untreated conditions, immunoblot analyses of GPA2 subunit alone reveals two bands at 16 and 13 kDa. Upon treatment with PNGase, the higher molecular weight band disappears and the 13 kDa band is intensified. A thick, additional band at ~32 kDa appears when GPA2 protein is cross-linked with DSS, and this band migrates slightly lower to ~30 kDa when GPA2 protein is treated with both DSS and PNGase. (B) A band at 24 kDa is observed in lanes loaded with untreated GPB5 subunit alone. Upon PNGase treatment, the 24 kDa band is not affected; however, upon treatment with DSS, a second faint band appears at 48 kDa that is not affected by deglycosylation. (C) Western blot analyses of co-expressed GPA2 and GPB5 subunits shows three distinct band sizes at 24 kDa, 16 kDa and 13 kDa, corresponding to the GPB5 subunit and two forms of GPA2 subunit protein. Similar to (A), upon treatment with PNGase, the higher molecular weight form of GPA2 is abolished and the 13 kDa band intensifies. When GPA2/GPB5 protein is treated with the DSS cross-linker, two additional bands are detected at ~48 kDa and ~ 32 kDa. Upon treating protein samples with DSS and PNGase, the ~32 kDa band migrates to 30 kDa whereas the ~48 kDa band is not affected.

4.4.3 Heterodimerization of mosquito and human GPA2/ GPB5

Using yeast two-hybrid analyses and cross-linking experiments, it was previously shown that *Homo sapiens* (human) GPA2 (hGPA2) and GPB5 (hGPB5) subunits are capable of heterodimerization (Nakabayashi et al. 2002). As a result, to verify whether *A. aegypti* GPA2 and GPB5 subunit proteins are heterodimeric candidates, experiments were first performed alongside hGPA2/hGPB5 subunit proteins, using the latter as a positive control.

To begin, single-promoter expression constructs with differentially-tagged subunits were designed to incorporate a FLAG-tag and His-tag on the C-terminus of (human and mosquito) GPA2 and GPB5 subunits, respectively (i.e. GPA2-FLAG and GPB5-His). After transfecting HEK 293T cells, media containing secreted proteins was collected, concentrated, and cross-linked with DSS. Upon gel electrophoresis, immunoblots were probed with either a monoclonal anti-FLAG or anti-His antibody to detect tagged subunit partners. When probed with an anti-His antibody, no bands were detected in lanes containing only GPA2-FLAG (human and mosquito) protein (Fig. 4-4A, B). Similarly, no bands were detected when an anti-FLAG antibody was used to detect GPB5-His protein (human and mosquito) (Fig. 4-4A, B). Lanes loaded with cross-linked human GPB5-His protein revealed two bands at 18 kDa (monomer) and 36 kDa (homodimer) (Fig. 4-4A). Similarly, in lanes loaded with cross-linked human GPA2-FLAG protein, two bands at 20 kDa (monomer) and 40 kDa (homodimer) were detected (Fig. 4-4A). A combination and subsequently cross-linking of separately-produced human GPA2-FLAG and human GPB5-His protein revealed an intense 38 kDa band size that correlated to the molecular weight of the hGPA2-FLAG/hGPB5-His heterodimers; detected with both anti-His and anti-FLAG primary antibody solutions (Fig. 4-4A).

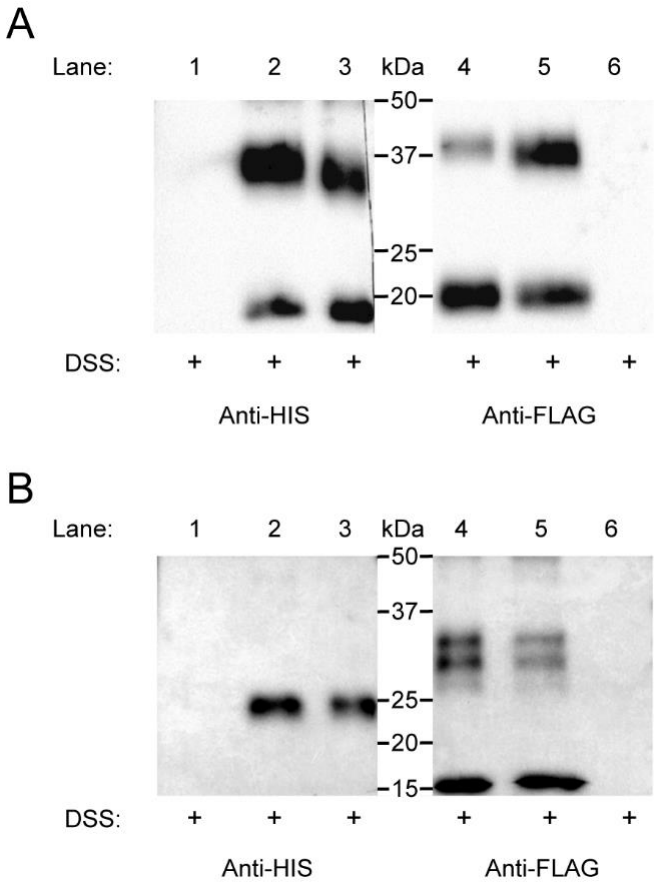

Figure 4-4: Elucidating heterodimerization of *H. sapiens* (human) (A) and *A. aegypti* (mosquito) (B) GPA2 and GPB5 subunits. Single promoter expression constructs for human and mosquito GPA2-FLAG and GPB5-His were transiently transfected in HEK 293T cells. Protein was subsequently concentrated, treated with DSS cross-linker, and probed with an anti-His or an anti-FLAG antibody after gel electrophoresis. (A, B) No bands were detected in lanes loaded with cross-linked GPA2-FLAG protein (Lane 1) or cross-linked GPB5-His protein (Lane 6), probed with an anti-His antibody or anti-FLAG antibody respectively. (A) Bands corresponding to the monomeric form (18 kDa) and homodimer (36 kDa) of cross-linked human GPB5-His (Lane 3) and to the monomeric form (20 kDa) and homodimer (40 kDa) of cross-linked human GPA2-FLAG protein (Lane 4). A combination, and subsequently cross-linking, of separately-produced human GPA2-FLAG and GPB5-His protein (Lane 2, 5) revealed a band size correlating to the human GPA2/ GPB5 heterodimer (38 kDa), detected using an anti-His (Lane 2) or anti-FLAG (Lane 5) antibody. (B) Bands corresponding to the mosquito GPB5 monomer (24 kDa) (Lane 3), mosquito GPA2 glycosylated monomer (16 kDa) and homodimer pairs (30 kDa and 32 kDa) (Lane 4). No detection of bands correlating to mosquito heterodimer-like pairs (37-40 kDa) were observed, when probed with either anti-His (Lane 2) or anti-FLAG (Lane 5) antibodies.

The same experiments were replicated using *A. aegypti* GPA2-FLAG/ GPB5-His protein but using three-fold higher concentration of DSS cross-linker to help improve inter-subunit interactions. Results showed that lanes loaded with DSS-treated GPB5-His protein resulted in a 24 kDa monomer band (Fig 4-4B), whereas lanes containing cross-linked GPA2-FLAG detected a 16 kDa (glycosylated monomer), 30 kDa and 32 kDa (homodimers) band size (Fig. 4-4B). As a result, unlike immunoblots containing hGPA2-FLAG and hGPB5-His protein (Fig. 4-4A), lanes loaded with cross-linked *A. aegypti* GPA2-FLAG and GPB5-His failed to provide evidence of bands correlating to the predicted molecular weight of an *A. aegypti* GPA2/GPB5 heterodimer (expected at 37-40 kDa) (Fig. 4-4B).

