Characterization and functional deorphanization of CCHamides and their receptors in the yellow fever mosquito, *Aedes aegypti*

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Abstract

Studying the neuroendocrine system of mosquitoes, which transmit various pathogens leading to human diseases, allows us to better understand their physiology. Novel CCHamide neuropeptides, CCHa1 and CCHa2, and their associated receptors, CCHa1R and CCHa2R, were recently identified across insects, but they have not been studied in mosquitoes. This study aims to quantify and localize expression of CCHamides and CCHa2R to determine their physiological role in the mosquito, *Aedes aegypti*. Results revealed adults express the most *CCHamide* transcripts while *CCHa2R* transcript was enriched in pupa. The midgut was found to be the primary source for both *CCHamide* transcripts while higher *CCHa2R* transcript abundance was detected in the hindgut and the reproductive system. Both CCHamide receptors, the CCHa1R and CCHa2R, were deorphanized, revealing CCHamides exhibited the strongest ligand activity. *CCHa2* and *CCHa2R* transcripts were unresponsive to either sucrose ingestion or starvation stress while *CCHa1* and *CCHa2* transcript levels responded to a blood-meal.

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I would also like to say thanks to Salwa for being so patient in teaching me research techniques and answering my silly questions when I just joined the lab, which allowed me to have a smooth transition. I am looking forward to work with you in the coming years.

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Chapter I: General Introduction

1.1 Neuropeptides

Sophisticated and collaborative nervous, endocrine and other organ systems of both invertebrates and vertebrates require a large variety of signaling molecules ranging from gaseous, aminergic and fatty acid molecules, peptidergic substances and proteins to perform normal functions (Burbach, 2011). Neuropeptides are classified as one of the most diverse and versatile groups of signaling molecules that are employed as i) neurotransmitters, which relay signals to other neurons via synapses; ii) neuromodulators, which regulate the intensity or effects of other signaling molecules and neurotransmission processes; and iii) neurohormones that influence targets found at a distance from their origin as they are circulated throughout the body (Nässel, 2002; Mercier et al., 2007; Predel et al., 2010; Burbach, 2011; Elphick et al., 2018; Agrawal et al., 2019). Neuropeptides are small proteinaceous substances ranging in size from 5 to 80 amino acid residues that are produced and secreted by neurons, including neurosecretory cells, and endocrine cells in other organs of the body, including the gut (Predel et al., 2010). Neuropeptides often are released into circulation where they can then act on corresponding receptors in target tissues and organs to trigger downstream responses, for example, activating various signaling cascades (Katz and Frost, 1996; Nässel, 2002; Burbach, 2011; Agrawal et al., 2019; Abou El Asrar et al., 2020).

Neuropeptides and signaling networks are widespread in many levels of organisms, from placozoans, the simplest structure of animals to more complex vertebrates, and from animals without developed neurons, for example, *Trichoplax adhaerens*, to animals with an intricate nervous system (Nässel, 2002; Yañez-Guerra et al., 2022). Studies have shown that the common ancestor of deuterostomes and protostomes was the evolutionary origin of more than thirty

neuropeptide signaling systems (Elphick et al., 2018). In fact, studies have identified two conservative prepropeptides, which are unprocessed neuropeptide precursors with a signal sequence (Wiren et al., 1988), phoenixin (PNX) and nesfatin in bilaterians, cnidarians, ctenophores, sponges and choanoflagellate. This indicated that some prepropeptides have premetazoan origin and can be traced back before neurons had even evolved (Yañez-Guerra et al., 2022).

At the molecular and cellular levels, production and release of active neuropeptides is similar to other proteins, which starts with gene encoded prepropeptides and precursor proteins in certain cells. Prepropeptides consist of a signal peptide, which normally contains 15 to 30 amino acids at the N-terminus of the protein and is responsible for the translocation of prepropeptides to the secretory pathway, and an inactive propeptide sequence requiring further processing to yield biologically active peptides. The signal peptide will be cleaved off once prepropeptides are transported through the endoplasmic reticulum membrane to release propeptides with the help of a small class of enzymes called signal peptidases at the cleavage site that is located in the carboxy-terminal domain of the signal sequence (von Heijne, 1990; Srivastava and Howell, 2012). Resulting propeptides are then translocated through the Golgi apparatus and further proteolytically processed by proteases in secretory vesicles to produce biologically active neuropeptides (Creuzenet et al., 1993; Nässel, 2002; Hook et al., 2008). Multiple neuropeptides can be produced from a single propeptide precursor by direct enzymatic cleavage at normally dibasic (and occasionally monobasic) cleavage sites (Scheller et al., 1983; Scheller and Kirk, 1987; Veenstra, 2000; Hook et al., 2008). Following this cleavage that liberates peptides from the propeptide precursor, further post-translational modifications take place to allow neuropeptides to mature prior to their release from large dense-core vesicles, which are a less

abundant type of secretory vesicle that typically contains substances that interact with G protein-coupled receptors (Zupanc, 1996; Strand, 1999; Zhang et al., 2011; Birinci et al., 2020). Common post-translational modifications of neuropeptides include formation of disulphide bridges to give the neuropeptide a cyclical structure, N-terminal pyroglutamate formation, C-terminal amidation and tyrosyl group sulfation (Zupanc, 1996; Strand, 1999; Karhunen et al., 2001). Neuropeptides enclosed in vesicles can be released to the haemolymph due to depolarization at two locations, the normal synaptic site or the non-synaptic site, where there is no direct contact with another axon terminal (Nässel, 2002; Elphick et al., 2018). Neuropeptides can be inactivated if their signaling is no longer needed by one of the two mechanisms: 1) reducing the sensitivity of the receptor by down-regulating the receptor expression or phosphorylating the receptor (Garland et al., 1996; McConalogue et al., 1998); 2) degrading the neuropeptides with peptidases and endosomal enzymes (McKelvy and Blumberg, 1986; Nässel, 2002). For example, insulin degrading enzyme (IDE) or insulinase is expressed globally in *Drosophila melanogaster* and was suggested to regulate ILP-2 expression and associated signaling pathways by inactivating the peptides. Furthermore, IDE found in mammals was also shown to degradate other peptides intracellularly (Fernández-Gamba et al., 2009; Galagovsky et al., 2014; Strand et al., 2016).

At the tissues/organs level, neuropeptides in insects such as *D. melanogaster* and *Ae. aegypti* are produced and stored in neurosecretory cells within the central nervous system (CNS) including the brain and ventral nerve cord, the latter of which contains the suboesophageal ganglion (SOG) located inside the head and positioned below the oesophagus, three thoracic ganglia and eight abdominal ganglia (Nässel, 1996; Hellmich et al., 2014; Strand et al., 2016). Additionally, insect endocrine glands, the corpus allatum (CA) and the corpus cardiacum (CC)

that are located posterior to the brain, are well studied and confirmed to release many types of neuropeptides in insects, such as adipokinetic hormone (AKH), diuretic peptides and antidiuretic peptides (Spring and Hazelton-Robichaux, 2003; Predel et al., 2010; Strand et al., 2016). The insect midgut is another site where production and release of various peptides takes place. Jiang and Edgar indicated that cells differentiated from the intestinal stem cells within the epithelium of the midgut were not only responsible for nutrient absorption, but also for secreting digestive enzymes and neuropeptides (Jiang and Edgar, 2012). More than ten neuropeptides were studied in different regions of *Drosophila* midgut (Chen et al., 2016; Wu et al., 2020). These neuropeptides are involved in regulating food intake, digestion and maintaining homeostasis post feeding. Furthermore, a special group of gut-produced neuropeptides that are also produced in the CNS are considered as brain-gut peptides that are not only acting locally but also targeting other tissues. Studies have shown that these brain-gut neuropeptides are important in modulating growth and development, reproduction, excretion and addiction behaviours across insects (Chung et al., 1999; McBrayer et al., 2007; Pérez-Hedo et al., 2010; Vadnie et al., 2014; Vanderveken and O'Donnell, 2014; Abou El Asrar et al., 2020; Wu et al., 2020).

Following the purification and amino acid sequence identification of the first neuropeptide, vasopressin, in vertebrates in 1951 (Turner et al., 1951), the first neuropeptide in insects, proctolin, was identified and sequenced in 1975 (Starratt and Brown, 1975). It was firstly isolated from the cockroach *Periplaneta americana* as a neurotransmitter that enhances contraction of hindgut visceral muscles (Starratt and Brown, 1975; Lange and Orchard, 2013). Further studies revealed that proctolin influenced many other biological processes, such as digestion, reproduction and haemolymph circulation in a variety of insects and other arthropods (Lange and Orchard, 2013). Subsequent studies found that even in invertebrates, there are a large

number of neuropeptides belonging to different families that categorized based on their structure and functions (Nässel, 2002). As an excellent model in neuroendocrine study, approximately 40 neuropeptide genes were identified in *D. melanogaster* (Nässel and Zandawala, 2019), such as adipokinetic hormone (AKH), which mobilizes energy storage molecules such as carbohydrates and lipids under starvation condition (Sehadova et al., 2020); insulin-like peptides (ILPs) that are involved in regulation of insect development, growth, metabolism and aging (Géminard et al., 2006; Kannan and Fridell, 2013; Okamoto and Yamanaka, 2015); FMRFamide-like peptide that modulate feeding behaviour, regulate water homeostasis and cardioacceleratory function (Dockray, 2004; Peymen et al., 2014); as well as structurally diverse diuretic and antidiuretic hormones (Agrawal et al., 2019). Similarly, in other insects such as the yellow fever mosquito, *Aedes aegypti*, up to 50 peptide hormones have been identified such as head peptides, allatostatins and ILPs that are well-studied at the level of gene expression, signaling and physiological function (Matsumoto et al., 1989; Veenstra et al., 1997; Stracker et al., 2002; Li et al., 2006; Riehle et al., 2006; Brown et al., 2008; Strand et al., 2016).

In addition to neuropeptide-encoding genes, a number of novel G protein-coupled receptors were also identified and functionally deorphanized in the last decades (Nässel and Zandawala, 2019). As a result of the diversity of neuropeptides, their associated receptors and signaling pathways, they have been found to be involved in all essential physiological processes in insects, including biosynthesis of other proteins, cardiac and intestinal muscle contraction, osmoregulation, ecdysis and eclosion, reproduction, growth and development, and feeding behaviours (Nässel, 2002).

1.2 Neuropeptide receptors

G protein-coupled receptors (GPCRs), the largest class of membrane proteins in both vertebrates and invertebrates, are the most common receptors that elicit cellular responses to neurotransmitters, hormones and neuropeptides (Rosenbaum et al., 2009; Kamato et al., 2015). Assuredly, in addition to the ligands mentioned above, there are a wide range of molecules that can bind to GPCRs to initiate downstream signaling pathways, including photons, ions, odorants, growth factors and other stimuli (Liu et al., 2021). As the most abundant cell membrane receptor family, 5% of genes in the human genome code for GPCRs (Kamato et al., 2015; Liu et al., 2021).

The majority of GPCRs share key structural characteristics including seven hydrophobic α-helical transmembrane domains, three intracellular and extracellular loops along with an intracellular carboxyl terminus (C-terminus) and an extracellular amino terminus (N-terminus) (Kobilka, 2007; Oldham and Hamm, 2008; Rosenbaum et al., 2009; Liu et al., 2021). Across the GPCR superfamily, transmembrane segments tend to be more highly conserved, and more variations are observed in the intracellular and extracellular loops between transmembrane domains and at both the N- and C-termini(Kobilka, 2007). The amino acid sequence length of the N-terminus in GPCRs is related to the types of corresponding ligands, from the shortest 10 amino acid residues up to 600 residues. The N-terminus of neuropeptide and monoamine associated GPCRs are usually small, for example, serotonin and histamine receptors, while the N-terminus of glycoprotein hormone receptors such as thyrotropin receptor (TSHr) and follicle stimulating hormone receptor (FSHr) are relatively long (Smits et al., 2003; Kobilka, 2007).

There are three subunits, Gα, Gβ and Gγ, that tightly interact with the GPCR at the intracellular side. Among them, Gβ and Gγ subunits form a heterodimer, which interacts with the

Gα subunit that is able to bind with guanosine triphosphate (GTP) and guanosine diphosphate (GDP) (McCudden et al., 2005). These three subunits are crucial for the signal transduction pathway specificity and functionality of the receptor. Gα subunit is further classified into four subtypes: α_i, α_s, $\alpha_{12/13}$ and α_q (Kamato et al., 2015). Each α subtype activates different secondary messengers and downstream signaling cascades. α_I regulates ion channels, phospholipases and phosphodiesterase, and inhibits cyclic adenosine monophosphate (cAMP); α_s regulates the level of cAMP in the opposite way, it increases the synthesis of cAMP by up-regulating adenylyl cyclase activity; GTPase activity is affected by $\alpha_{12/13}$; lastly, one of the most common signaling pathways, PIP_2-IP_3 pathway, is governed by the α_q subunit (Fig 1.1) (Kamato et al., 2015). Phosphatidylinositol 4, 5-biphosphate (PIP_2) is hydrolysed by the phospholipase Cβ (PLCβ) that is activated by α_q to produce 1, 4, 5-inositol triphosphate (IP_3) and diacylglycerol (DAG). Subsequently, calcium influx into the cytosol is triggered by the binding of IP_3 and IP_3 receptors (IP_3Rs) on the endoplasmic reticulum (ER) that open up Ca^{2+} channels and then affect important physiological processes (Kamato et al., 2015; Kania et al., 2017).