4.4.4 A. aegypti GPA2/GPB5 fail to activate LGR1-mediated Gs and Gi/o signalling pathways

Bioluminescent assays were employed to confirm LGR1 interacts with mosquito GPA2 and/or GPB5 subunits and elucidate downstream signalling pathways upon receptor activation. Since human GPA2/GPB5 has previously been shown to successfully bind and activate a human thyrotropin receptor (hTSHR)-mediated stimulatory G protein (Gs) signalling pathway (Nakabayashi et al. 2002), our experimental design was first tested using hGPA2/GPB5 and hTSHR as a positive control. Recombinant hGPA2 and hGPB5 subunit proteins were produced in HEK 293T cells with single promoter expression constructs containing the hGPA2 or hGPB5 sequences. Culture media containing secreted proteins were subsequently concentrated, and crude extracts of either hGPA2, hGPB5 or a combination of hGPA2 and hGPB5 proteins were

used as ligands for incubations with HEK 293T cells expressing the hTSHR and a mutant luciferase biosensor (luciferase), which emits light upon interacting with cAMP. Control treatments involved the incubation of hTSHR/ luciferase-expressing cells with concentrated media collected from red fluorescent protein (mCherry)-transfected cells (negative control), and data was normalized to treatments with 250 nM forskolin (positive control) (Fig. 4-5A). Results showed incubations with a combination of both hGPA2 and hGPB5, rather than each subunit alone, were required to stimulate a cAMP-mediated luminescent response from hTSHR-expressing cells compared to control treatments (Fig. 4-5A). We next sought to perform similar experiments using *A. aegypti* GPA2/GPB5 and LGR1. Given the availability of a dual promoter plasmid, an additional treatment was performed whereby LGR1/ luciferase-expressing cells were incubated with GPA2/GPB5 protein that was co-expressed within the same cells. Unlike hGPA2/hGPB5 subunit homologs (Fig. 4-5A), no combination of mosquito GPA2 and/or GPB5 subunit proteins elevated a cAMP-mediated luminescent response above negative control treatments with mCherry secreted proteins (Fig. 4-5B).

Using an *in silico* analysis to predict coupling specificity of *A. aegypti* LGR1 and human TSHR to different families of G-proteins (Sgourakis et al. 2005), *A. aegypti* LGR1 was strongly predicted to couple to inhibitory (Gi) G proteins (Table 4-S3). As a result, to determine whether *A. aegypti* GPA2 and/or GPB5 activate Gi/o signalling pathway, various combinations of GPA2 and GPB5 were tested for their ability to inhibit a forskolin-induced rise in cAMP luminescence (Fig. 4-5C, D). Results showed that sole treatments of GPB5 proteins alone significantly inhibited a forskolin-induced luminescent response, relative to control treatments with mCherry proteins, when incubated with cells expressing LGR1 (Fig. 4-5C). However,

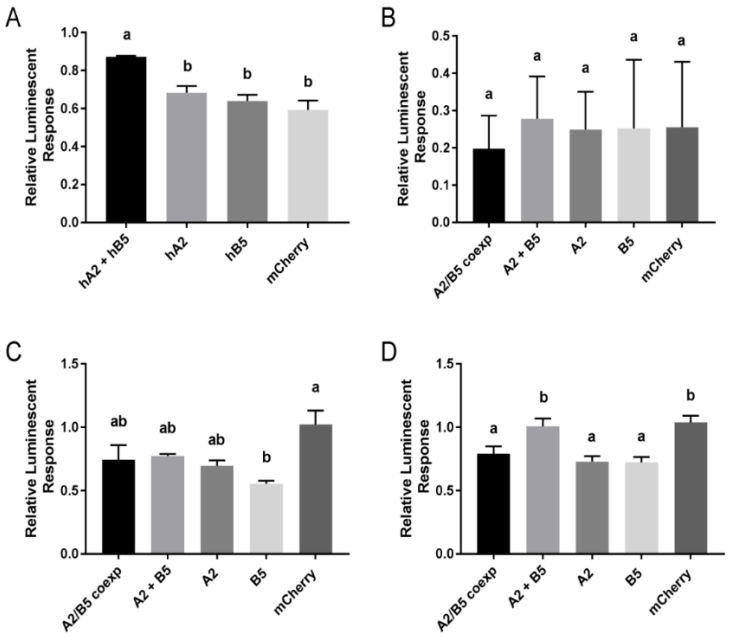

Figure 4-5: cAMP-mediated bioluminescent assays to determine the effects of GPA2, GPB5 and GPA2/ GPB5 on G-protein signalling of *H. sapiens* (human) TSHR (A), *A. aegypti* (mosquito) LGR1 (B, C), or cells expressing a red fluorescent protein, mCherry (D). Secreted protein fractions for each subunit were prepared separately from the culture media of HEK 293T cells expressing human GPA2 (hA2), human GPB5 (hB5), mosquito GPA2 (A2), mosquito GPB5 (B5), mcherry (mCherry), or co-expressing mosquito GPA2 and GPB5 in a dual promoter plasmid (A2/B5 coexp). Secreted protein fractions were then separately added, or combined (A2 + B5) and then incubated with cells co-expressing the pGlosensor^{TM} cAMP biosensor -22F reporter construct and (A) human TSHR, (B, C) mosquito LGR1 or (D) mCherry that was used as a negative control in the functional assay and served also as a control to verify transfection efficiency of HEK 293T cells. (A-D) Luminescent values were recorded and normalized to luminescence response from treatments with 250 nM forskolin. (A) Unlike treatments with human GPA2 (hA2) or human GPB5 (hB5) applied singly, a significant increase in cAMP-mediated luminescence was observed when TSHR-expressing cells were incubated with culture media containing both human GPA2 and human GPB5 (hA2 + hB5), relative to incubations with mCherry-transfected media. (B) No differences in luminescence were observed when LGR1-expressing cells were incubated with media containing mosquito proteins, compared to mCherry treatments. (C) The addition of B5 on LGR1-expressing cells significantly inhibited FSK-induced luminescent response, compared to treatments with mCherry fractions; (D) however, this inhibition was also observed when A2 and B5 proteins were incubated with cells not expressing mosquito LGR1. Mean ± SEM of three (A, B, D) or six (C) biological replicates. Columns denoted with different letters are significantly different from one another. Multiple comparisons one-way ANOVA test with Tukey's multiple comparisons ($P<0.05$).

Table 4-S3. Normalized scores for predicted G-protein coupling specificity for *A. aegypti* LGR1 and *H. sapiens* TSHR by PRED-COUPLE 2.0 (Sgourakis et al. 2005). Leucine-rich repeat-containing G protein-coupled receptor 1 (LGR1), thyroid-stimulating hormone receptor (TSHR).

G Protein	***Aedes aegypti* LGR1**	***Homo sapiens* TSHR**
Gs	0.36	0.9
Gi/o	0.97	0.94
Gq/11	0.9	0.84
G12/13	0.92	0.92

similar inhibitive effects of GPA2 and GPB5 proteins were also observed with cells not expressing LGR1 (Fig. 4-5D).

4.4.5 Characterization of tethered A. aegypti GPA2/GPB5

Receptor activation was only observed when both human GPA2 and GPB5 subunits were available for receptor binding (Fig. 4-5A) and, unlike human GPA2/GPB5 subunit heterodimerization that was observed in our experiments, mosquito GPA2/GPB5 lacked evidence of heterodimerization using the heterologous expression system (Fig. 4-4). As a result, it was hypothesized that the activation of glycoprotein hormone receptors, like *A. aegypti* LGR1, is dependent on subunit heterodimerization. To mimic GPA2/GPB5 heterodimers, both GPA2 and GPB5 mosquito subunits were expressed as a tethered, single-chain polypeptide by fusing the C-terminus of the GPB5 prepropeptide sequence with the N-terminus of the GPA2 propeptide sequence using a tagged linker sequence composed of twelve amino acids, involving three glycine-serine repeats and six histidine residues.