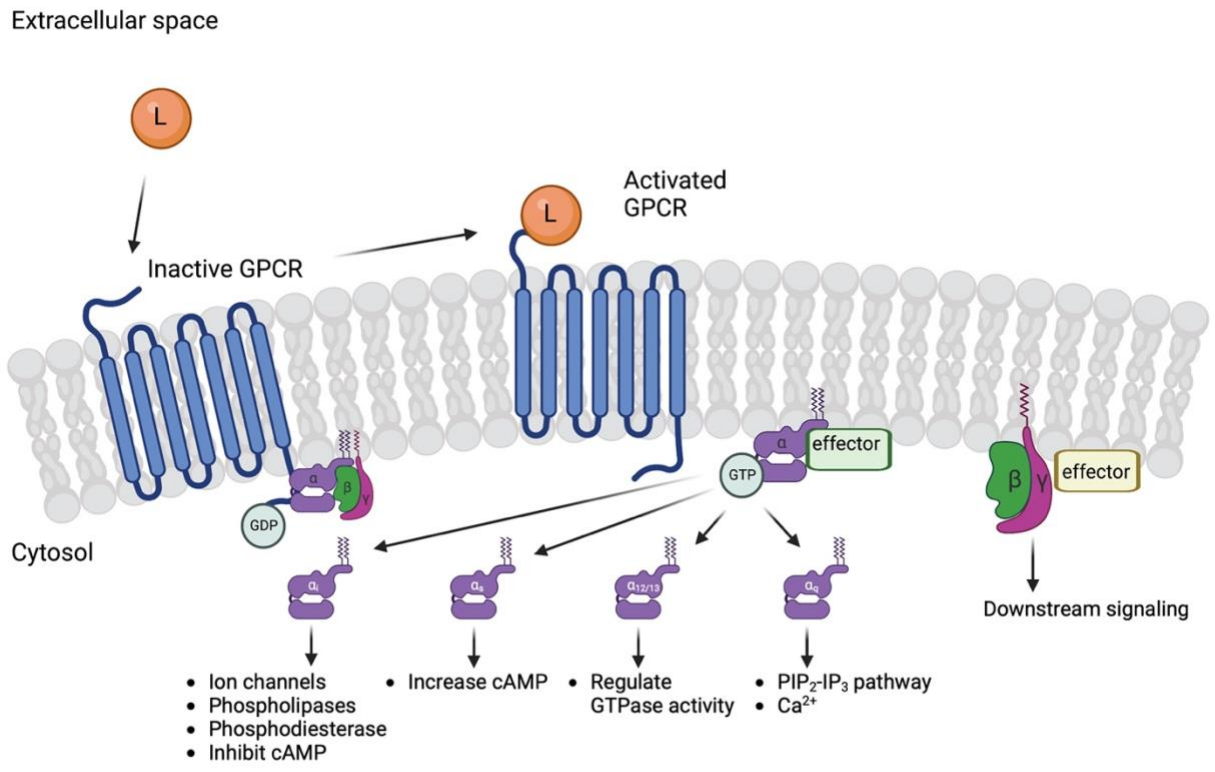

Figure 1.1 Schematic representation of GPCR activation and general downstream signaling. Figure was created using BioRender.com.

In addition to the α subtype classification, GPCRs are also characterized by the sequence and function of the receptor called A-F system (Liu et al., 2021). In the insect research area, the A-F classification system has been applied. The largest GPCRs family, the rhodopsin-like family, is grouped as Class A, followed by the secretin receptor family as Class B; the metabotropic glutamate family and receptors for the fungal mating pheromone are known as Class C and D, respectively. All cAMP receptors are organized as Class E; and lastly, Class F contains the frizzled/smoothened receptors (Hu et al., 2017; Liu et al., 2021).

As one of the most important and revolutionary models in the field of insect research and the first insect with a fully sequenced genome, there are a total of 200 genes that code for GPCRs in *D. melanogaster* (Hanlon and Andrew, 2015; Liu et al., 2021). In the human disease vector, mosquito *Ae. aegypti*, 135 GPCR genes were identified and the majority of them (89 genes) belong to Class A, the rhodopsin-like family (Nene et al., 2007; Strand et al., 2016). Since the 21st century, due to the rapid improvement of various biotechnology and statistical tools, the research on insect GPCRs has flourished. Numerous studies range from quantitative analysis of gene expression to localization of receptors by knocking down or knocking out GPCR genes via RNA interference (RNAi) and CRISPR-Cas 9 system; from receptor deorphanization and examining their associated physiological functions to characterization of possible signaling pathways that are induced by the receptors. Studies have shown that GPCRs are responsible for almost all essential physiological processes of insects, including biosynthesis of vital substances such as peptides and proteins, growth and development, mating and reproduction, regulation of nutrients and energy, animal behaviours, coping mechanisms in response to stress and insecticide resistance (Terhzaz et al., 2012; Jiang et al., 2013; Li et al., 2014; Kang et al., 2019; Petruccelli et al., 2020; Liu et al., 2021).

Activation of GPCRs and their associated signaling pathways starts with binding of the ligand and the corresponding GPCR. GDP, which originally tightly interacts with the Gα subunit is now exchanged for GTP due to a conformational change of the subunit that is induced by the guanine nucleotide exchange factor (GEF). β and γ subunits work jointly to inhibit the exchange of GDP to GTP when there is no ligand, but once Gα-GDP becomes Gα-GTP, the heterodimer Gβγ detaches from the α subunit and then activates the downstream effectors. Besides, Gα-GTP is also able to trigger downstream messengers. The GPCR and the signaling pathway is deactivated when Gα associated GTP is hydrolyzed back to GDP with the help of regulator G-protein signaling (RGS) proteins (McCudden et al., 2005) allowing the heterodimer Gβγ to return to its original position associated with the α subunit.

1.3 Using an insect model to study comparative neuroendocrinology: *Aedes aegypti*

There are several reasons to use an insect model as a tool to study the nature of the neuroendocrine system. First of all, genomes of insects are less complex compared to vertebrates. Secondly, they are easier to access due to a relatively short reproduction cycle and development period, and also budget friendly. Thirdly, they are more applicable for some experiments, for example, parabiosis (Scharrer, 1987). More importantly, studying insect neuroendocrinology puts the comparative approach into practice. Structural and functional parallels related to neuroendocrinology have been found between insects and vertebrates. Neurosecretory cells in the protocerebrum of insects resemble the hypothalamic neurosecretory centre of vertebrates (Scharrer, 1987). Many neuropeptide genes in insects have analogues in vertebrates as well, for instance, allatostatin verses somatostatin; tachykinin III and IV that were isolated from *Locusta migratoria* are homologues of the vertebrate tachykinins; also, the invertebrate neuropeptide F

(NPF) was identified as the orthologs of vertebrate neuropeptide Y (NPY) (Scharrer, 1987; Schoofs et al., 1990; Yañez-Guerra et al., 2020). On the one hand, insects play a pivotal role in the ecosystem because they act as primary or secondary consumers and decomposers to provide biological foundations to other organisms. On the other hand, some insect species pose challenges to agriculture, forestry and the health of human communities. Therefore, it is necessary to use insect models for endocrine research, because it allows us to collect more information about the physiology of these highly-diverse species, and better respond to challenges that are related to human health, the environment and ecological balance.

The focus of this thesis is on the yellow fever mosquito, *Aedes aegypti*. Characteristics of *Ae. aegypti* include white dots on the dorsal side of the thorax and white stripes on all legs. As a medium-sized mosquito species, adults can reach 4 to 7mm, and males are half or even two-thirds smaller than females (Carpenter and La Casse, 1974). Moreover, male antennas are bushy compared to female short-hair antennas. Due to the difference in feeding behaviour, male mouthpart is only suitable for feeding on nectar, while female proboscis is longer and can be used for blood feeding (Clemons et al., 2010). In terms of mosquito development, there are four distinct stages including the egg, larva, pupa and adult stage (Fig. 1.2). Females usually lay eggs near water surface or moist location, but studies suggested that the egg have high tolerance to desiccation for several months (Clements, 1992). Except for the adult stage, which is the terrestrial stage, larval and pupal stages are aquatic. There are four instar larval stages, and each instar stage needs at least 4 days to grow, but the single pupal stage is a non-feeding and mobile stage, that takes around 2 to 3 days before adults emerge. The life span of adult mosquito can be up to one month depending on the environmental conditions, including for example, the temperature and humidity levels (Christophers, 1960; Clements, 1992; Clemons et al., 2010).

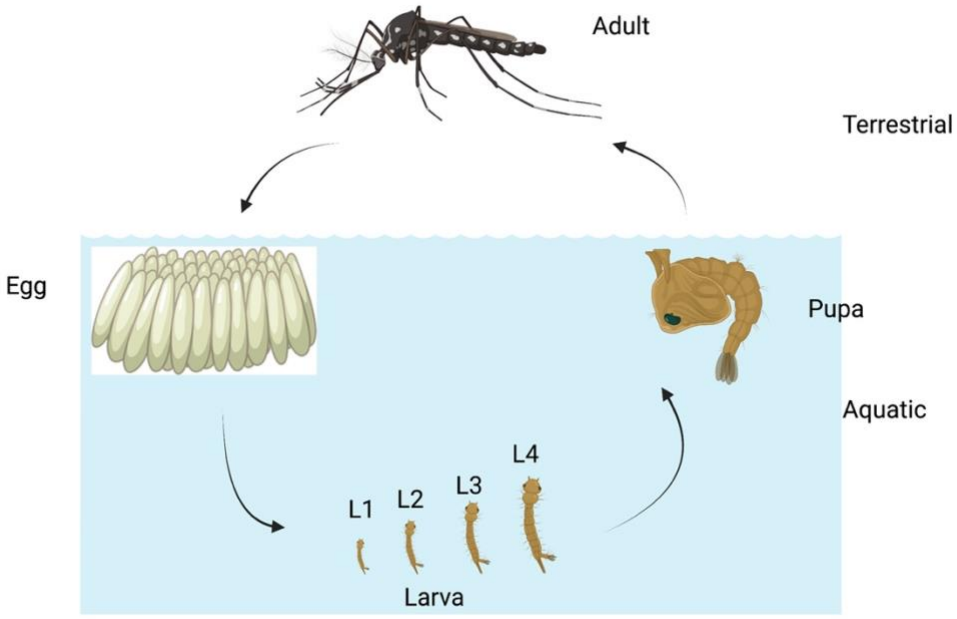

Figure 1.2 Schematic representation of *Ae. aegypti* development. Figure was created using BioRender.com.

Ae. aegypti originated from Africa and is now widespread throughout the world, especially in tropical and sub-tropical regions (Carvalho and Moreira, 2017). As a competent human disease vector, it is able to transmit various diseases, such as yellow fever, dengue, Zika, chikungunya and other disease agents through its blood feeding behaviour (Kotsakiozi et al., 2017). The World Health Organization (WHO) considers dengue fever as one of the most important mosquito-borne viral infections in over 100 countries. Approximately 400 million people get infected each year and around 50% of the global population is threatened by the virus (WHO, 2022). In the last 30 years, Brazil experienced a serious and prolonged dengue epidemic (Carvalho and Moreira, 2017). All four types of dengue viruses can cause mild or severe symptoms, including fever, vomiting, rash and muscle or bone pain. The specific medicine has not been discovered, and current treatments can only relieve symptoms. Even though one vaccine is available, the usage is restricted to personnel who are seropositive (Reinhold et al., 2018). Furthermore, the incidence of dengue fever is significantly higher than 25 years ago (Jansen and Beebe, 2010). The increased threat of mosquito-borne diseases may be due to larger populations, faster reproduction rates and wider habitats. Furthermore, studies have indicated that *Ae. aegypti* has successfully expanded its distribution in recent decades, and there are signs of accelerating invasion towards more northern regions such as Canada and Europe (Carvalho and Moreira, 2017). Jansen *et al.* argued urbanization could be one of the reasons since it provides vast suitable habitats for this particular mosquito, because as the most successful synanthropic species, it is well adapted to human settlements and highly associates with human activities (Jansen and Beebe, 2010). Global warming is also likely responsible for this phenomenon to a certain extent due to more geographic areas falling into the ecological niche suitable for *Ae. aegypti* (Reinhold et al., 2018).

In the context of the increasing threat of mosquito-borne diseases, it is urgent to propose new and effective control methods. It is of interest to study the neuroendocrine system of *Ae. aegypti* and to characterize and examine neuropeptides and associated signaling pathways that may be involved in mosquito blood feeding behaviour because this may provide insight for future studies that aim to design new compounds that could interfere with mosquito physiology and lessen the burden they have as human disease vectors.

1.4 Mosquito midgut and association to the blood meal

1.4.1 Basic characteristics of the mosquito midgut

The insect midgut is the primary organ responsible for food (i.e. blood meal) digestion, nutrient absorption and is also an important site of neuropeptide and hormone secretion (Cui and Franz, 2020). The mosquito midgut is a 2 to 3 mm tubular organ that is composed of a monolayered epithelium, and a basal lamina with collagen fibrils that embeds with muscle tissues, nerve fibrils and tracheoles surrounding it (Fig. 1.3) (Brown et al., 1985; Billingsley and Lehane, 1996; Cui and Franz, 2020). It begins from the cardiac sphincter in the prothorax region and ends at the pyloric valve, which is the junction between the midgut and the hindgut and connects to Malpighian tubules (MTs). The mosquito midgut extends across the thoracic cavity and the first two segments of abdomen into the bulb-shaped abdominal area (Brown et al., 1985). There are two regions, anterior midgut and posterior midgut in adult mosquitoes that are responsible for distinct functions. The anterior midgut plays a role in breaking down sugar and carbohydrate molecules, while the posterior midgut participates in blood meal digestion as well as regulating ion homeostasis post-blood meal in females (Billingsley, 1990; Foster, 1995;

Pullikuth et al., 2006; Dixon et al., 2017; Cui and Franz, 2020). However, the specialized functionality of this region in male mosquito remains unclear.