HEK 293T cells were transiently transfected to express a single promoter plasmid construct containing the tethered GPA2/GPB5 sequence, or the red fluorescent protein (mCherry) as a negative control. After transfection, cell lysates and the culture media that bathed cells, the latter of which contains secreted proteins, were collected for immunoblot analysis. No bands were detected in lanes containing cell lysate or secreted protein fractions of mCherry transfected cells (Fig. 4-6A). However, a strong band was detected at 32-40 kDa in the lysates of cells transfected to express tethered GPA2/GPB5 (Fig. 4-6A). Moreover, two bands were

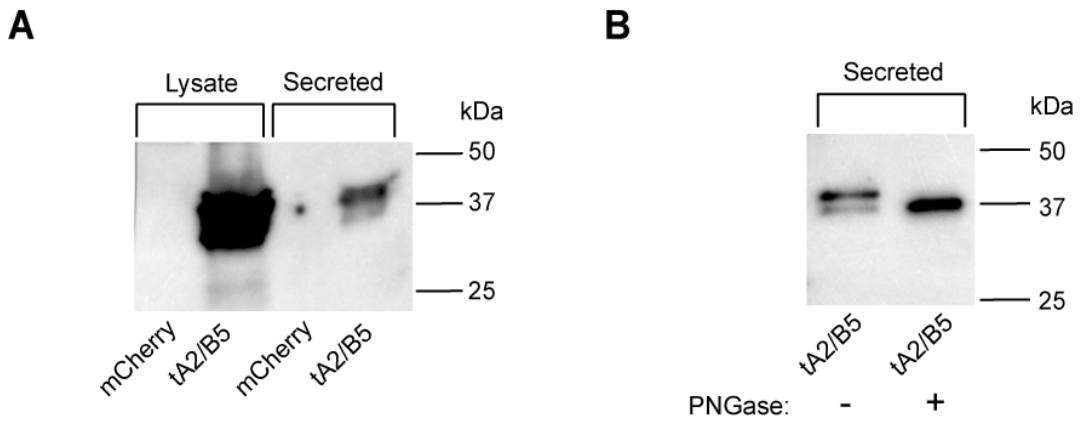

Figure 4-6: Western blot analysis and verification of *A. aegypti* GPA2/GPB5 tethered protein expressed in HEK 293T cells. (A) Western blot analysis of secreted (Secreted) or cell lysate (Lysate) protein fractions of HEK 293T expressing tethered GPA2/GPB5 (tA2/B5) or red fluorescent protein (mCherry) as a control. Tethered GPA2/GPB5 is represented as a strong 32-40 kDa band in cell lysate fractions, and as two relatively less intense 37 kDa and 40 kDa bands in secreted fractions; however, no bands were detected in lanes loaded with proteins from mCherry transfected cells. (B) Upon treatments of tethered GPA2/GPB5 secreted protein fractions with PNGase, the 40 kDa band disappears and the 37 kDa band intensifies, indicating removal of N-linked oligosaccharides.

detected at 37 kDa and 40 kDa in lanes loaded with secreted fractions of tethered GPA2/GPB5-transfected cells, that correlated to the predicted molecular weights of *A. aegypti* GPA2/GPB5 heterodimers corresponding to either non-glycosylated (13 kDa) or glycosylated (16 kDa) GPA2 plus GPB5 (24 kDa) producing bands of 37 kDa or 40 kDa, respectively (Fig. 4-6A). After treatments of secreted protein extracts containing tethered GPA2/GPB5 proteins with PNGase, the higher 40 kDa molecular weight band disappears and the 37 kDa band size intensifies confirming the observed shift in molecular weight is a result of removal of N-linked oligosaccharides (Fig. 4-6B).

4.4.6 Tethered A. aegypti GPA2/GPB5 activates LGR1

The effects of tethered GPA2/GPB5 proteins on LGR1 activity was examined. Cell lysates or secreted protein fractions collected from mCherry- or tethered GPA2/GPB5-transfected cells were incubated with HEK 293T cells co-expressing the cAMP luciferase biosensor and either *A. aegypti* LGR1 or mCherry (i.e. not expressing LGR1). Whether tethered GPA2/GPB5 proteins could elevate cAMP or inhibit a forskolin-induced rise in cAMP was assessed and compared to control treatments with proteins harvested from mCherry-transfected cells.

Overall, the relative luminescent response was greater in LGR1-transfected cells compared to mCherry-transfected cells (Fig. 4-7). However, neither secreted protein fractions nor cell lysates of tethered GPA2/GPB5-transfected cells were capable of eliciting an increase in the cAMP-mediated luminescent response relative to controls (Fig. 4-7A, B). Secreted protein fractions containing tethered GPA2/GPB5 protein had no effect on the forskolin-induced cAMP-mediated luminescence, compared to control treatments with mCherry secreted proteins in LGR1-expressing cells (Fig. 4-7C). Notably, however, treatments of LGR1-transfected cells, but

not cells lacking LGR1,with cell lysates containing tethered GPA2/GPB5 protein significantly inhibited the forskolin-induced rise in cAMP relative to treatments with mCherry cell lysates (Fig. 4-7D).