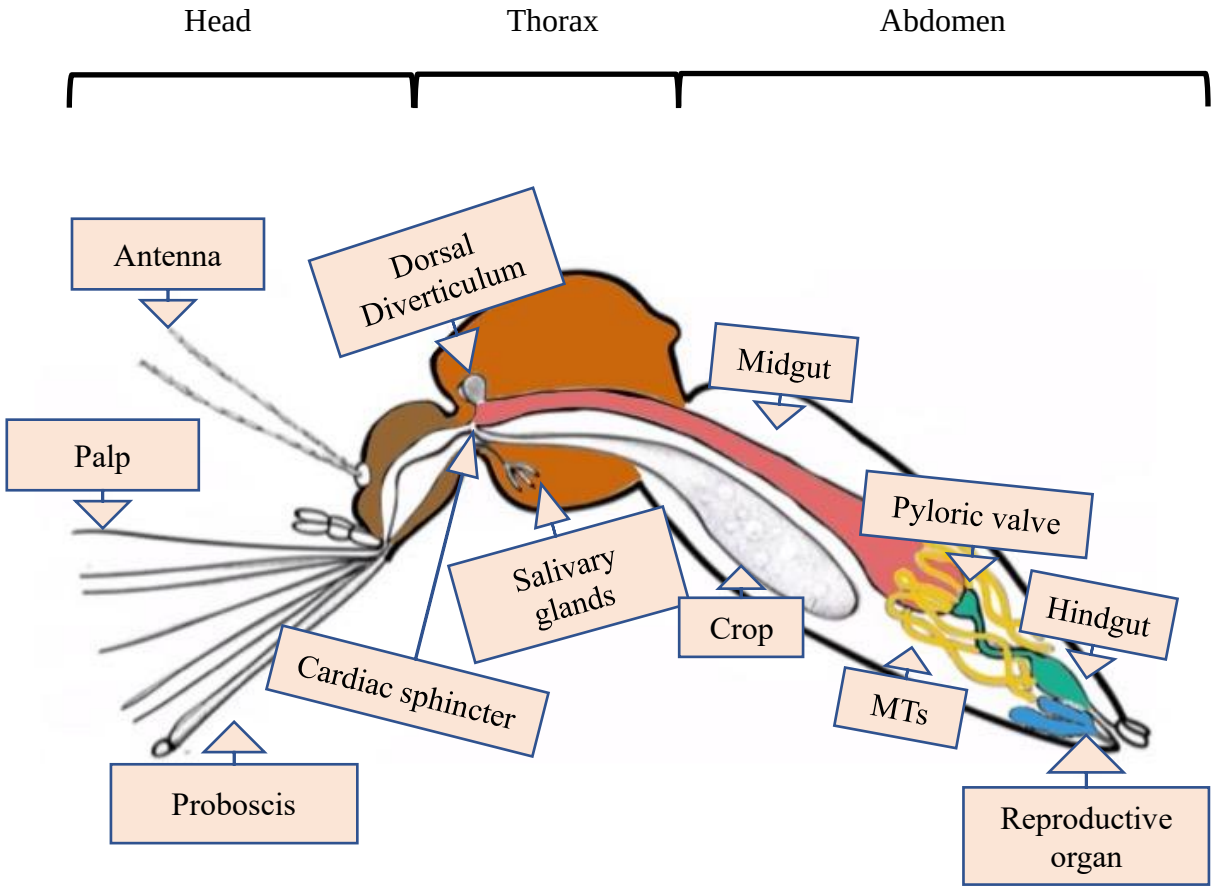

Figure 1.3 Schematic representation of adult mosquito anatomy. There are three major segments including the head, thorax and abdomen. Antenna: organs that gather information from external environment (i.e. level of CO_2). Palp: organs that sense odor. Proboscis: female mosquitoes use it for piercing host skin and feeding on blood. Diverticulum (dorsal diverticulum and crop): selectively store sugar solution (i.e. nectar). Salivary gland: secretes saliva contains bioactive components during the mosquito feeding process. Midgut: the digestive organ that begins from the cardiac sphincter in the prothorax region and ends at the pyloric valve. Hindgut: the organ involved in modifying urine before excretion. Malpighian tubules (MTs): tissues that are involved in ion and fluid transport. Reproductive organ: ovaries (female) or testes (male).

There are up to 20 distinct cell clusters in the midgut epithelium of mosquitoes, including 8 identified cell types, such as cardia and visceral cells (VM), intestinal stem cells (ISC), enteroblasts (EB) and differentiating enteroblasts (dEB), enteroendocrine cells (EE), enterocytes (EC) and enterocytes-like cells (Cui and Franz, 2020). According to studies on *D. melanogaster*, secretion of digestive enzymes and absorption of nutrients are handled by the most abundant cell type, EC. Production and release of peptide hormones and neuropeptides is carried out by EE based on changes in the extracellular environment and cell signaling. The ISC have the ability to divide into two new stem cells or differentiate into EC and EE via the undifferentiated progenitor EB in the process of asymmetric division (Nászai et al., 2015; Caccia et al., 2019; Cui and Franz, 2020). Brown *et al.* estimated there were 500 EE in adult mosquito midgut that have comparable ultrastructure to vertebrate gastro-intestinal endocrine cells. Additionally, EE are more abundant in the posterior midgut and less distributed in the anterior and middle region of the midgut. Unlike the columnar shaped EC with a labyrinth basal membrane, these endocrine cells are cone-shaped and have a smaller size with a flatter and smoother plasma membrane at the basal side (Brown et al., 1985). Among them, some cell bodies, which are further classified as open cells, have microvilli directly connecting to the lumen of the midgut allowing them to sense chemical and nutrient contents in the lumen and providing information to regulate neuropeptide and hormone secretion (Lartigue and Raybould, 2012; Lee and Owyang, 2019), while closed EE cells are located near to the basal membrane without microvilli extending to the lumen, which allows them to be modulated either by neural or humoral pathways rather than intestinal contents (Latorre et al., 2016). Many secretory granules that range from 60 to 120 nm were observed inside EE, and their cytoplasm ranged from a light density (transparent) to very dense (dark) that may correlate to contents inside the individual cell (Brown et al., 1985).

Numerous neuropeptides produced in the insect midgut region engage in various physiological processes (Wu et al., 2020). For example, the head peptide secreted by midgut endocrine cells in *Ae. aegypti* inhibits the host-seeking behaviour of females (Stracker et al., 2002). Insulin-like peptides (e.g. ILP2, ILP4, ILP 5) and tachykinin-related peptides expressed in *D. melanogaster* midgut as well as the midgut of other insects regulate development, growth, lifespan and response to nutritional conditions (Brogiolo et al., 2001; Grönke et al., 2010; Van Loy et al., 2010; Yamagishi et al., 2018). Allatostatin A enriched in the endocrine cells located at the posterior midgut of several insect species affects K^+ absorption and gut contraction (Spit et al., 2012; Vanderveken and O'Donnell, 2014). Moreover, diuretic hormone 31 was also determined to control the frequency in midgut and hindgut contraction of larval *D. melanogaster* (Reiher et al., 2011; Vanderveken and O'Donnell, 2014). It is not difficult to see that physiological functions of neuropeptides that originate in the midgut are often related to development, feeding behaviour, nutritional status, along with digestive processes including intestinal myoactivity and absorption.

1.4.2 Mosquito midgut post-blood feeding

As a haematophagous insect, a blood meal is essential to female *Ae. aegypti* because it provides the nutritional components needed to reproduce, which nectar-derived carbohydrates cannot provide. Ingestion of blood promotes vitellogenesis and oogenesis in female mosquitoes resulting in production of eggs (Nayar and Sauerman, 1975; Billingsley, 1990; Clements, 1992; Cui and Franz, 2020). As mentioned previously, the midgut is an important site for digesting blood meals, so it is not surprising studies have shown that cell composition ratio in the whole midgut undergoes tremendous changes post-blood meal (Cui and Franz, 2020). The ratio of cell cluster that can differentiate into other cells, such as the stem cells and EB increased 1.5% as a

proportion of the total cells. The total distribution of EC and EC-like cells increases approximately 13.5% in the female midgut by 24h post-blood feeding. Notably, a blood meal has been shown to have no major impact on the composition of EE cluster since it only reduced by 0.5%. Changes in the distribution of cardia and VM cells were greater, with a 4% and 10% reduction in cell composition post-blood feeding, respectively. Researchers suggested that the decreased in distribution of cardia and VM cells was due to their minor role in digestion of blood (Cui and Franz, 2020).

Since mosquitoes ingest a large volume of blood, the internal environment undergoes tremendous changes, for example, excess water, Na^+ and K^+ ions enter the midgut and haemolymph of female mosquitoes when they engorge on blood (Sanders et al., 2003). In response to challenges of maintaining homeostasis, as well as other stresses and the needs to alternate feeding and general behaviours, the transcriptome of the entire body changes dramatically after blood feeding. Studies have reported that the abundance of up to 50% of transcripts produced within a gonotrophic cycle were affected in the malaria mosquito, *Anopheles gambiae* (Dana et al., 2005; Marinotti et al., 2006; Bonizzoni et al., 2011). As the primary organ of blood meal digestion and nutrient absorption, gene expression in the midgut was also largely affected. Sanders *et al.* found that expression of 333 genes in *Ae. aegypti* midgut were varied post-blood meal. First of all, enzymes such as proteases and aminopeptidases that are responsible for breaking down proteins, amino acids and fats were up-regulated, resulted in an increased rate of metabolism (Billingsley and Hecker, 1991; Noriega et al., 1997; Sanders et al., 2003). In addition, the expression of the apical membrane transport protein V-ATPase and an amino acid transporter were also up-regulated (Sanders et al., 2003). These findings were supported by other studies that found the transcript abundance of between 26 to 201 genes in 18

out of 20 identified cell clusters in the mosquito midgut increased (Cui and Franz, 2020). Most of the genes among them play roles in various metabolic processes and ion transport, which is consistent with a previous study (Sanders et al., 2003). Notably, a significant amount of genes expressed in EE cells that are involved in cellular homeostasis, synthesizing receptors and signaling pathways showed increased transcript abundance. Some genes were found to be down-regulated, such as hydrolase and aquaporin, but these genes were found in EC, cardia and VM cell clusters and were not in the midgut endocrine cells (Cui and Franz, 2020).

Neuropeptide gene expression profiles in mosquitoes were also affected by blood meal engorgement. The haemolymph concentration of NPF, which is secreted by the head and midgut endocrine cells of *Ae. aegypti*, was found to be significantly higher 24h before and after blood feeding than other time points (Stanek et al., 2002). Another study revealed that inhibition of host-seeking behaviour of female mosquitoes post-blood meal associated with reduced expression of short neuropeptide F-2 (sNPF-2), allatostatin-A-5 (AstA-5) and neuropeptide-like precursor-1-5 (NPLP-1-5), even though changes were transient (Christ et al., 2017).

1.5 CCHamides and CCHamide receptors

1.5.1 General background of CCHamides

The neuropeptides CCHamides are considered as brain-gut neuropeptide that have been discovered in arthropods, such as crustaceans, centipedes and insects (Ida et al., 2012; Chipman et al., 2014; Christie, 2014; Veenstra and Ida, 2014; Ren et al., 2015). Two separate genes were identified in insects that belong to CCHamide family, one gene codes for CCHamide 1 (CCHa1) and another codes for CCHamide 2 (CCHa2) (Ren et al., 2015). Both neuropeptide structures were originally described by Zdárk *et al* in 2000, and subsequent studies indicated that

CCHamides were expressed in the midgut of flies including *D. melanogaster* and *Delia radicum* and isolated from them via capillary offline RP-HPLC coupled with MALDI-TOF MS/MS (Zdárek et al., 2000; Reiher et al., 2011; Zoephel et al., 2012).

Both neuropeptides were extracted from whole *D. melanogaster* and their primary amino acid sequences were identified by Ida *et al*. (Ida et al., 2012). The prepropeptide encoded by CCHa1 cDNA contains 182 amino acid residues (CG14358) with a signal peptide at the N-terminus. The deduced active peptide sequence is SCLEYGHSCWGAH-NH_2. The prepropeptide encoded by CCHa2 gene consists of 136 residues (CG14375), which is smaller compared to the CCHa1 prepropeptide. Interestingly, however, the CCHa2 precursor was found to produce three slightly different peptides as a result of differential processing over the N-terminal region. Their amino acid sequences are: 1). AQQSQAKKGCQAYGHVCYGGH-NH_2 (the longest); 2). KKGCQAYGHVCYGGH-NH_2; 3). GCQAYGHVCYGGH-NH_2 (the shortest). The N-terminal signal peptide cleavage site was located right before the longest CCHa2 active peptide. CCHa1 and CCHa2 active peptides are structurally similar in that they contain 13 amino acid residues with a disulfide bond between two cystines, which allows them to form a cyclical structure. Additionally, they all have a YGH motif and a C-terminus GXH-NH_2 motif, where X=A or G (Ida et al., 2012).

1.5.2 General background of CCHamide receptors

In 2010, two orphan GPCRs, CG14593 and CG30106, that were originally cloned in *Drosophila* a decade earlier (Frederiksen, 2004), were deorphanized as specific receptors of the novel endogenous ligands CCHa1 and CCHa2, and named CCHa1 receptor (CCHa1R) and CCHa2 receptor (CCHa2R) (Hansen et al., 2011; Ida et al., 2012). Studies demonstrated that CCHa1 and CCHa2 could specifically activate CCHa1R and CCHa2R at below nanomolar

concentrations. Moreover, around 100- to 1000-fold higher concentration of the peptides, CCHa1 and CCHa2, could induce a response by promiscuous binding to CCHa2R and CCHa1R, respectively (Hansen et al., 2011; Ida et al., 2012; Tian et al., 2021). Evidence suggested there were two essential structural features in CCHa1 and CCHa2 required to activate their receptors within the physiological concentration range: 1) the amide group at C-terminus and 2) the intrachain disulfide bond (Ida et al., 2012; Tian et al., 2021). Investigating the signaling cascade mediated by CCHamides and their receptors revealed that CCHa1R linked to $G\alpha_q$ and $G\alpha_s$ subunits, whereas CCHa2R only linked to the $G\alpha_q$ subunit. Furthermore, activation of both receptors resulted in releasing intracellular Ca^{2+} and phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK1/2), but increasing of cAMP can be only induced by binding of CCHa1 to CCHa1R (Tian et al., 2021).

Although typical GPCRs consist of seven transmembrane domains, structural studies discovered that CCHa2R has an incomplete 7th transmembrane domain with a short intracellular amino acid sequence (Tran et al., 2019). An N-glycosylation motif located within the 2nd extracellular loop is present in both receptors. According to the classification of GPCRs, CCHa1R and CCHa2R were both categorized as Class A, rhodopsin-like receptors (Tran et al., 2019). Subsequent evidence further suggested that they belong to the phylogenetic subgroup III-B within the Class A, bombesin/gastrin releasing peptide receptors, which are then sub-divided into gastrin releasing peptide-preferring receptor (GRPR), the neuromedin B-preferring receptor (NMBR) and bombesin receptor subtype 3 (BRS3). BRS3 is a group of orphan receptors, and it is where CCHa1R and CCHa2R was phylogenetically grouped (Hewes and Taghert, 2001; Ida et al., 2012). In addition, an earlier phylogenetic study indicated that CCHamide receptors and the vertebrate bombesin/GRP receptor lineages shared a common ancestor (Hewes and Taghert,

2001). This may provide insights into the functionality of CCHamide receptors and the corresponding signaling pathway because mammalian BRS3 was known to be expressed in the hypothalamus and participate in the onset of obesity and diabetes (Ohki-Hamazaki et al., 1997; Hansen et al., 2011; Ida et al., 2012; Veenstra and Ida, 2014).

1.5.3 Previous studies of CCHa1 and CCHa1R

Since CCHa1 is a relatively recently discovered neuropeptide, its developmental and tissue-specific expression and functionality have not been intensively studied in multiple insects, except a few studies on *D. melanogaster* and the beet armyworm *Spodoptera exigua*. Over development of *D. melanogaster,* CCHa1 and CCHa1R transcripts were found to be expressed in every developmental stage from first instar larva to adult flies. Eggs had no sign of either CCHa1 or CCHa1R transcripts. Notably, both transcripts reached the highest level of abundance in adulthood with male flies in particular exhibiting a significantly higher peptide and receptor transcript level than female adult flies. Differential expression between CCHa1 and CCHa1R was observed in four instar larva stages. CCHa1 was expressed relatively unchanged throughout the larval phase, but the receptor transcript was significantly enriched in the last instar larval stage compared to earlier larval stages (Li et al., 2013).

In terms of tissue-specific expression, Llopis-Giménez *et al.* determined that CCHa1 transcript was highly enriched in the brain of both larva and adult *S. exigua* as well as in the gut region of larva under standard laboratory conditions. Moreover, they found CCHa1 mRNA level was up-regulated in the brain of larva after 24h starvation (Llopis-Giménez et al., 2019). In *Drosophila* larva, only the gut region was found to produce a large amount of CCHa1, and only relatively small amounts of receptor was expressed in both brain and gut. However, expression profiles changed in adult flies, with both sexes exhibiting high levels of *CCHa1* and *CCHa1R*

transcripts in the brain and gut, even though females produced noticeably less of each transcript relative to males (Li et al., 2013). Another study also confirmed that CCHa1 was in the CNS and the midgut of adult *Drosophila* (Titos and Rogulja, 2020). Specifically in the anterior dorsal neuron 1 (DN1a), which was classified as one of the *Drosophila* clock neurons that is responsible for expressing clock genes based on circadian rhythms. In addition to the dorsal neurons, five other lateral neuron clusters (LPN, LN_d, 5^{th} s-LNv, I-LNv, and s-LNv) are also involved in the circadian clock network (Fujiwara et al., 2018). Based on the evidence of immunohistochemistry using rabbit anti-CCHa1 antibody, CCHa1 was detected in the s-LNv neuron cluster with weaker staining suggesting less expression and in the I-LNv neuron cluster with stronger staining suggesting higher expression. Furthermore, the projection of CCHa1 immunoreactivity from DN1a to the dorsal s-LNv was revealed (Fujiwara et al., 2018).

The level of cAMP was increased when CCHa1 activated its receptor (CCHa1R) suggesting a possible downstream signaling pathway involving this second messenger (Fujiwara et al., 2018). Researchers observed that knocking down or knocking out *CCHa1* expression reduced morning activity of flies and changed their circadian activity patterns. More specifically, contrary to the control group, CCHa1 mutant flies experienced either an advanced or a delayed morning activity under normal light to dark cycle or under constant dark conditions, respectively (Fujiwara et al., 2018). Also, a longer siesta was observed in the CCHa1 mutant flies. Fujiwara *et al.* suggested that this change in behaviour might relate to altered expression of pigment dispersing factor (PDF) in mutant flies. Lack of CCHa1 would result in up-regulating the secretion of PDF and then subsequently enhance the amplitude of PAR domain protein 1 (PDP1) cycling within s-LNv neurons that was known to increase the siesta of the flies with high level of expression in previous study (Fujiwara et al., 2018; Kistenpfennig et al., 2018).

As mentioned previously, *CCHa1* expression was detected in the posterior midgut of adult *D. melanogaster* (Titos and Rogulja, 2020). This study also discovered that a protein-rich meal increased the secretion of CCHa1 via midgut endocrine cells; however, other nutrient components, for example, sugar and fat had no effect on the *CCHa1* expression (Titos and Rogulja, 2020). Furthermore, increased secretion of CCHa1 is a gradual and prolonged process as indicated by a higher transcript level of *CCHa1* that was observed 24h post protein ingestion compared to 6h post ingestion. Subsequent experiments indicated that a single or multiple specific amino acid components were not sufficient to trigger this up-regulation, instead a high concentration of amino acid mixture would generally increase the transcript expression of *CCHa1* (Titos and Rogulja, 2020). Since food satiety associates with the duration and the depth of sleep, the authors considered that CCHa1 produced by intestinal endocrine cells following a protein-rich meal was used to regulate arousability of flies (Titos and Rogulja, 2020). With the help of RNAi, they noticed that flies lacking either CCHa1 or its corresponding receptor (CCHa1R) were much easier to be woken up (~85% of experimental group) by low amplitude stimulation than the wild type flies (~20% responded). Furthermore, CCHa1 knockdown resulted in a more fragmented sleep rather than a continuous one and knockdown of the receptor resulted in less sleep duration (Titos and Rogulja, 2020). To further pinpoint where in the nervous system CCHa1-CCHa1R signaling that was responsible for this physiological process, *CCHa1R* expressed in different neuron clusters was knocked down individually. Results revealed that the receptor localized within dopaminergic neurons in the protocerebral anterior medial (PAM) cluster was involved in modulating arousability. The PAM is close to the anterior region of the brain in the anterior inferiormedial protocerebrum which is known to be tightly associated with appetitive memory and reward signals of flies (Yamagata et al., 2015; Yamagata et al., 2021).

Even though CCHa1R was also localized in other neuron clusters, such as cholinergic, octopaminergic, glutamatergic and circadian neurons, they appeared to have no correlation with arousability (Titos and Rogulja, 2020).

In addition to playing a role in regulating circadian rhythm and arousability, CCHa1R may also play an important role in modification of starvation induced olfactory responses in *D. melanogaster* (Farhan et al., 2013) that like many other organisms, forage for food using their olfactory system. Flies up-regulate their olfactory sensitivity to food odorants as well as exhibit a stronger and more active behavioural response when they encounter starvation stress. Simultaneously, expression of CCHa1R in the fly brain was found to increase 2.5-fold. These starvation-induced activities were no longer displayed after knocking out CCHa1R revealing that the receptor and its associated signaling pathway participated in the olfactory augmentation process in sensory neurons that express olfactory receptor OR59b (Farhan et al., 2013).

1.5.4 Previous studies of CCHa2 and CCHa2R

Similar to CCHa1 expression, previous studies in *D. melanogaster* suggested that *CCHa2* and its receptor (*CCHa2R*) transcript levels fluctuate over the development of the organism. *CCHa2* transcript level remains relatively low until flies reached adulthood (Li et al., 2013). Comparatively, *CCHa2R* levels increased drastically from egg to the first and second instar larval stage and dropped thereafter until the last larval stage. *CCHa2R* transcript increased again in the pupa stage and maintained high expression in adulthood (Li et al., 2013). Notably, a sexually dimorphic expression profile for both peptide and receptor transcript was detected, which was also observed for *CCHa1* and *CCHa1R*. Male adults generally had a higher abundance of mRNA transcripts than female adults (Li et al., 2013).

Similar to *CCHa1* discussed above, *CCHa2* was found particularly enriched in the midgut of both larva and adults, whereas the brain was determined as a nearly exclusive site for *CCHa2R* expression (Li et al., 2013; Ren et al., 2015). However, Sano *et al.* presented some contradictory data since their real-time quantitative PCR (RT-qPCR) results showed there was nearly no *CCHa2* transcript detected in *D. melanogaster* larval midgut; instead the fat body was found to be the exclusive site having high levels of *CCHa2* transcript (Sano, 2015). This was also supported by the immunohistochemistry results, since staining detection using an antibody that specifically targets CCHa2 peptide was absent in the fat body of CCHa2 knockout flies. However, in partial contradiction of their own RT-qPCR results, clear CCHa2 staining can be seen in the midgut of wild type larva in contrast to the knockout group (Sano et al., 2015). In another insect, the kissing bug *Rhodius prolixus*, *CCHa2* was not only detected in the anterior midgut, but also in the CNS and Malpighian tubules (MTs), while the receptor is expressed in both anterior and posterior midgut as well as MTs, but not in the brain (Capriotti et al., 2019). Distinct expression profiles in different insects suggests that CCHa2 and its associated signaling pathway might possess species-specific physiological functions.

In *R. prolixus*, CCHa2 exhibited a dual function in the regulation of diuresis during the post-prandial condition. Serotonin-stimulated secretion by the MTs was strengthened and reabsorption via the anterior midgut was largely reduced by CCHa2 (Capriotti et al., 2019). As the result of depleting CCHa2 neuropeptide by RNAi, the amount of urine excreted at each time point over 6h, two different observations were made. Before 60 min, lack of CCHa2 neuropeptide led to increase urine production, but after 60 min, the amount of urine produced was noticeably less than negative control animals with normal levels of CCHa2 expression (Capriotti et al., 2019).

However, this function does not apply to *D. melanogaster* where CCHa2 was found to be involved in regulating feeding behaviour and responding to starvation stress. Studies indicated *CCHa2* transcript levels were down-regulated when flies were starved, and the expression was rescued post-feeding (Sano, 2015; Sano et al., 2015). Further experiments tested the effect of various nutrient components to the expression of *CCHa2*, including yeast, peptone, leucine and multiple types of sugar. Results revealed that CCHa2 was a general sugar responsive neuropeptide, which notably increased in response to glucose, fructose and trehalose, despite one exception that it also responded to yeast ingestion (Sano, 2015; Sano et al., 2015). Ren *et al.* noticed that compared to wild type flies, CCHa2 knockout flies showed a significant reduction in food consumption and feeding and foraging activities. On the one hand, the volume of sucrose solution and yeast ingested per hour by mutant adult flies and larva reduced by half. On the other hand, the feeding activity of mutant larva, the number of movements of their mouth hook, decreased by one third. Moreover, adult flies lacking CCHa2 were less active during their typical foraging time period (Ren et al., 2015). Effects of CCHa2/CCHa2R on feeding behaviour are not limited to *D. melanogaster*, but have also been observed in the pea aphid, *Acyrthosiphon pisum*. When starved, *CCHa2R* transcript was increased, which was opposite to the expression of *CCHa2* in starved *D. melanogaster*. However, knocking down *CCHa2R* reduced food consumption in aphids as observed in flies that lacked *CCHa2* expression (Shahid et al., 2021).

In addition to regulating these behaviours, CCHa2 also seems to play a role in development of flies, which is not surprising due to changes in expression observed over the course of development. One study found that the average pupariation time was delayed approximately 70 hours due to depletion of CCHa2 in mutant flies. Furthermore, all influences can be rescued to the level close to the wild type condition by re-introducing the CCHa2 gene (Ren et al., 2015).

Since ILPs are well known to be deeply involved in processes of feeding, energy circulation and development in insects, CCHa2 was hypothesized to have interactions with them and their associated signaling pathways. As predicted, Ren *et al.* illustrated that *Drosophila* ILP2 (dilp2) and ILP3 (dilp3) were remarkably down-regulated in CCHa2 mutant larva and pupa (Ren et al., 2015). In contrast, another group suggested otherwise by knocking out the receptor in larva rather than the neuropeptide. Both dilp2 and dilp3 expression were not affected by the loss of CCHa2R, but they noticed that dilp5 that was normally enriched in the brain of fed larva drastically decreased due to CCHa2R mutation. Similar results were obtained by interfering with CCHa2 expression via RNAi (Sano et al., 2015). Thus, signaling involving CCHa2/CCHa2R results in the control of various physiological processes and supports a pleiotropic role for this neuropeptide in insects.

1.6 Research objectives and hypotheses

The structure of CCHamide neuropeptides were initially reported in a study of parturition hormone in *Glossina morsitans* (Zdárek et al., 2000). Subsequent studies have identified CCHamides and their authentic receptors in many arthropods (Chipman et al., 2014; Christie, 2014; Veenstra and Ida, 2014; Ren et al., 2015). However, currently, expression profiles and possible physiological roles and associated signaling pathways of CCHamides and their receptors in many insects, including *Ae. aegypti* remain elusive. This research is focussed on functionally characterizing CCHamide receptors and elucidating possible functions of these neuropeptides based on their molecular distribution in adult *Ae. aegypti*. Therefore, the aim of this project is to examine, quantify and localize transcript expression profiles of CCHamides and

CCHamide receptors, and functionally deorphanize receptors to help deduce their physiological roles.

The first objective is to quantitatively determine expression profiles of *Ae. aegypti* CCHa1 and CCHa2 and CCHa2R in different developmental stages and in various tissues/organs of adult males and females. Based on expression profiles on *D. melanogaster* (Li et al., 2013), it was hypothesized that 1) *CCHa1*, *CCHa2* and *CCHa2R* expression would vary over development and reach the highest abundance in the adult stage; and 2) *CCHa1* and *CCHa2* would be enriched in midgut of adult mosquitoes of both sexes; and 3) *CCHa2R* would be enriched in the head.

The second objective is to localize and visualize CCHa2 producing neurons or endocrine cells and determine their qualitative expression profiles via immunohistochemistry. It was hypothesized that *Ae*CCHa2 antibody staining would be observed in enteroendocrine cells in the midgut of adult male and female mosquitoes, which would correspond to the CCHa2 expression profile revealed in RT-qPCR.

The third objective is to examine the functional activation of *Ae. aegypti* CCHa1R and CCHa2R in order to deorphanize both receptors and examine their responses to CCHamides along with multiple structurally distinct neuropeptides. Previous studies reported CCHa1 and CCHa2 were the ligands that specifically activate CCHa1R and CCHa2R, respectively in *D. melanogaster* (Hansen et al., 2011; Ida et al., 2012), land crab *Gecarcinus lateralis* (Tran et al., 2019) and silkworm *Bombyx mori* (Tian et al., 2021) with physiologically relevant concentrations. Cross activation was also observed when applying 100-1000 fold higher concentrations of these neuropeptides. Therefore, it was hypothesized that *Ae. aegypti* CCHa1 and CCHa2 would have the highest activity towards the orphan mosquito receptors, CCHa1R

and CCHa2R, respectively, but would also activate the other receptor at a much higher concentration due to similar peptide structures between these two CCHamide neuropeptides. Lastly, it was hypothesized that peptides lacking the CCHamide structural characteristics such as the disulfide bond, a YGH motif and a C-terminal GXH-NH_2 motif, would not activate the receptors at all.