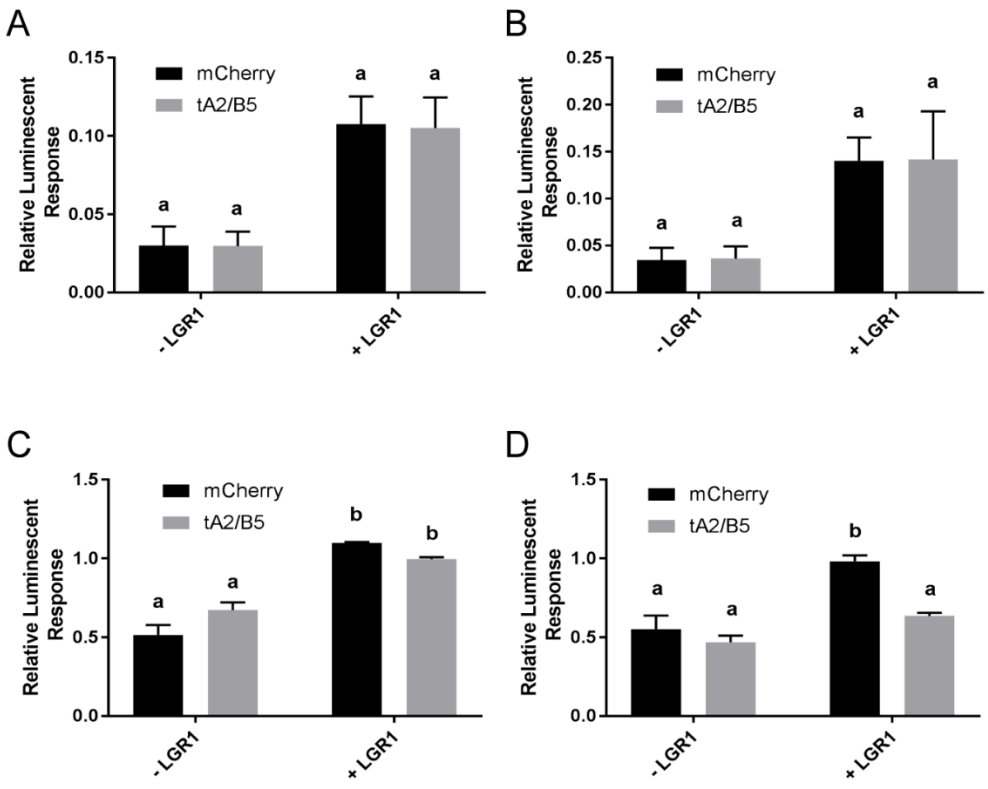

Figure 4-7: cAMP-mediated bioluminescent assays to determine the effects of tethered GPA2/GPB5 on receptor activation and G protein signalling of LGR1. Secreted protein fractions (A, C) and cell lysates (B, D) derived from cells transiently transfected to express tethered GPA2/GPB5 (tA2/B5) or red fluorescent protein (mCherry) were tested for their ability to stimulate (Gs signalling) (A, B) or inhibit 1 µM forskolin-induced (Gi/o signalling) (C, D) cAMP-mediated luminescence, upon incubations with recombinant cells co-expressing the -22F cAMP biosensor variant and LGR1 (+ LGR1) or red fluorescent protein (- LGR1). (A-D) Luminescent values were recorded and shown as normalized to the luminescence from sole treatments with 1 µM forskolin on LGR1-expressing cells. (C, D) In treatments involving mCherry proteins, the relative luminescent response was greater in LGR1-transfected cells (+ LGR1) compared to cells not expressing LGR1 (- LGR1). (A, B) Incubations of LGR1-expressing cells with tA2/B5 secreted (A) or cell lysate (B) proteins, failed to increase cAMP-mediated luminescence above incubations with mCherry proteins. (C, D) 1 uM forskolin and mCherry proteins or tA2/B5 proteins was added to wells expressing or not expressing LGR1. The ability for each ligand treatment to reduce a forskolin-induced increase in cAMP luminescence was examined. (C) The tA2/B5 secreted protein samples incubated with LGR1-expressing cells did not significantly affect the forskolin-induced cAMP luminescence, compared to incubations with mCherry secreted proteins. (D) Relative to incubations with mCherry cell lysate proteins, cell lysates containing tA2/B5 protein significantly inhibited forskolin-induced elevations of cAMP-mediated luminescence, and this inhibition was observed to be specific to LGR1-transfected cells. Mean ± SEM of three biological replicates. Columns denoted with different letters are significantly different from one another. Multiple comparisons two-way ANOVA test with Tukey's multiple comparisons ($P<0.05$).

4.5 Discussion

4.5.1 Co-expression of A. aegypti GPA2/GPB5 in neuroendocrine cells of the abdominal ganglia

The central nervous system (CNS) of adult mosquitoes is comprised of a brain and a ventral nerve cord, consisting of three thoracic ganglia and six abdominal ganglia. GPA2 and GPB5 transcript is significantly enriched in the abdominal ganglia of adult mosquitoes relative to peripheral tissues and other regions of the CNS, with no sex-specific differences. Though GPA2 and GPB5 transcript was identified in the thoracic ganglia and brain, fluorescence *in situ* hybridization (FISH) techniques used to localize GPA2 and GPB5 transcript, as well as GPB5 immunoreactivity, did not identify specific cells in these regions of the nervous system. Instead, GPA2 and GPB5 transcript, as well as GPB5 immunoreactivity was identified in two-three bilateral pairs of neuroendocrine cells within the first five abdominal ganglia. These findings are consistent with previous findings in the fruit fly *Drosophila melanogaster*, where GPA2 and GPB5 subunit transcript was localized to four bilateral pairs of neuroendocrine cells in the fused ventral nerve cord, that were distinct from cells expressing other neuropeptides including leucokinin, bursicon, crustacean cardioactive peptide or calcitonin-like diuretic hormone (Sellami et al. 2011).

Both FISH and immunohistochemical techniques revealed GPA2 and GPB5 expression within two bilateral pairs of neuroendocrine cells, that were positioned slightly posterior to where the lateral nerves emanate. In some mosquitoes, a third bilateral pair of cells expressing GPB5 protein was detected; however these additional cells were not detected using FISH, which suggests GPB5 transcript may be differentially regulated between different bilateral pairs of cells. Given that the same number of cells were observed to express GPA2 transcript, and these cells localized to similar positions as GPB5 expressing cells, GPA2 and GPB5 were hypothesizd to be co-expressed in the same cells. To verify cellular co-expression of GPA2/GPB5, abdominal

ganglia were treated with GPA2- and GPB5-targeted RNA anti-sense probes simultaenously. Given that only two bilateral pairs of cells were detected, and that these cells were more intensly stained compared to preparations subjected to only GPA2 or GPB5 probe alone, our results confirm that GPA2 and GPB5 are co-expressed within the same neurosecretory cells of the abdominal ganglia in adult mosquitoes. The cellular co-expression of GPA2 and GPB5 proteins implies that, upon a given stimulus, both subunits are simultaneously released and regulated in a similar manner. Importantly, since co-expression and heterodimerization of the classic glycoprotein hormone subunits takes place within the same cells (Galet et al. 2004; Xing et al. 2004), these findings indicate that the GPA2/GPB5 subunits are likely produced and released as heterodimers.

4.5.2 Heterodimerization and Homodimerization of GPA2/GPB5

To study the interactions of *A. aegypti* GPA2 and GPB5 subunits *in vitro*, hexa-histidine tagged proteins secreted into the culture media of HEK 293T cells were collected and analysed under denaturing conditions after cross-linker treatments, which had been utilized previously to show GPA2/GPB5 heterodimerization in other organisms (Nakabayashi et al. 2002; Sudo et al. 2005; Sun et al. 2010). Cross-linked protein samples were then deglycosylated to identify whether the removal of N-linked sugars affected dimerization. Sole treatments of GPA2 and GPB5 protein with cross-linker resulted in the detection of bands with sizes corresponding to homodimers of GPA2 (~32 kDa) and GPB5 (48 kDa). GPA2 homodimer bands migrated lower to ~30 kDa upon a subsequent treatment with PNGase. However, experiments performed with cross-linked GPA2/GPB5 protein were not able to confirm heterodimeriation since the detected band sizes could also reflect GPA2 and GPB5 homodimeric interactions. Previous cross-linking studies that

demonstrated GPA2/GPB5 heterodimerization in human (Nakabayashi et al. 2002; Sun et al. 2010) and fruit fly (Sudo et al. 2005) did not provide evidence on the interactions of each subunit alone to determine if homodimerization is possible. In these studies, molecular weight band sizes that were identified as heterodimers, could have been the result of homodimeric interactions (Nakabayashi et al. 2002; Sudo et al. 2005; Sun et al. 2010). As a result, additional experiments are required to confirm GPA2/GPB5 heterodimerization in these organisms.

To clarify whether *A. aegypti* (mosquito) GPA2/GPB5 heterodimerize, each subunit was differentially tagged (GPA2-FLAG and GPB5-His), and immunoblots containing various combinations of cross-linked proteins were probed with either anti-FLAG or anti-His antibody. As a positive control, experiments were first performed using *H. sapiens* (human) GPA2/GPB5 subunit proteins. Similar to mosquito GPA2 and GPB5 subunits, results showed human GPA2 and GPB5 subunits are capable of homodimerization. To study heterodimeric interactions, GPA2 and GPB5 subunit proteins were expressed separately in HEK 293T cells. Upon combining and treating protein samples with DSS, a molecular weight band size at 38 kDa, corresponding to the molecular weight of GPA2/GPB5 heterodimers (human GPA2-FLAG/ GPB5-His), was detected and migrated differently than bands corresponding to GPA2 (40 kDa) and GPB5 (36 kDa) homodimers. Taken together, our results confirm human GPA2 and GPB5 are capable of heterodimerization *in vitro*. Further, an induction of cAMP was observed when both subunits were present for TSHR activation, whereas treatments with individual subunits showed small, insignificant increases of cAMP-mediated luminescence relative to negative control treatments. As a result, human GPA2/GPB5 is capable of heterodimerization, and since a combination of both subunits were required to signal a TSHR-mediated elevation in cAMP, these heterodimers are required to functionally activate its cognate glycoprotein hormone receptor (TSHR) *in vitro*.