The last objective was to investigate possible physiological functions of the CCHamide neuropeptides. Previous studies in other insects revealed that CCHa2 was a sugar-responsive neuropeptide and mostly involved in regulating feeding and related behaviours (Ren et al., 2015; Sano, 2015; Sano et al., 2015). It was hypothesized that starvation would reduce *CCHa2* expression in adult mosquitoes of both sexes. Moreover, since female mosquitoes also acquire a blood meal to obtain essential nutrients needed for egg production and their feeding behaviour is largely associated with blood ingestion, it was hypothesized that engorging a blood meal would affect the level of *CCHa1* and *CCHa2* transcripts in female mosquitoes.

As a widely distributed anthropophilic mosquito species, *Ae. aegypti* is able to transmit various pathogens leading to human diseases. Studying CCHamides and their receptors allows us to better understand mosquito biology and could help pave new directions for preventing mosquito-borne diseases since CCHamides are likely to participate in the regulation of feeding activities.

Chapter II: Characterization and functional deorphanization of CCHamides and CCHamide receptors in the yellow fever mosquito, Aedes aegypti

2.1 Materials and methods

2.1.1 Animal rearing

Ae. aegypti colony maintenance was achieved by regular blood feeding of female mosquitoes with sheep blood in Alsever's solution (Cedarlane Laboratories, Burlington, ON), which allows them to produce eggs. Dampened Whatman filter papers (GE Bioscience) were placed in cages housing the *Ae. aegypti* colony (Liverpool strain) to allow a suitable substrate for egg laying by females. The collected eggs were then placed in a plastic container with distilled water to hatch and rear under a 12: 12hr (light to dark) cycle in an incubator at 25°C as previously described (Wahedi and Paluzzi, 2018). Hatched larva were fed once every two days with several drops of liquid larval food containing 2% beef liver powder and 2% brewer's yeast (NOW foods, Bloomingdale, Illinois) in distilled water. Unless noted otherwise, all mosquitoes post-eclosion were provided a 10% sucrose solution in a microcentrifuge tube fitted with a cotton ball. Pupa were isolated and transferred into a plastic mesh-covered container and the resulting adult mosquitoes were either used for subsequent experiments or transferred back to the main colony.

2.1.2 CCHamide and receptor expression profiles

2.1.2.1 Primer design and examination

Primers for expression profiling of CCHamides and their receptors as well as preparing constructs for mammalian cell line expression were designed using Geneious Pro Bioinformatics Software (see Table 2.1). Primers of *CCHa1* (737F-901R) and *CCHa2* (247F-474R) were designed over different exons; primers of *CCHa2R* (334F-560R) were located at exon boundaries; other primers were located outside the open reading frames (see section 2.3.4.1). The

amplification efficiency of primers was examined by a standard curve analysis via real time-quantitative PCR (RT-qPCR) with two-fold serial dilutions of male midgut cDNA. Amplification specificity of primers was confirmed by observing no detection in negative control groups, which included a no reverse transcriptase control and a no template control group. The first negative control group accounts for false positive amplification from potential genomic DNA contamination, and the second negative control determined the absence of primer-dimers and any other non-specific amplification. Correctness of amplicon was also confirmed by Sanger sequencing results that were then compared to the predicted sequence of *Ae. aegypti* CCHa1 (GenBank accession: XM_021854293.1), CCHa1R (GenBank accession: XM_021851237.1), CCHa2 (GenBank accession: XM_021838098.1) and CCHa2R (GenBank accession: XM_021852958.1). Primer pairs with the best efficiency were chosen for transcript expression profiling by RT-qPCR.

2.1.2.2 Sample collection and tissue dissection

Whole mosquitoes over several developmental stages, including 4th instar larva, early stage pupa (transparent/light colour), late stage pupa (dark colour), one-day old post-eclosion males and females and four-day old post-eclosion males and females, were collected (n=10) and stored in DNA/RNA protection reagent (New England Biolabs, Whitby, On) prepared with sterile nuclease-free water (Wisent Inc., St. Bruno, Quebec, Canada) in 1 : 1 ratio and stored at -20°C for further processing.

One-day old male and female mosquitoes (n=35-40) were anaesthetized via brief CO_2 gas exposure and kept on ice. Individual mosquitoes were anchored onto a silicone-lined petri dish by a dissection pin impaling the thorax region and submerged in 3-4 drops of nuclease-free Dulbecco's phosphate-buffered saline (DPBS; Wisent Inc., St. Bruno, QC) ready for dissection.

The head, thorax, midgut, MTs, hindgut, reproductive system including ovaries (female) or testes (male) and accessory reproductive organs (oviducts and spermathecae), and carcass, which contains skeletal muscles, fat body, trachea, integument and abdominal ganglia, were dissected and stored in DNA/RNA protection solution (as above) for subsequent experiments.

2.1.2.3 RNA isolation, cDNA synthesis and RT-qPCR

Whole body samples and dissected tissues/organs that were stored in DNA/RNA protection solution were mechanically homogenized with a polypropylene pestle. Total RNA was then extracted using a Monarch® Total RNA Miniprep Kit (New England Biolabs, Whitby, On) following the manufacturer's protocol. The concentration of purified RNA was quantified using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) with a Take3 micro-volume plate. Following the manufacturer's protocol, 500ng whole insect RNA or 80ng tissue-specific RNA was used as template to synthesize cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad, Mississauga, ON) followed by 11-fold dilution prior to RT-qPCR analysis.

Expression profiles of *CCHa1*, *CCHa2* and *CCHa2R* were assessed by amplifying a 164bp, 227bp and a 226bp DNA fragment, respectively, with optimized primers described above (see section 2.3.2.1) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, United States) using PowerUP™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, United States). RT-qPCR cycling condition started with 50°C for 2 minutes followed by 95°C for 20 seconds, and then 40 cycles of (i) 95°C for 3 seconds and (ii) 62°C for 30 seconds. Two reference genes, ribosomal protein 49 (rp49) and rpS18, were used as optimal endogenous controls to normalize the expression of target genes of interest via $\Delta\Delta C_T$ method using primers described previously (Paluzzi et al., 2014). Quantitative determination of developmental expression profiles of *Aedae*CCHamides and *CCHa2R* analyzed 5 biological replicates with each

containing triplicates of technical replicates and a no-template control group. Between 3 to 4 biological replicates were used to determine tissue-specific expression profiling. Post-blood meal expression of *CCHa1* and *CCHa2* in female mosquitoes was analyzed using 5 biological replicates (see section 2.3.5.2 for details). In the starvation-stress assay (see section 2.3.5.1), *CCHa2* and *CCHa2R* expression patterns were examined in 3 biological replicates.

2.1.3 Localization and visualization of CCHa2 producing neurons and endocrine cells

2.1.3.1 Whole mount immunohistochemistry

In order to localize the distribution of CCHa2 producing neurons and endocrine cells, and in light of the CCHa2 transcript expression profile, the entire gut region, including the midgut, pyloric valve (i.e. the junction between the midgut and hindgut), the hindgut, and abdominal ganglia of one-day old male and female sugar-fed mosquitoes and 24 to 48h starved mosquitoes were dissected to perform the whole mount immunohistochemistry, following a protocol described previously (Sajadi et al., 2020). Dissected tissues were fixed in 4% paraformaldehyde (PFA) overnight at 4ºC. Tissues were washed 10 minutes with DPBS for three times and followed by 1h incubation at room temperature with 4% Triton X-100, 2% bovine serum albumin (BSA) and 10% normal sheep serum (NSS) for tissue permeabilization. Primary antibody (5ug/mL), anti-*Aedae*CCHa2 (Biomatic Co., Kitchener, ON), was prepared in 0.4% Triton solution 12h prior use, and then incubated with permeabilized tissues for 48h at 4ºC on a rocking platform with gentle agitation. For the negative control treatments, 10μM of either CCHa1 or CCHa2 synthetic peptide (PepMic, Suzhou, China) was pre-incubated overnight along with the primary antibody and used on permeabilized tissues as described above. After washing three times with DPBS, tissues were then incubated for 24h at 4ºC on a rocking platform with the secondary antibody, goat-produced anti-rabbit Alexa 568 (Life Technologies, Burlington, ON)

that was diluted in 10% NSS and DPBS (1:200 dilution). Tissue samples were rinsed three times with DPBS again and then mounted onto microscope slides in mounting buffer containing glycerol and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR) to observe cell nuclei in prepared tissues and examined under a Lumen Dynamics XCite™ 120Q Nikon fluorescence microscope (Nikon, Mississauga, ON, Canada).

2.1.3.2 Enzyme-linked immunosorbent assay (ELISA)

A custom rabbit polyclonal affinity-purified antibody, anti-*Aedae*CCHa2 (antigen sequence: Cys- GCAAFGHACYGGH-NH_2) (Biomatic Co., Kitchener, ON) from two individual rabbits, RB3155 and RB 3156, was used to target *Aedae*CCHa2 neuropeptide in mosquito tissues via whole mount immunohistochemistry. An ELISA test was conducted to determine the optimal antibody concentration, examine the binding specificity to the CCHa2 neuropeptide and identify potential cross reactivity with other neuropeptides including CCHa1.

Serial dilutions of synthetic of *Aedae*CCHa1, *Aedae*CCHa2, *Aedae*RYamide1 (Amino acid sequence: PVFFVASRY-NH_2) (Ida et al., 2011) and *Aedae*CAPA-PK1 (Amino acid sequence: AGNSGANSGMWFGPRL-NH_2) (Predel et al., 2010) (PepMic, Suzhou, China) were prepared ranging from 0.05μmol/100μl to 10μmol/100μl in carbonate buffer that consisted of 15mmol/L Na_2CO_3-H_2O (pH 9.4) and 35mmol/L $NaHCO_3$ (pH 9.4), and loaded onto a 96-well high binding ELISA plate (Sarstedt Inc. Montreal, QC) covered with plastic wrap, and then incubated at 4°C for 48h. After three washes using wash solution (346mmol/L NaCl, 2.7mmol/L KCl, 1.5mmol/L KH_2PO_4, 5.1mmol/L Na_2HPO_4-$7H_2O$ and 0.5% Tween-20), separate wells of the plate were incubated with primary antibodies (1 : 1000 dilution), anti-CCHa2 (RB3155 and RB3156), which were prepared in carbonate block solution that contains DPBS, 0.5% (w/v) skimmed milk powder and protease-free bovine serum albumin (BSA), for 2h at room temperature on a

shaking platform. Following primary antibody incubation, each well underwent three washes and this was followed by application of a secondary antibody (1:1500 dilution) anti-rabbit-HRP (Bio-Rad Laboratories (Canada) Ltd, Mississauga, ON) and incubated for 1h at room temperature on a rocker. This was followed by three washes and then tetramethylbenzidine (TMB) solution (Sigma-Aldrich Canada Co., Oakville, ON) was loaded onto the plate, and 2N HCl solution was added once the colour developed. A Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) was used to measure the absorbance of each well at 450 nm.

2.1.4 Heterologous functional activity bioluminescence assays

2.1.4.1 Preparation of mammalian expression constructs with *Ae. aegypti* CCHa1R and CCHa2R

The complete open reading frames of *Aedae*CCHa1R and *Aedae*CCHa2R were amplified by PCR using Q5 high-fidelity DNA polymerase (New England Biolabs, Whitby, ON) with primers located upstream of the start codon and downstream of the stop codon (Table 2.1) from *Ae. aegypti* predicted receptor sequences (CCHa1R GenBank accession: XM_021851237.1; CCHa2R GenBank accession: XM_021852958.1). Following the recommended protocol, using a Monarch PCR purification kit (New England Biolabs, Whitby, ON), the amplicons comprising the open reading frame of CCHa1R and CCHa2R were purified. Open reading frame sequences that contain the consensus Kozak translation initiation sequence prior to the start codon (Kozak, 1986) and restriction sites *Hind*III and *Xba*I at 5' and 3' ends, respectively, were obtained by PCR reamplification as above with modified primers (see Table 2.1) based on the earlier cloned receptor cDNA sequences. Reamplified PCR products were A-tailed and then ligated to pGEM-T Easy vector (Promega, Madison, WI, USA), and then transformed into high efficiency competent *E. coli* cells (New England Biolabs, Whitby, ON). Harvested plasmid DNA miniprep

were confirmed by Sanger sequencing (The Centre for Applied Genomics, Sick Kids, Toronto, ON) to verify the accuracy of cloned open reading frame sequences. Restriction enzyme digested CCHa1R and CCHa2R construct sequences were ligated into pcDNA3.1+ (selectable marker: ampicillin) and pBudCE4.1 (selectable marker: zeocin) mammalian expression vectors followed by bacterial transformation and plasmid DNA extraction to obtain a large quantity of receptor constructs using ZymoPURE II Plasmid Midiprep Kit (Zymo Research, Tustin, CA, USA) following the manufacturer recommendation. The concentration and purity of vector constructs were determined by UV spectrometry using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). All prepared constructs (pcDNA-CCHa1R and CCHa2R, pBud-CCHa1R and CCHa2R) were tested and validated by running a preliminary bioluminescence assay with BSA media and ligands including CCHa1 or CCHa2, RYa1 and CAPA-PK1 as described in the methods below (see section 2.1.4.3). Ligands were prepared at the potential saturation concentration (10^{-6}M) based on previous study (Lajevardi and Paluzzi, 2020). pBud-CCHa1R and pcDNA-CCHa2R were selected to complete further experiments based on their higher signal to noise ratio and better performance.

2.1.4.2 Cell culture and colony maintenance

A recombinant Chinese hamster ovary cell (CHO-K1) line stably expressing aequorin (Paluzzi et al., 2012; Gondalia et al., 2016) stored in the liquid nitrogen was thawed and cultured into a T-25 cell culture flask (Sarstedt Inc. Montreal, QC) containing complete growth media that was prepared with Dulbecco's modified eagles medium: nutrient F12 (DMEM:F12) basal media (Wisent Inc., St. Bruno, QC), 10% heat-inactivated fetal bovine serum (HI-FBS; Wisent, St. Bruno, QC), 200µg/mL geneticin, and Gibco™ Antibiotic-Antimycotic (100X) (Anti-anti)

(Thermo Fisher Scientific, Mississauga, ON). Cells were grown at 37°C in a water-jacketed incubator with 5% CO_2 to maintain physiological pH of the bicarbonate-based growth media.

Cell colony maintenance for subsequent experiments was achieved by cell sub-culturing. Cells grown to approximately 100% confluency were detached from the culture flask surface using 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Mississauga, ON) for ~2min. The combined media that contains old growth media and Trypsin-EDTA solution was centrifuged at 400 rpm for 7 minutes to obtain a cell pellet. The supernatant was then discarded and the cell pellet was gently resuspended with fresh complete cell growth media and cultured into two new cell culture flasks and kept in an incubator at 37°C.

2.1.4.3 Receptor construct transfections and bioluminescence assay

Cells just reaching full confluency were used for transfection. The procedure started with normal sub-culturing protocol to increase transfection efficiency by reducing the number of cells to approximately 90% confluency. After being incubated at 37°C for 6h, cells were transfected with either pcDNA3.1+ receptor construct, pBudCE4.1 receptor construct containing also the murine promiscuous $G_{alpha}15$ (Paluzzi et al., 2014) or pBud-EGFP, the latter of which acted as a control treatment, using Lipofectamine 3000 or Lipofectamine LTX reagent (Invitrogen, Burlington, ON) following the manufacture guidelines. Both Lipofectamine 3000 and Lipofectamine LTX reagent serve the same function, but Lipofectamine 3000 delivered a slightly higher transfection efficiency and hence might result in greater recombinant protein expression. Cells transfected with the plasmid constructs were expected to produce and express either CCHa1R, CCHa2R or enhanced green fluorescent protein (EGFP) with the latter acting as a positive control for transfection and negative control for the bioluminescence assay.

Cells were harvested 48h post-transfection by dislodging with DPBS-EDTA solution, centrifugating and resuspending in assay media (BSA media), which was prepared with DMEM:F12 media, 10% Bovine Serum Albumin (BSA) and Anti-anti, with 5μM coelenterazine-h. Following 3h incubation at room temperature with stirring in the dark, cells were further diluted 10-fold in assay media and incubated for an additional 45 minutes. In order to examine the selectivity of responsiveness of *Aedae*CCHa1R and *Aedae*CCHa2R to multiple neuropeptides, a library of commercially synthesized insect neuropeptides were prepared (10^{-6}M and 10^{-7}M final titre) in assay media and loaded on 96-well luminescence plates (Greiner Bio-One, Germany) in quadruplicates following a palindromic loading scheme. To generate dose-response curves of *Aedae*CCHa1 and *Aedae*CCHa2 to both receptors, serial dilutions ranging from concentrations of 10^{-5}M to 10^{-14}M were prepared in BSA media and loaded on 96-well luminescence plates. BSA media alone was used to determine any background noise, which acted as a negative control; 50μM or 100μM ATP was used as a positive control to normalize bioluminescent response of each well due to a known Ca^{2+} flux triggered by endogenous purinoceptors expressed in CHO-K1 cells (Iredale and Hill, 1993). The Ca^{2+} kinetic luminescent response that was generated by injecting prepared cells to each well via an automated injector apparatus connected to the Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) was measured for 20 seconds followed by additional measurements for 10 seconds after ATP injection to the same well.

2.1.5 Physiological assays

2.1.5.1 Starvation assay

Starved mosquitoes of both sexes between 0 to 6h post-eclosion were isolated and randomly divided into 6 containers with different diet conditions in order to examine effects of starvation

stress on the transcript abundance of CCHa2 and CCHa2R. Test conditions included 10% sucrose-fed for 24 and 48h, water-fed for 24 and 48h and starved for 24 and 48h. Mosquitoes were collected and dissected for the whole mount immunohistochemistry or stored in the DNA/RNA protection reagent for subsequent RT-qPCR analysis.

2.1.5.2 Blood feeding assay

Hatched mosquito females were reared under normal laboratory conditions with males as previously described until reaching the age of 5-7 day-old without a single blood meal. They were then provided with pre-warmed (~37°C) sheep blood (Cedarlane Laboratories, Burlington, ON) for 20 minutes using an artificial feeding membrane as described previously (Rocco et al., 2017). Blood-fed females, which can be easily distinguished by the red colour of their ballooned abdomen, were isolated and kept in containers with 10% sucrose solution for 1h, 6h, 12h and 24h post-blood feeding before collection and stored in the DNA/RNA protection reagent for subsequent RT-qPCR analysis.

2.1.6 Statistical analysis

All log-transformed RT-qPCR data and luminescence responses of multiple ligands in the heterologous functional assay were analyzed by one-way ANOVA with Bonferroni's multiple comparisons test where $p < 0.05$ was considered significant. Dose-response curves and EC_{50} values were generated using the sigmoidal dose-response model. Statistical analysis was carried out by GraphPad Prism 9 (GraphPad Software, San Diego, USA).

Table 2.1 Primer information for oligonucleotides used for functional assay CCHamide receptor construct amplification and RT-qPCR analysis of CCHa1, CCHa2 and CCHa2R to determine developmental and adult tissue-specific transcript levels.

Oligo Name	Sequences (5' > 3')	Function
CCHa1-737F	CTCCGTCTGTTTCGTTAGTGGA	RT-qPCR amplification of *Aedae*CCHa1
CCHa1-901R	GTTGTCGAAGCAGATCATCACG	RT-qPCR amplification of *Aedae*CCHa1
CCHa2-247F	AGTAAAACATTCGATGACGCCTCC	RT-qPCR amplification of *Aedae*CCHa2
CCHa2-474R	CAGTGATGGTGCCTGTTGC	RT-qPCR amplification of *Aedae*CCHa2
CCHa2R-334F	AACACCTACATTTTCTCGCTGG	RT-qPCR amplification of *Aedae*CCHa2R
CCHa2R-560R	GTCTGCAGCTTTCGTAAAGG	RT-qPCR amplification of *Aedae*CCHa2R
CCHa1R-Res-756F	AAAAAGCTTgccaccATGTTGGACCCAGGG	ORF cloning for functional receptor assay
CCHa1R-Res-2154R	AAATCTAGAATGTGAAAATAGTGCTCCTA	ORF cloning for functional receptor assay
CCHa2R-29F	gccaccATGGAACTGTGGCGCTTGATAC	ORF cloning for functional receptor assay
CCHa2R-1336R	GAAATACACCATCAGCCGCAC	ORF cloning for functional receptor assay
CCHa2R-Res-29F	AAGCTTgccaccATGGAACTGTGGCGCTTGATAC	ORF sub-cloning for functional receptor assay
CCHa2R-Res-1336R	TCTAGAGAAATACACCATCAGCCGCAC	ORF sub-cloning for functional receptor assay

2.2 Results

2.2.1 Expression profiles of transcripts encoding CCHamides and the CCHa2 receptor

2.2.1.1 Developmental expression profiles

RT-qPCR primers of *CCHa1* (737F-901R), *CCHa2* (247F-474R) and *CCHa2R* (334F-560R) were chosen based on the amplification specificity and efficiency of 101.6%, 95.2% and 89.5%, respectively, for transcript expression profiling in various developmental stages, tissues and organs of adult *Ae. aegypti* and animals under different conditions or stresses.

Results demonstrated that the transcript abundance of *CCHa1*, *CCHa2* and *CCHa2R* varied over the course of mosquito development (Fig. 2.1). In general, both neuropeptide transcripts showed a gradually increasing trend from pupal stage to adulthood despite that in the 4th instar larval stage, *CCHa1* had relatively higher transcript abundance (Fig 2.1A) while *CCHa2* was absent (Fig. 2.1B). Moreover, the highest CCHa2 expression was detected in the early adulthood of both sexes (Fig. 2.1B), but *CCHa1* transcript was enriched in 4-day old males (Fig. 2.1A). Noticeably, differential expression of both neuropeptide transcripts between sexes of adult mosquitoes was observed; males had significantly higher *CCHa1* and *CCHa2* transcript abundance than 4-day old females (Fig. 2.1A-B). In contrast, *CCHa2* receptor transcript expression was found to be more stable with similar abundance throughout development (Fig. 2.1C). Even though the highest expression of CCHa2R was detected in the late pupa stage, it was not significantly different from most of the other stages, except for the 4-day old female mosquitoes.

2.2.1.2 Spatial expression profiles

Tissue and organ specific expression patterns of CCHamide transcripts (*CCHa1* and *CCHa2*) and CCHa2R were examined in 1-day old male and female mosquitoes.

CCHa1 transcript was distributed mainly in three tissues including the head (comprised mainly by the brain), midgut and carcass containing skeletal muscle, fat body and cuticle (Fig. 2.2A-B). The midgut was determined to have a highly enriched *CCHa1* transcript in both sexes; however, differential expression patterns in other tissues between males and females were observed. Abundance of *CCHa1* in the head of male mosquitoes was lower than the midgut region (Fig. 2.2A), but it was expressed as much in the female head as in the midgut (Fig. 2.2B). Even though the carcass was also enriched with *CCHa1* transcript compared to Malpighian tubules (MTs), its abundance was only a fraction of the levels detected in midgut of both males and females.

Similar to *CCHa1* expression, the midgut was determined to be a major site of *CCHa2* transcript that was significantly higher than all other examined tissues with the exception of the female carcass, which also had high abundance of *CCHa2* transcript (Fig. 2.2C-D). Unlike the high *CCHa1* transcript levels in the head, the *CCHa2* expression was low.

Compared to the peptide transcripts that demonstrated enrichment in a subset of the examined tissues, the distribution of the CCHa2 receptor transcript (*CCHa2R*) was unique with enrichment in a different subset of tissues in males only (Fig. 2.3A), although there was an apparently higher level of transcript in the female hindgut (Fig. 2.3B). Unlike the observed *CCHa1* and *CCHa2* enrichment in the midgut, *CCHa2R* was not found to be enriched in this tissue. Instead, the male hindgut tended to be the site that demonstrated the most *CCHa2R* abundance followed by the reproductive organs, the head and the carcass. In females, while there was no significant enrichment of *CCHa2R* in any examined tissue, the data trended in support of higher *CCHa2R* abundance in the hindgut (Fig. 2.3B) and trace amounts detected in other tissues.

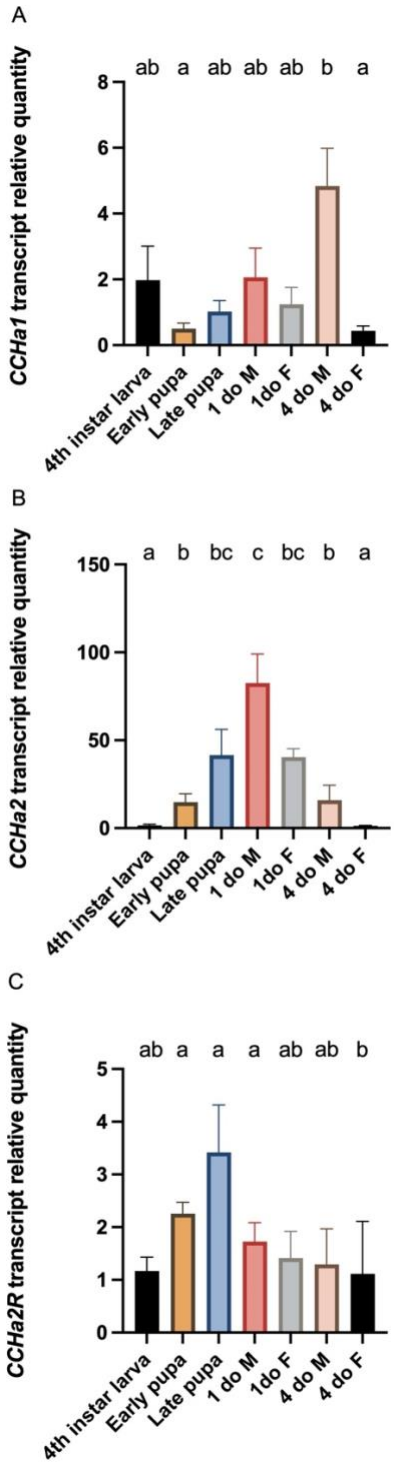

Figure 2.1 Expression patterns of *Ae. aegypti CCHa1* (A), *CCHa2* (B) and *CCHa2R* (C) in different developmental stages measured by RT-qPCR. Transcript relative quantity was normalized to the quantity of reference genes rp49 and rpS18 (for details see materials and methods section). Statistical differences are denoted with different letters, as determined by a one-way ANOVA on log-transformed data ($p<0.05$). Data represent the mean ± SEM (n=6).