The classic glycoprotein hormones, which encompass FSH, LH, TSH and CG, are formed by a common alpha subunit non-covalently linked to a hormone-specific beta subunit. Each subunit is co-expressed and assembled as a heterodimer in the endoplasmic reticulum within cells before being secreted as heterodimer into circulation (Xing et al. 2004). Heterodimerization is required for secretion and receptor binding activity of each hormone (Pierce and Parsons 1981; Brownstein 1985; Szkudlinski et al. 2002). Our results confirm human GPA2/GPB5 heterodimerizes, as detected following chemical cross-linking treatments and, even without cross-linking, both subunits are required to activate TSHR in our studies. In these experiments, human GPA2 and GPB5 subunits were not co-expressed within the same cells using a dual promoter expression construct. Rather, subunits were separately expressed by different batches of cells and mixed post harvesting of conditioned media to demonstrate heterodimerization and TSHR activation. Thus, unlike FSH, TSH, CG, and LH (Pierce and Parsons 1981), our results provide novel information that demonstrate co-expression is not necessarily required for heterodimerization of human GPA2/GPB5 *in vitro*.

Analogous experiments using the same (data not shown) as well as three-fold higher concentrations of DSS cross-linker were performed with mosquito GPA2-FLAG and GPB5-His subunit proteins; however, no bands corresponding to the expected molecular weights of GPA2/GPB5 heterodimers (37-40 kDa) were observed (Fig. 4-4B). Rather, only bands resembling GPA2 and GPB5 homodimers were detected (Fig. 4-4B). The inability for mosquito GPA2 and GPB5 subunits to successfully heterodimerize could result from improper protein folding of insect-derived secretory proteins in HEK 293T cells, a mammalian cell model. As a result, future experiments should test the heterologous expression and heterodimerization of *A.*

aegypti GPA2/GPB5 in insect cell lines that could provide a more appropriate environment for tertiary and quaternary protein structure formation.

Mosquito GPA2/GPB5 (each subunit alone, mixed from different cell batches or co-expressed in the same cells using a dual promoter vector) was not capable of stimulating a cAMP-mediated luminescent response in LGR1 expressing cells. Since LGR1 is predicted to couple a Gi/o signalling pathway (Table 4-S3), we next sought to determine if mosquito GPA2/GPB5 could inhibit a forskolin-induced cAMP response. Sole treatments of GPA2 and GPB5 subunit proteins inhibited a forskolin-induced rise in cAMP, however these inhibitory actions were not owed to G protein signalling events related to *A. aegypti* LGR1, since inhibition was observed in control cell lines without LGR1. These results suggest mosquito GPA2 and GPB5 subunit proteins may non-specifically interact with other endogenously expressed proteins, like the orphan glycoprotein hormone receptors LGR4 and LGR5 that are highly expressed in HEK 293T cells (Atwood et al. 2011).

4.5.3 A. aegypti GPA2/ GPB5 heterodimers activate LGR1 and initiate a switch from Gs to Gi coupling

Activation of the human thyrotropin receptor was only observed when both human GPA2 and GPB5 subunits were present which, unlike results involving mosquito subunits, demonstrated human GPA2/GPB5 subunit heterodimerization *in vitro* using the mammalian heterologous system. Nonetheless, given the observed co-localization of the subunits within 2-3 bilateral pairs of cells in the first five abdominal ganglia in *A. aegypti*, and also shown earlier in *D. melanogaster* (Sellami et al. 2011), it was hypothesized that *A. aegypti* GPA2/GPB5 heterodimers are required to functionally activate LGR1 *in vitro*. To test this hypothesis, a

tethered construct was designed that linked the C terminus of GPB5 to the N-terminus of GPA2 using a histidine tagged glycine/serine-rich linker sequence. Natural and synthetic linkers function as spacers that connect multidomain proteins, and are commonly used to study unstable or weak protein-protein interactions (Priyanka et al. 2013). The incorporation of a linker sequence between glycoprotein hormone subunits has been performed previously, and does not affect the assembly, secretion or bioactivity of human FSH (Fares et al. 1992), TSH (Joshi et al. 1995) and CG (Furuhashi et al. 1995). The conversion of two independent glycoprotein hormone subunits to a single polypeptide chain using a glycine-serine repeat linker sequence has been performed previously with lamprey GPA2/GPB5 (Sower et al. 2015), and in this study, tethered GPA2/GPB5 was shown to induce a cAMP response. Interestingly, similar proteins involving TSH alpha fused to TSH beta with carboxyl-terminal peptide (CTP) as a linker promoted a three-fold higher induction of cAMP compared to wild-type TSH, likely because the addition of a CTP linker increases protein stability and flexibility (Azzam et al. 2012).

HEK 293T cells were transiently transfected to express tethered *A. aegypti* GPA2/GPB5 and cell lysates and secreted protein fractions were collected for expression studies. Immunoblot analysis revealed the detection of a strong 32-40 kDa band in lanes loaded with cell lysate proteins, and two less intense distinct bands at 40 kDa and 37 kDa in secreted protein fractions of GPA2/GPB5-transfected cells. Molecular weight band sizes at 40 kDa and 37 kDa in secreted protein fractions matched the expected band sizes of tethered GPA2/GPB5 heterodimers, corresponding to nonglycosylated 13 kDa or glycosylated 16 kDa GPA2 plus 24 kDa GPB5. Moreover, treatments with PNGase revealed tethered GPA2/GPB5 proteins are glycosylated, as observed for GPA2 expressed independantly in earlier experiments and in previous studies (Paluzzi et al. 2014). Highly intense bands in cell lysates indicate the retention of immature and

mature GPA2/GPB5 heterodimers. For this reason, cell lysates and secreted protein fractions of tethered GPA2/GPB5-expressing cells were individually tested on their ability to activate LGR1.

In humans, GPA2/GPB5-TSHR signalling stimulates adenylyl cyclase activity to increase cytosolic cAMP via interaction with a Gs protein (Nakabayashi et al. 2002; Sun et al. 2010; Huang et al. 2016), and these results were confirmed in our studies. Comparatively, GPA2/GPB5 signalling was also shown to increase levels of cAMP upon binding LGR1 in *D. melanogaster* (Sudo et al. 2005). Interestingly, our experiments demonstrate low level constitutive basal activity of adenylyl cyclase in LGR1 expressing cells since cAMP luminescent response was moderately greater in LGR1-transfected cells compared to cells not expressing LGR1. One possible limitation in our experimental design was the lack of a control for transient transfection, since greater cAMP-mediated luminescence from LGR1-transfected cells compared to cells not expressing LGR1, demonstrating constitutive basal activity of LGR1, could be a result of different quantities of recombinant cells or variable luciferase activity. However, to avoid bias, cells were transfected in *en masse* and equally distributed amongst wells just before the luciferase assay, and error was further minimized by repeating experiments multiple times. Moreover, our results parallel previous findings since constitutive activity of glycoprotein hormone receptors is well known and has been demonstrated to be stronger for the thyrotropin receptor than the LH/CG receptor (Cetani et al. 1996). Surprisingly, our experiments indicate that incubations of LGR1-expressing cells with the fusion GPA2/GPB5 tethered protein triggers a switch from low level constitutive Gs coupling to Gi coupling for *A. aegypti* LGR1, given that tethered GPA2/GPB5 signficantly inhibited the forskolin-induced increase in cAMP in LGR1-transfected cells but not in cells lacking LGR1 expression . Promiscuous G protein coupling has been reported for glycoprotein hormone receptors like the TSH receptor (Gs and Gq) and LH/CG

(Gi and Gs) (Herrlich et al. 1996; Grasberger et al. 2007; Kleinau et al. 2010).