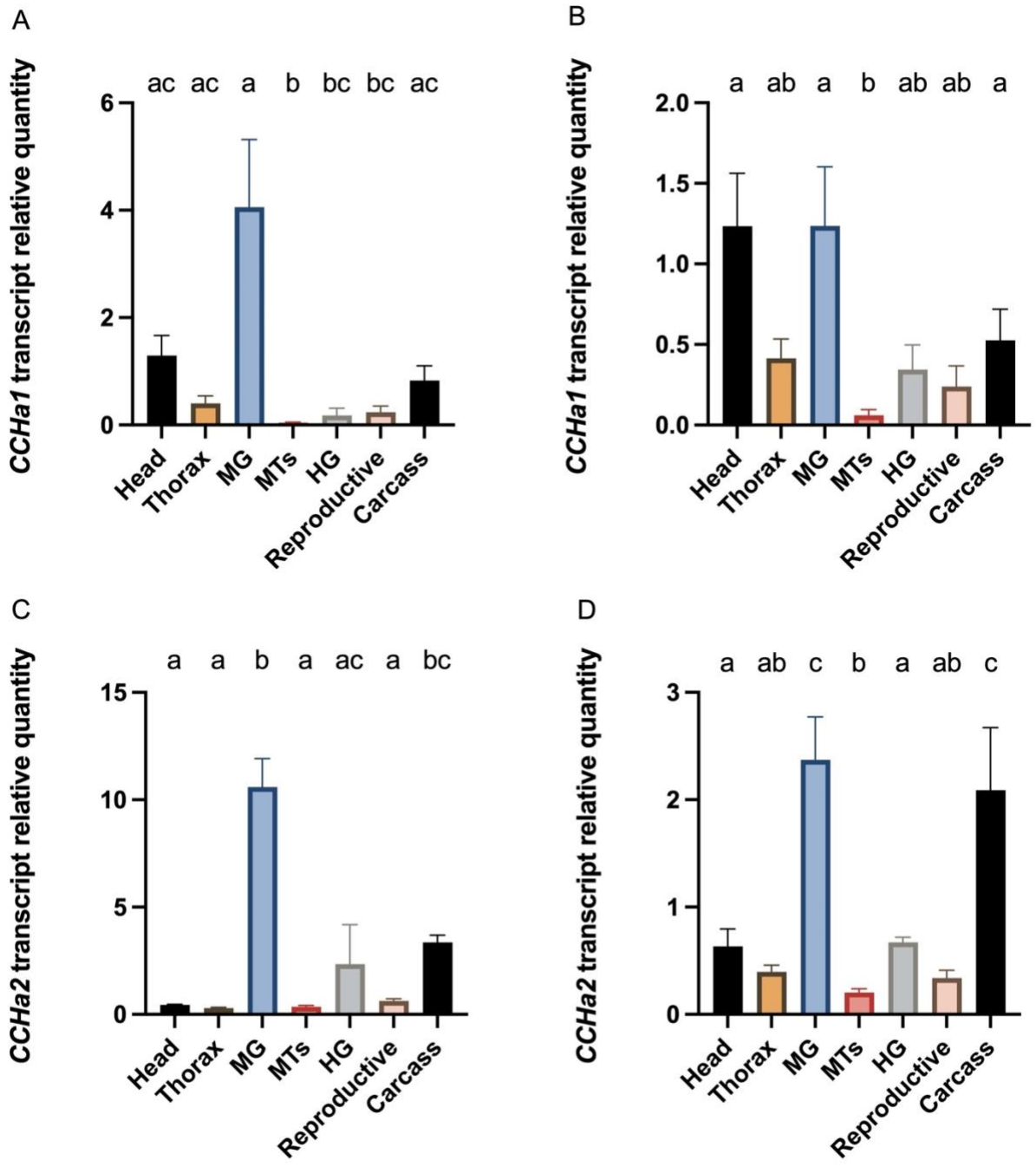

Figure 2.2 Tissue-specific expression patterns of *CCHa1* in male (A) and female (B) and *CCHa2* in male (C) and female (D) 1-day old adult of *Ae. aegypti* measured by RT-qPCR. Tissue/organ abbreviations: head, thorax, midgut (MG), Malpighian tubules (MTs), hindgut (HG), reproductive system and carcass, which includes skeletal muscle and fat body. Transcript relative quantity was normalized to the quantity of reference genes rp49 and rpS18 (for details see materials and methods section). Statistical differences are denoted with different letters, as determined by a one-way ANOVA on log-transformed data ($p<0.05$). Data represent the mean ± SEM (n=4).

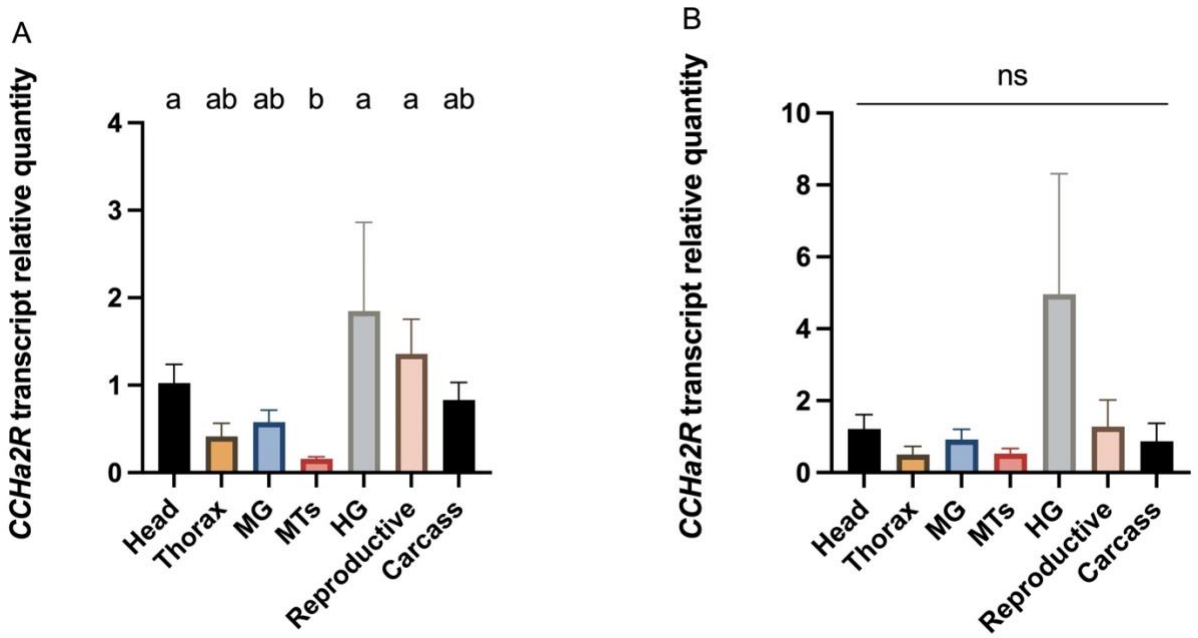

Figure 2.3 Tissue-specific expression patterns of *CCHa2R* in male (A) and female (B) 1-day old adult *Ae. aegypti* measured by RT-qPCR. Tissue/organ abbreviations: head, thorax, midgut (MG), Malpighian tubules (MTs), hindgut (HG), reproductive system and carcass, which includes skeletal muscle and fat body. Transcript relative quantity was normalized to the quantity of reference genes rp49 and rpS18 (for details see materials and methods section). Statistical differences are denoted with different letters, as determined by a one-way ANOVA on log-transformed data ($p<0.05$). Data represent the mean ± SEM (n=4).

2.2.2 Anti-*Aedae*CCHa2 antibody specificity and localization of CCHamide immunoreactivity

In order to examine the binding specificity as well as potential cross-reaction of the affinity purified anti-*Aedae*CCHa2 primary antibody to the neuropeptides CCHa1 and CCHa2 and other structurally unique peptides, an ELISA test was conducted. Results showed that with the exception of CCHamides, other mosquito neuropeptides, including RYamide-1 and CAPA-PK1, were not recognized by the antibody. Both CCHa1 and CCHa2 were recognized by the antibody. Moreover, the antibody showed over a two-fold higher binding affinity towards CCHa1 (EC_{50}=59.29nM) compared to CCHa2 (EC_{50}=134.97nM) (Fig. 2.4).

In light of these observations regarding antibody specificity, whole mount immunohistochemistry was conducted to localize CCHamide immunoreactive neurons or endocrine cells in one-day old mosquitoes using the antibody described above. In negative control treatments where the primary antibody was blocked by pre-incubating with the CCHa2 peptide, no immunoreactive staining was observed (Fig. 2.5A). In experimental treatments with unblocked antibody, 15-20 immunoreactive endocrine cells were located in the posterior midgut region adjacent to the midgut-hindgut junction known as the pyloric valve (Fig. 2.5B-C). Immunoreactive neurons were also observed in the ventral nerve cord of mosquitoes. Specifically, at least 1-3 neurons in the terminal ganglia were found to be CCHamide-immunoreactive (Fig. 2.6) with axonal projections localized over the posterior end of the ganglion that may innervate the reproductive system of female mosquitoes (Fig. 2.7A). Bilaterally-paired axonal projections were also detected in pre-terminal abdominal ganglia (Fig. 2.7B).

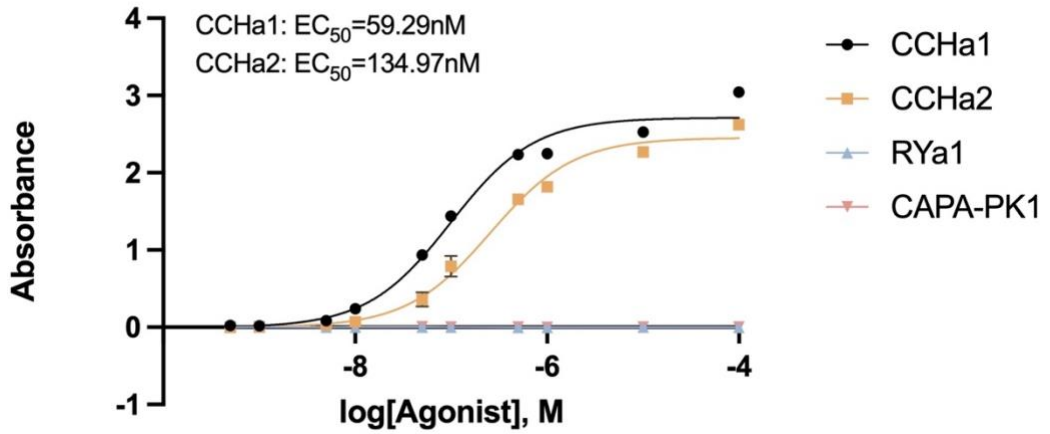

Figure 2.4 Binding affinities of customized antibody (RB3155) to synthetic neuropeptides CCHa1, CCHa2, RYa-1 and CAPA-PK1 measured by ELISA test. The antibody anti-*Aedae*CCHa2 was designed to target the *Aedae*CCHa2 sequence GCQAYGHVCYGGH-NH_2. The antibody had high affinity to *Aedae*CCHa1 (EC_{50} = 59.29nM) and *Aedae*CCHa2 (EC_{50} = 134.97nM), and was not able to recognize *Aedae*RYa-1 and *Aedae*CAPA-PK1. Figure is the representative of one experimental trail. Another antibody (RB3156) exhibited similar results (data not shown). EC_{50} values represent average values of all trials (n=2), which were analyzed by sigmoidal dose-response model.

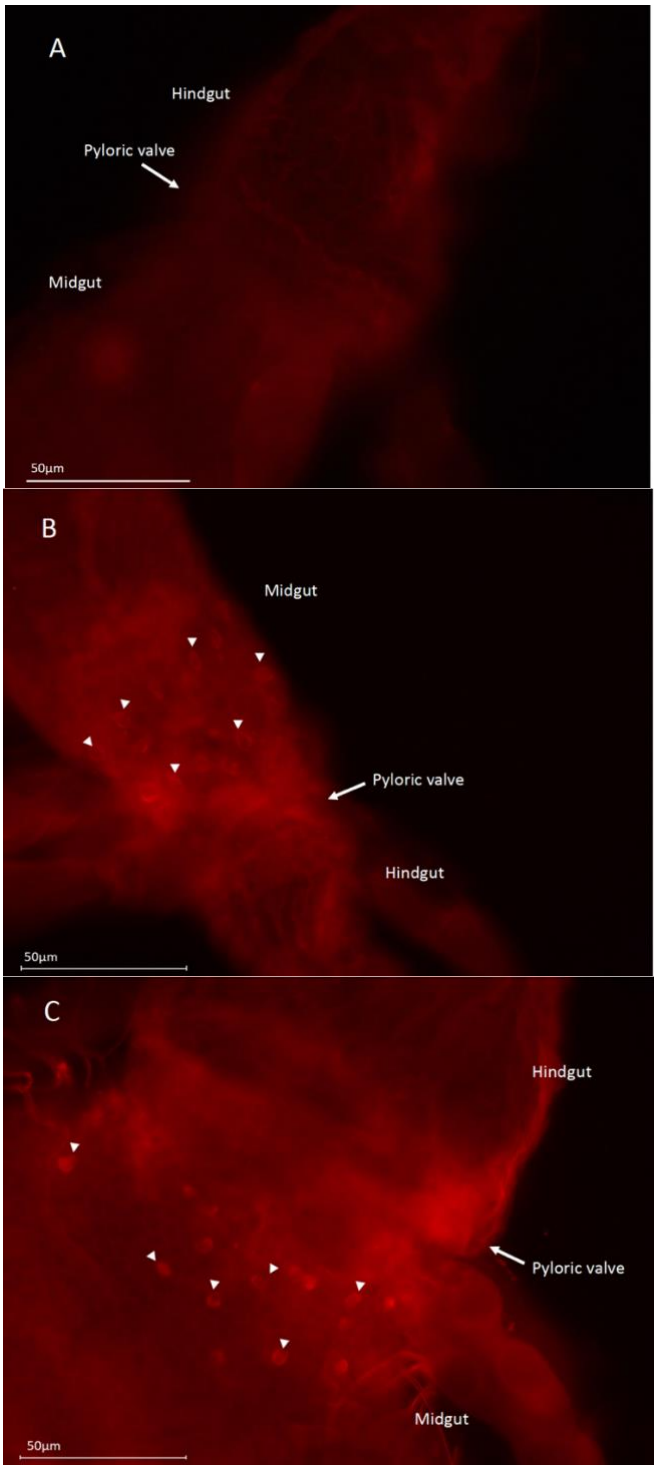

Figure 2.5 Immunoreactive endocrine cells were localized in the posterior midgut of 1-day-old *Ae. aegypti* reared under standard laboratory conditions via custom anti-*Aedae*CCHa2 antibody. (A) Negative control of male gut: pre-incubated CCHa2 (10µM) overnight with anti-*Aedae*CCHa2 primary antibody. Multiple immunoreactive endocrine cells (denoted by white arrowhead) localized in the posterior midgut adjacent to the pyloric valve of 1-day old male (B) and female (C).