4.5.4 Intersubunit interaction and evidence for GPA2/GPB5 heterodimers

To help stabilize heterodimerization, the beta subunit sequences of the classic glycoprotein hormones (FSH, LH, TSH and CG) contain two additional cysteine residues that form an additional disulfide bridge which wraps around and "buckles" the alpha subunit (Alvarez et al. 2009). Though heterodimerization can occur with mutated forms of this "seatbelt" structure, there is a dramatic decrease in heterodimer stability (Galet et al. 2004; Xing et al. 2004). GPB5 in vertebrates and invertebrates lack the seatbelt structure required to stabilize heterodimerization (Alvarez et al. 2009). Thus, the hypothesis that GPA2/ GPB5 functions as a heterodimer in a physiological situation (i.e. without chemical cross-linking) is challenged. The dissociation constant (Kd) associated with heterodimerization of the classic glycoproteins hormone subunits and GPA2/GPB5 is 10^{-7} M to 10^{-6} M (i.e. heterodimeric interactions are favoured at these concentrations) (Ingham et al. 1976; Strickland and Puett 1982). However, since the classic beta subunits contains an additional disulfide bridge that strengthens its association with the common alpha subunit, heterodimeric interactions are stabilized in circulation at physiological concentrations as low as 10^{-11} M to 10^{-9} M (Galet et al. 2004). Without this seatbelt structure, GPA2/GPB5 heterodimeric interactions are posssible but only at micromolar concentrations, not typically observed in circulation (Galet et al. 2004; Alvarez et al. 2009). Together, the evidence so far suggests that endocrine regulation by GPA2/GPB5 heterodimers is not likely and rather, these subunits may function independently or regulate physiology as a heterodimer in a paracrine/ autocrine fashion.

In rats, GPA2/GPB5 is expressed in oocytes and may act as a paracrine regulator of TSHR-expressed granulosa cells in the ovary to regulate reproductive processes (Sun et al. 2010). Similarly, it was recently shown that LGR1 is expressed in immature sperm of the adult mosquito testis, and is implicated in regulating flagaller development and coordinating spermatogenesis (Rocco et al. 2019). Given that our results demonstrate only GPA2/GPB5 heterodimers can activate LGR1 *in vitro*, and that heterodimers may regulate physiology in a paracrine/ autocrine manner rather than being secreted into circulation, future studies should examine the expression profile of GPA2/GPB5 in more detail in sites outside the nervous system such as the testes to determine if GPA2/GPB5 heterodimers act as a paracrine/ autocrine regulator of *A. aegypti* spermatogenesis.

4.5.5 GPA2 and GPB5 homodimerization

Our results established human and mosquito GPA2 and GPB5 subunits can homodimerize. However, whether these homodimers have a physiological function *in vivo* is unknown. Sole treatments of recombinant cells with mosquito/ human GPA2 and GPB5 subunits alone did not stimulate specific downstream signalling in either LGR1 or TSHR-expressing cells supporting that only GPA2/GPB5 heterodimers can activate their cognate glycoprotein hormone receptors. However, it is possible that GPA2 and GPB5 homodimers could activate other receptors. In insects, the neuropeptide bursicon is a heterodimer of two subunits called burs and pburs. Burs/pburs heterodimers act via a glycoprotein hormone receptor called LGR2 to regulate developmental processes such as tanning and sclerotization of the insect cuticle as well as wing inflation (Luo et al. 2005). Recently, it was demonstrated that bursicon subunits can homodimerize (i.e. burs/burs and pburs/pburs) and these homodimers mediate actions

independently of LGR2 to regulate immune responses in *A. aegypti* and *D. melanogaster* (An et al. 2012; Zhang et al. 2017).

In addition to the human/ mosquito GPA2/GPB5 homodimers observed in our studies, human GPA2 was also shown to interact with the beta subunits of CG and FSH (Nakabayashi et al. 2002). Lastly, the expression patterns of GPA2 and GPB5 in a number of organisms do not always co-localize, since GPA2 expression exhibits a much wider distribution and is expressed more abundantly than GPB5 in a number of vertebrate and invertebrate organisms (Nagasaki et al. 2006; Okada et al. 2006; Dos Santos et al. 2009; Tando and Kubokawa 2009b; Trudeau 2009; Heyland et al. 2012). Taken together, it can be suggested that GPA2 and GPB5 subunits could interact with other unkown proteins, and may possibly activate different receptors or signalling pathways of presently unknown origin.

4.5.6 Concluding remarks

Though much is known about the classic glycoprotein hormones LH, FSH, TSH and CG and their cognate receptors in vertebrates, little progress has been made thus far towards better understanding GPA2/GPB5 function, signalling and subunit interactions, especially in the invertebrates. To our knowledge, this is the first study to demonstrate *A. aegypti* and *H. sapiens* GPA2 and GPB5 subunit homodimerization *in vitro*. Our results also confirm that both subunits of *A. aegypti* and *H. sapiens* GPA2/GPB5 are required for the activation of their cognate receptors LGR1 and TSHR respectively. Unlike previous reports that show GPA2/GPB5-induced LGR1 activation elevates cellular cAMP by coupling a Gs pathway, the current results provide novel information that *A. aegypti* LGR1 couples to a Gi protein to inhibit cAMP levels following application of the GPA2/GPB5 fusion polypeptide. Further, our results suggest that

LGR1 may be constitutively active in the absence of its ligand, GPA2/GPB5, utilizing a Gs signalling pathway since LGR1-transfected cells did demonstrate higher overall levels of cAMP levels. GPA2 and GPB5 subunits are co-expressed within the the same neurosecretory cells of the first five abdominal ganlgia in mosquitoes where their coordinated release and regulation are likely. As a result, whether GPA2/GPB5 are secreted as heterodimers, like the classic glycoprotein hormones, and/or as homodimers remains to be determined *in vivo*. Similar to bursicon homodimers and the bursicon receptor LGR2, these results show GPA2/GPB5 homodimers do not act via LGR1 and TSHR signalling. Whether these homodimers are functional *in vivo* and what physiological role they play (if any) is a research topic that should be addressed in future studies. All in all, this investigation has provided novel information for GPA2/GPB5 and LGR1 signalling in the invertebrates and contributes towards a better understanding and functional elucidation of this ancient glycoprotein hormone signalling system common to nearly all bilaterian organisms.

4.6 References

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CHAPTER 5:

GENERAL DISCUSSION: SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

The GPA2/GPB5 glycoprotein hormone signalling system is present in nearly all bilaterally segmented metazoans with a fully sequenced genome and, since its initial discovery, this system has been implicated in a variety of physiological roles. However, in any given organism, its exact function is unclear, and many characteristics of its signalling remain elusive. Since the beginning of my research, GPA2/GPB5 signalling and its potential importance, even to humans, has gathered some attention (Karponis and Ananth 2017). Most research has been related to understanding its function in vertebrate organisms and only a handful of literature describes GPA2/GPB5 signalling systems in the invertebrates (Rocco and Paluzzi 2016). The collection of studies outlined in the chapters of this thesis describe properties of the GPA2/GPB5 glycoprotein hormone signalling system in detail and establish a physiological role for GPA2/GPB5 and its receptor LGR1 in the adult mosquito *A. aegypti*, a potent disease vector. A summary and integration of the major findings of the specified objectives outlined in the first chapter of this thesis are described below.

5.1.1: A role for GPA2/GPB5 signalling in hydromineral balance and reproduction in mosquitoes

To help identify a specific physiological role for GPA2/GPB5 and its receptor LGR1 in the adult mosquito *A. aegypti*, the organ- and tissue-specific, along with cellular expression patterns of LGR1 was mapped and potential targets for GPA2/GPB5 signalling were revealed. LGR1 transcript and immunoreactivity was found distributed along basolateral surfaces and detected as punctate intracellular staining in epithelial cells of various gut regions – many of which are known to have established functions in the maintenance of ion and water homeostasis. Thus, my

results supported an earlier established iono- and osmoregulatory function for GPA2/GPB5 in adult mosquitoes (Paluzzi et al. 2014) and fruit flies (Chintapalli et al. 2007; Sellami et al. 2011; Vandersmissen et al. 2014). Interestingly, receptor expression was also found associated with follicular epithelia and nurse cells in the female ovaries, suggesting a potential role related to female reproductive biology. In addition, LGR1 immunoreactivity was distinct and regionalized in the adult male testes, suggesting that GPA2/GPB5 could also regulate physiological processes associated with male reproductive biology in mosquitoes. Taken together, results of these findings were the first of their kind to study the protein-level distribution of LGR1, comprehensively characterizing its tissue-specific distribution in mosquitoes and provide evidence that GPA2/GPB5, like its vertebrate gonadotropin homologs FSH and LH, could potentially hold a reproductive role by regulating gametogenesis in mosquitoes.

5.1.2: The regulation of A. aegypti spermatogenesis by GPA2/GPB5 signalling

Using immunohistochemical techniques, LGR1 expression was characterized further in the testes of adult male *A. aegypti* by examining its subcellular localization throughout spermatogenesis. In the mosquito testis, LGR1 immunoreactivity was found associated to the plasma membrane of immature developmental stages of sperm, including spermatogonia, spermatocytes and spermatids. In the mammalian testis, the glycoprotein hormones FSH and LH exert their functions on spermatogenesis by binding to their receptors expressed in the somatic Sertoli and Leydig cells respectively (Holdcraft and Braun 2004). Though less studied, there have also been reports demonstrating the expression of several G protein-coupled receptors in the plasma membrane of germ cells like sperm in vertebrates. For example, the membrane progestin receptor-alpha (mPRα), is localized on the plasma membrane of the midpiece region of teleost

(Zhu et al. 2003; Tubbs and Thomas 2008, 2009) and human (Thomas et al. 2009) sperm. Activation of mPRα by progestin induces sperm hypermotility (Tan and Thomas 2014, 2015; Tan et al. 2014) likely by increasing intracellular levels of cAMP (Tubbs and Thomas 2009). Similarly, expression of the LH/CG receptor has been reported in human sperm (Eblen et al. 2001) and activates differentiation of spermatids to spermatozoa in flatfish (Chauvigné et al. 2013). In our studies, LGR1 co-localized with gamma tubulin in the flagella centriole adjunct, where receptor downstream signalling events could be implicated in microtubule nucleating activity to coordinate flagella development. Interestingly, the LH/CG receptor, homologous to LGR1 (Hauser et al. 1998), in sperm couples a cAMP/protein-kinase A pathway and is also believed to upregulate genes involved in flagella formation (Chauvigné et al. 2013).

The centrosome, which is the main microtubule organizing center, is comprised of the centriole and electron-dense pericentriolar material; which contains proteins like gamma tubulin and CP190 that nucleate microtubules. Microtubule nucleation is important for both mitotic spindle assembly in proliferating cells and formation of the flagellar axoneme in spermatids (Riparbelli et al. 1997; Dallai et al. 2016). In our experiments, after utilizing RNA interference methods to knockdown LGR1 expression in mosquito larva and pupa, net quantities of spermatozoa in reproductive tracts and fertility were significantly perturbed in adult stages. Moreover, adult males demonstrated spermatogenic defects including shortened flagella. Given that LGR1 immunolocalized to the spermatid centriole adjunct, where microtubule nucleation occurs, and that knockdown of LGR1 led to spermatozoa defects like shortened flagella, it was hypothesized LGR1 signalling could help coordinate microtubule nucleating activity during spermatogenesis. Further, if LGR1 signalling is involved in flagellar development by regulating microtubule nucleating activity, in addition to aiding the development of the axoneme in

spermatid flagella, LGR1 signalling could be implicated in mitotic spindle assembly, which is required for the proliferation of spermatogonia and spermatocytes. The involvement of LGR1 signalling in sperm proliferation may explain why LGR1 knockdown males were rendered with 60% less spermatozoa compared to controls. Moreover, it is possible that this proliferative function could be the common denominator for GPA2/GPB5 function in other tissues like the alimentary canal, where LGR1 was found expressed in adult mosquitoes in our studies. In support of this, recent studies have demonstrated that thyrostimulin (vertebrate GPA2/GPB5) and its receptor are implicated in the proliferation of epithelial ovarian cancer cells by transactivating epidermal growth factor receptor (EGFR) (Huang et al. 2016).

In rats, a paracrine system involving oocyte-derived GPA2/GPB5 and granulosa-cell expressed TSH receptor, has been suggested in the female ovaries to coordinate oogenesis (Sun et al. 2010). Given that GPA2/GPB5 subunits are co-expressed in the testis (Nagasaki et al. 2006), a paracrine system could also exist in male gonads of rats. In the testis of mosquitoes, it is possible that GPA2/GPB5 could act as a paracrine regulator of microtubule nucleation upon binding LGR1 in sperm. Though expression of GPA2/GPB5 was enriched to the central nervous system, some GPA2 and GPB5 transcript was detected in male reproductive tissues, suggesting that components for a paracrine/ autocrine system are present in the male mosquito testes.

Collectively, these data were of the first to study hormone signalling pathways in mosquito spermatogenesis, and considerably contributed to advancing our understanding of how spermatogenesis is regulated in insects, a highly unexplored area. Importantly, my results demonstrated GPA2/GPB5 signalling functions to regulate mosquito spermatogenesis, and these results could aid in the functional elucidation of GPA2/GPB5 homologs in other organisms.

5.1.3: More detailed insights to the properties of GPA2/GPB5 signalling

Heterologous systems with mammalian cell lines were employed to produce recombinant *A. aegypti* GPA2/GPB5, as well as recombinant *Homo sapiens* GPA2/GPB5 for a comparative model, to study subunit dimerization and ligand-receptor interactions. Interestingly, unlike the classic glycoprotein hormones (Pierce and Parsons 1981; Brownstein 1985; Szkudlinski et al. 2002), my results demonstrated that cellular co-expression of *H. sapiens* GPA2 and GPB5 is not a requirement for heterodimerization, which could explain why GPA2 and GPB5 subunits are not always found co-expressed in the same cells in a variety of organisms (Nagasaki et al. 2006; Okada et al. 2006; Dos Santos et al. 2009; Tando and Kubokawa 2009; Heyland et al. 2012). In adult mosquitoes, GPA2 and GPB5 subunits were found co-expressed and enriched within two or three bilateral pairs of neurosecretory cells of the first five abdominal ganglia, however our experiments failed to demonstrate *A. aegypti* GPA2/GPB5 heterodimeric interactions using recombinant proteins produced from mammalian cell lines. Unlike *H. sapiens* GPA2/GPB5, it is possible that there is an additional component required for stabilizing *A. aegypti* subunit heterodimerization, such as a cofactor, that is not present in the HEK 293T cell line employed to produce recombinant *A. aegypti* GPA2/GPB5 proteins. This additional component could be specific to insect cells, or explicit to neurons which produce GPA2/GPB5 in mosquitoes. Future studies that examine heterodimerization of recombinant GPA2/GPB5 subunits produced in an insect primary cell culture, such as *Drosophila* Schneider 2 (S2) cells, are required. Moreover, though earlier cross-linking studies state recombinant fruit fly GPA2/GPB5, produced from HEK 293T cells, are capable of heterodimerization *in vitro*, these studies lacked appropriate controls to test homodimerization and as a result, future studies will need to re-assess the heterodimerization of *D. melanogaster* GPA2 and GPB5 subunits.

To mimic *A. aegypti* GPA2/GPB5 heterodimers, GPA2 and GPB5 were expressed in HEK 293T cells as a single polypeptide chain using a hexa-histidine tagged glycine-serine repeat artificial linker sequence. Comparable to *H sapiens* GPA2/GPB5-TSH receptor signalling, the results in this thesis established that tethered *A. aegypti* GPA2/GPB5 fusion protein, rather than individual subunits alone, were required to functionally activate *A. aegypti* LGR1. In organisms that possess GPA2/GPB5 signalling components, these data imply that only GPA2/GPB5 heterodimers can activate their cognate glycoprotein hormone receptors. Results also demonstrated LGR1 may, in the absence of its ligand, constitutively couple to a stimulatory G protein, and upon binding tethered *A. aegypti* GPA2/GPB5 fusion protein, activates an inhibitory G protein signalling pathway. Consequently, these results demonstrate that invertebrate glycoprotein hormone receptors, similar to the vertebrate TSH and LH/CG receptors, can promiscuously associate with and activate multiple G protein signalling pathways (Herrlich et al. 1996; Grasberger et al. 2007; Kleinau et al. 2010).

Together with my previous findings that LGR1 is implicated in mosquito spermatogenesis, collectively these results suggest that GPA2/GPB5 heterodimers, produced from the abdominal ganglia, or produced locally from the testes, may elicit their actions through inhibitory G protein signalling pathways upon binding LGR1, which then mediates spermatogenesis in adult *A. aegypti*.

Moreover, for the first time, my results demonstrate *A. aegypti* and *H. sapiens* GPA2 and GPB5 subunits are capable of homodimerization *in vitro*, and these homodimers do not activate LGR1 and TSH glycoprotein hormone receptors respectively. The neuropeptide hormone bursicon, is a heterodimer of two cysteine knot proteins called bursα and bursβ, and these heterodimers bind the receptor LGR2 to regulate physiological processes during insect

development like cuticle tanning and wing expansion (Dewey et al. 2004; Luo et al. 2005; Honegger et al. 2008; Bai and Palli 2010). Similar to GPA2 and GPB5 subunit expression in several vertebrate and invertebrate species (Nagasaki et al. 2006; Okada et al. 2006; Dos Santos et al. 2009; Tando and Kubokawa 2009; Heyland et al. 2012), the ability for bursicon to function solely as a heterodimer was questioned since bursα and bursβ are not always co-expressed in the same cells (Luo et al. 2005; Dai et al. 2007; Honegger et al. 2008; Wang et al. 2008) and the transcript abundance of bursβ can surpass that of bursα (Chung et al. 2012). Under non-reducing conditions, immunoblot analyses of recombinant *D. melanogaster* and *A. aegypti* bursicon subunit proteins, produced in HEK 293T cells, revealed bursα and bursβ can form heterodimers and homodimers (An et al. 2012; Zhang et al. 2017). Injections of neck-ligated flies (a technique to minimize the influence of brain endocrine factors) with recombinant bursα and bursβ homodimers, rather than heterodimers, were required to elevate the expression of genes encoding for antimicrobial peptides (AMPs) (An et al. 2012; Zhang et al. 2017). Interestingly, these effects were unaltered when experiments were repeated in LGR2 mutants, suggesting that the upregulation of AMP genes is LGR2-independent. Additionally, gene upregulation was lost in mutant loss-of-function flies for the transcription factor Relish. Taken together, bursicon homodimers mediate AMP transcript regulation by activating transcription factors in an LGR2-independent manner in mosquitoes and fruit flies (An et al. 2012; Zhang et al. 2017).

Additional research will be required to determine, (i) whether *A. aegypti* and *H. sapiens* GPA2 and GPB5 homodimers, if not an experimental artifact, are present *in vivo*, (ii) given that individual subunits were not capable of stimulating glycoprotein hormone receptors, which receptors bind GPA2 and GPB5 homodimers (if there are any), and (iii) elucidate their overall physiological relevance.

5.2 Future Directions

Though GPA2/GPB5 signalling systems are widely distributed amongst most bilaterian organisms, physiological roles amongst invertebrate and vertebrate organisms are unclear and the mechanism of its signalling is largely understudied. For organisms with this glycoprotein hormone system present, the current set of studies have drastically contributed to a better understanding and molecular dissection of the GPA2/GPB5 glycoprotein hormone signalling system. Significant insight to both function as well as mechanistic actions of its signalling in the adult mosquito *A. aegypti* were performed and provide strong incentive to characterizing GPA2/GPB5 signalling systems in other invertebrate and vertebrate organisms. Overall, some aspects of GPA2/GPB5 signalling are unique from the classic glycoprotein hormones (i.e. gonadotropins and TSH) in vertebrates and, though novel insights have been provided from the current collection of studies, additional research that further characterizes this evolutionarily ancient glycoprotein hormone signalling system in detail is warranted.

Future work examining the potential roles for GPA2/GPB5 in female *A. aegypti* reproduction would help to resolve whether GPA2/GPB5 functions are sex-specific in mosquitoes, and a functional characterization of its role in spermatogenesis and/or oogenesis in other invertebrates could help determine if these reproductive functions are conserved across multiple organisms. Additionally, future loss-of-function studies could be performed to clarify the involvement of GPA2/GPB5 in ion and water homeostasis and enlighten whether GPA2/GPB5 signalling is pleiotropic in adult *A. aegypti*. The current set of findings that GPA2/GPB5 signalling regulates mosquito spermatogenesis will hopefully motivate and direct research towards better understanding the regulation of male reproductive biology in insects, a severely understudied and potentially fruitful research avenue.

Unlike the classic glycoprotein hormones, GPA2/GPB5 subunits were demonstrated to form homodimers in our studies. Bursicon homodimers function independently of its glycoprotein hormone receptor LGR2 and occupy roles in immunoregulation (An et al. 2012; Zhang et al. 2017), whereas its heterodimeric counterpart functions in cuticle sclerotization and tanning in insects (Luo et al. 2005). As a result, future work should be geared towards understanding if the *in vitro* observed GPA2 and GPB5 subunit homodimerization occurs *in vivo*, and if they do, what function GPA2/GPB5 homodimers encompass in mosquitoes and humans.

Altogether, the results provided in this thesis support that the GPA2/GPB5 glycoprotein hormone signalling system is complex and atypical to that of other glycoprotein hormones, with potential to be implicated in critical physiological processes that govern both invertebrate and vertebrate physiology.

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