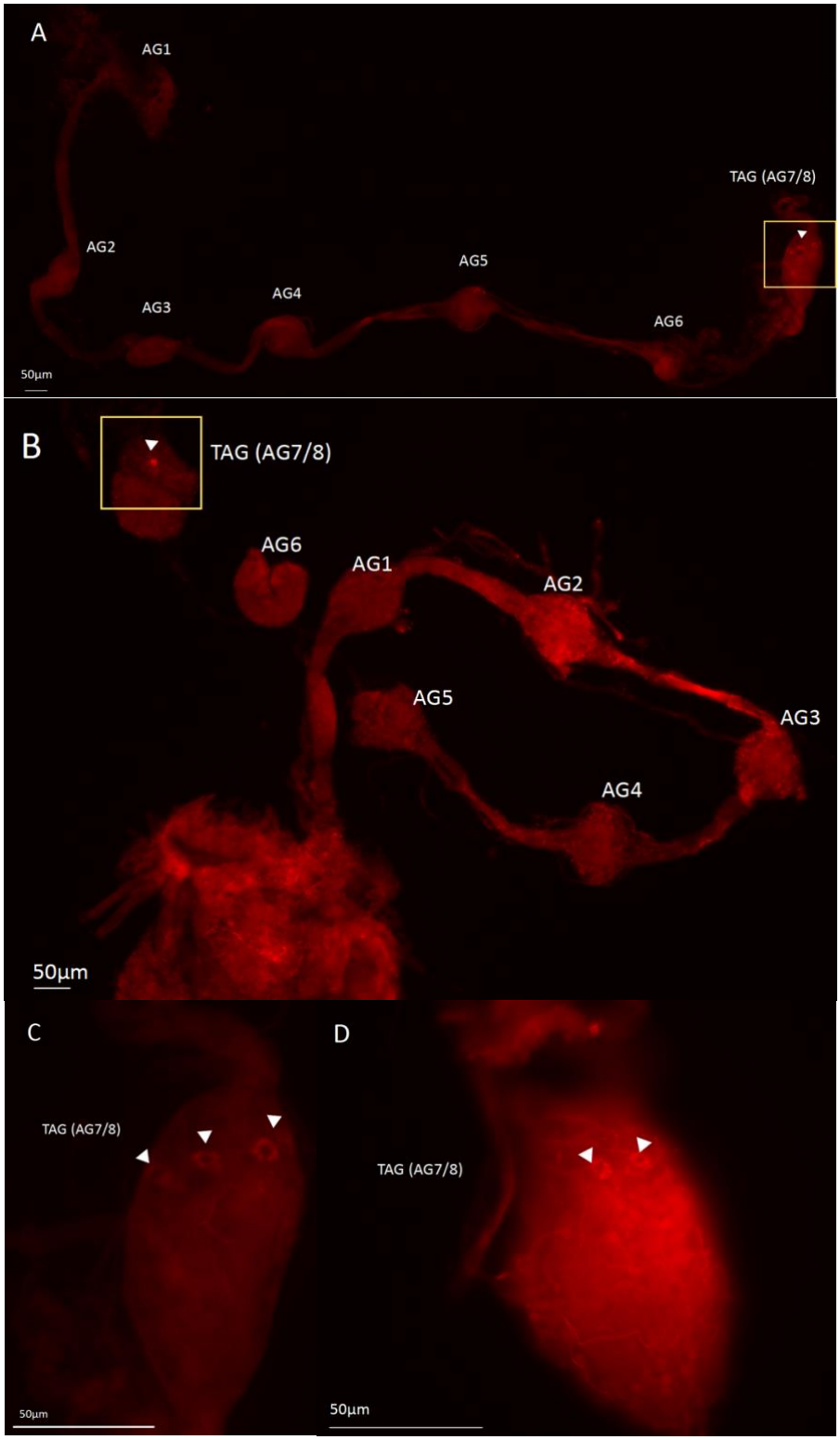

Figure 2.6 Immunoreactive neurons were localized in the terminal abdominal ganglion (TAG) 1-day-old *Ae. aegypti* reared under standard laboratory conditions via custom anti-*Aedae*CCHa2 antibody. Between 1-3 immunoreactive neurons (denoted by white arrowhead in the yellow box) within the TAG in a complete set of abdominal ganglia from 1-day old female (A) and male (B). Two magnified individual TAGs that represent all sample specimens (C, D).

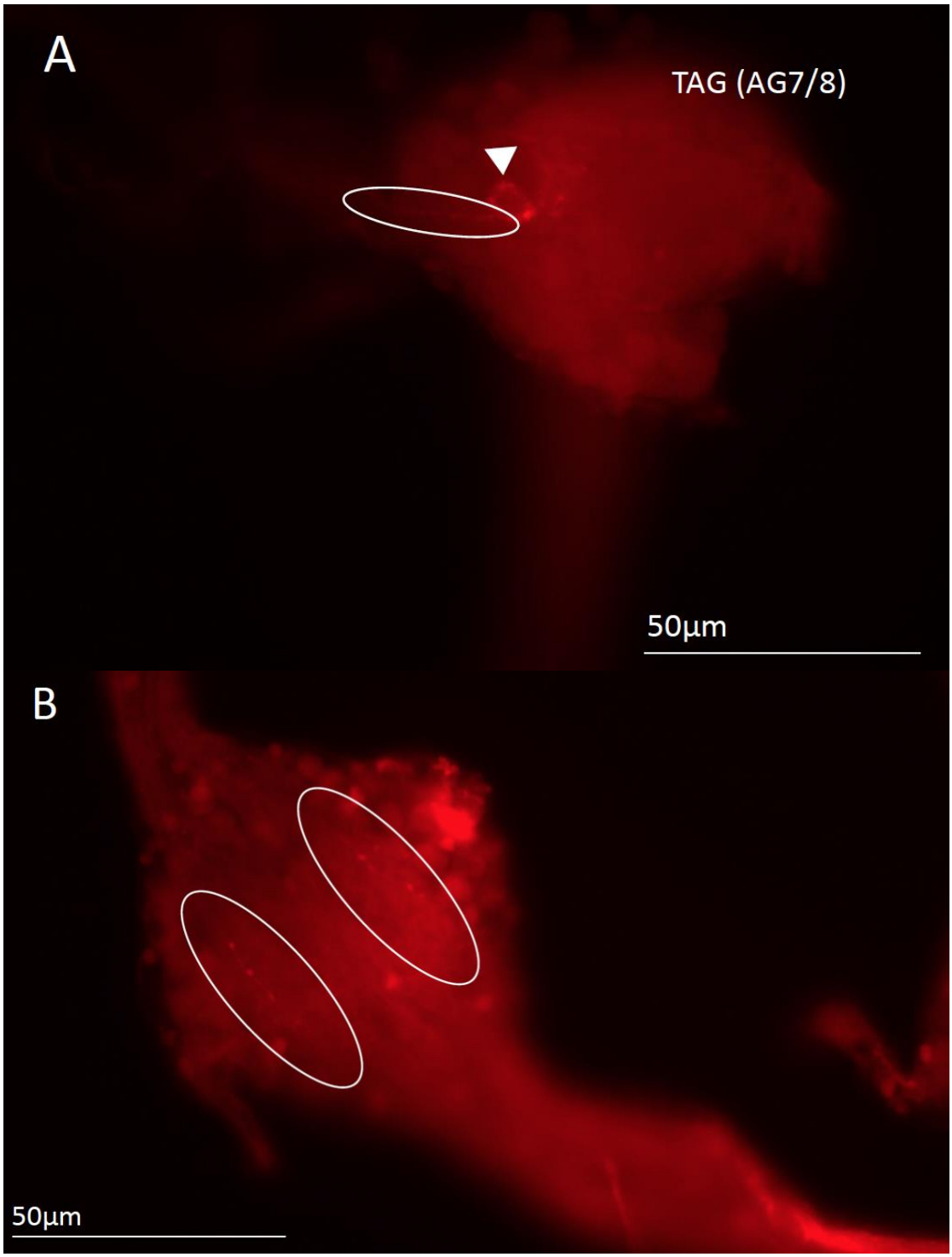

Figure 2.7 CCHamide immunoreactive processes observed in the abdominal ganglion and TAG. Axonal projection towards the posterior end of the ventral nerve cord detected in the TAG (A). Bilateral axonal projections detected in pre-terminal abdominal ganglion (B).

2.2.3 Deorphanization of CCHa1 and CCHa2 receptors

Validation of the two synthesized mammalian expression constructs for each CCHamide receptor and determination of the one with the highest activation efficiency were achieved by examining luminescent responses in a heterologous expression system. Specifically, CCHamide receptor open reading frames were ligated into pcDNA3.1+ and pBud mammalian expression plasmids, which contain different selective markers, ampicillin and zeocin, respectively. This avoids the pGEM vector to be carried over to the pBud construct since pGEM does not contain zeocin resistance. Ligands (10^{-6}M) including CCHa1 or CCHa2, RYa-1 and CAPA-PK1 were tested along with control treatments to standardize the luminescent responses. Cells expressing the control expression construct pBud-EGFP showed no response above background luminescence to any of the tested neuropeptides. This control was important since it validated that any luminescent response above background generated from the CCHamide receptor constructs resulted from activation of CCHa1R or CCHa2R (Fig. 2.8). Based on a series of preliminary experiments repeated multiple times, the pBud-CCHa1R and pcDNA-CCHa2R were selected to complete further experiments including dose-response profiles because of their higher activation efficiency and greater signal to background noise ratio.

In order to deorphanize CCHamide receptors in *Ae. aegypti*, eleven insect neuropeptides that represent a variety of neuropeptide families that participate in distinct physiological functions were prepared at 10^{-6}M and tested to examine the sensitivity and specificity of the CCHa1R and CCHa2R to different ligands. *Aedae*CCHa1 and *Aedae*CCHa2 were the only two neuropeptides that effectively elicited significantly elevated luminescent responses with both CCHamide receptors compared to the negative control, BSA assay media alone (Fig. 2.9A-B). Unlike the activity of CCHamides, the CCHa1R and CCHa2R were not responsive to any other

neuropeptides at the same concentration (10^{-6}M) having only background luminescent responses. Both CCHamide neuropeptides elicited similar high intensity activation of CCHa2R; however, a significantly stronger response, in other words, a higher binding affinity was recorded when *Aedae*CCHa1 was applied to CCHa1R compared to *Aedae*CCHa2.

A dose-response curve revealed a dose-dependent selective activation of CCHa2R evident by the luminescence signal (representative of Ca^{2+} signaling) by ligands *Aedae*CCHa1 and *Aedae*CCHa2 with EC_{50} values of 15.8nM and 8.5nM, respectively (Fig. 2.9D). In other words, CCHa2 was ~2-fold more potent than CCHa1 in activating CCHa2R. The relative luminescence plateaus of both CCHa1 and CCHa2 activation were at 80% because recorded luminescence response values were normalized to the response generated by applying ATP to CHO-K1 cells, which triggered maximum response due to the endogenous ATP receptor.

However, the dose-response curve of CCHa1R indicated a nearly 1000-fold difference in terms of receptor binding and activation efficiency between *Aedae*CCHa1 and *Aedae*CCHa2 with EC_{50} values of 0.42nM and 984.1nM, respectively (Fig. 2.9C). The relative luminescence plateaus of CCHa1 activation were at 85% when normalized to the response generated by applying ATP, and the plateaus of CCHa2 was not reached even at the highest concentration tested (10^{-5}M).

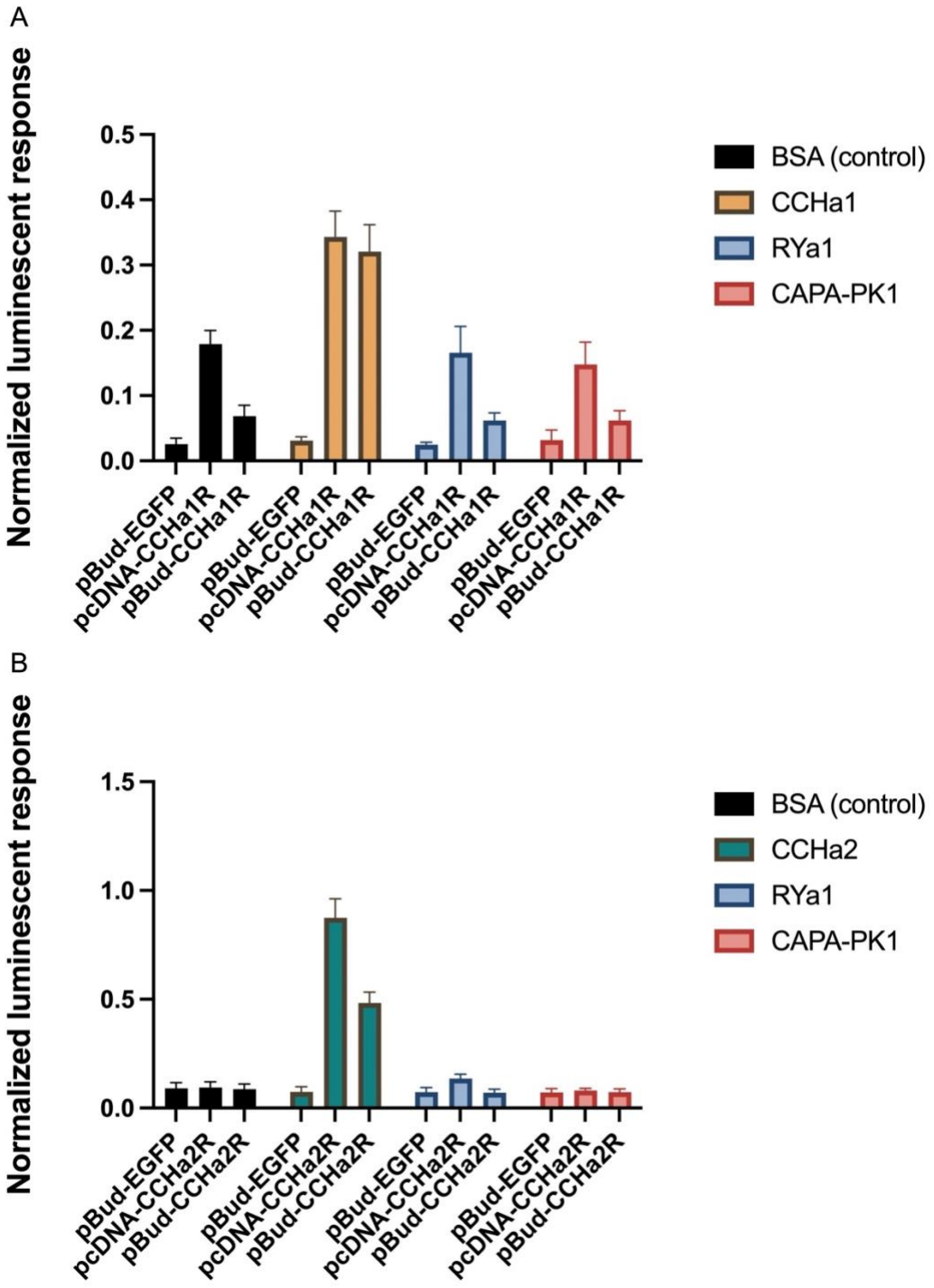

Figure 2.8 Normalized luminescent response of CHO-K1 cells expressing EGFP, *Ae. aegypti* CCHa1R (A) and CCHa2R (B) in pcDNA3.1+ and pBud vector. Assay media (BSA) alone and different ligands (10^{-6}M) were applied to cells expressing different expression constructs to validate the CCHamide receptor activity by comparing the luminescent responses generated via receptor activation. Figure is a single replicate that is representative of repeated experimental trials (n=3).

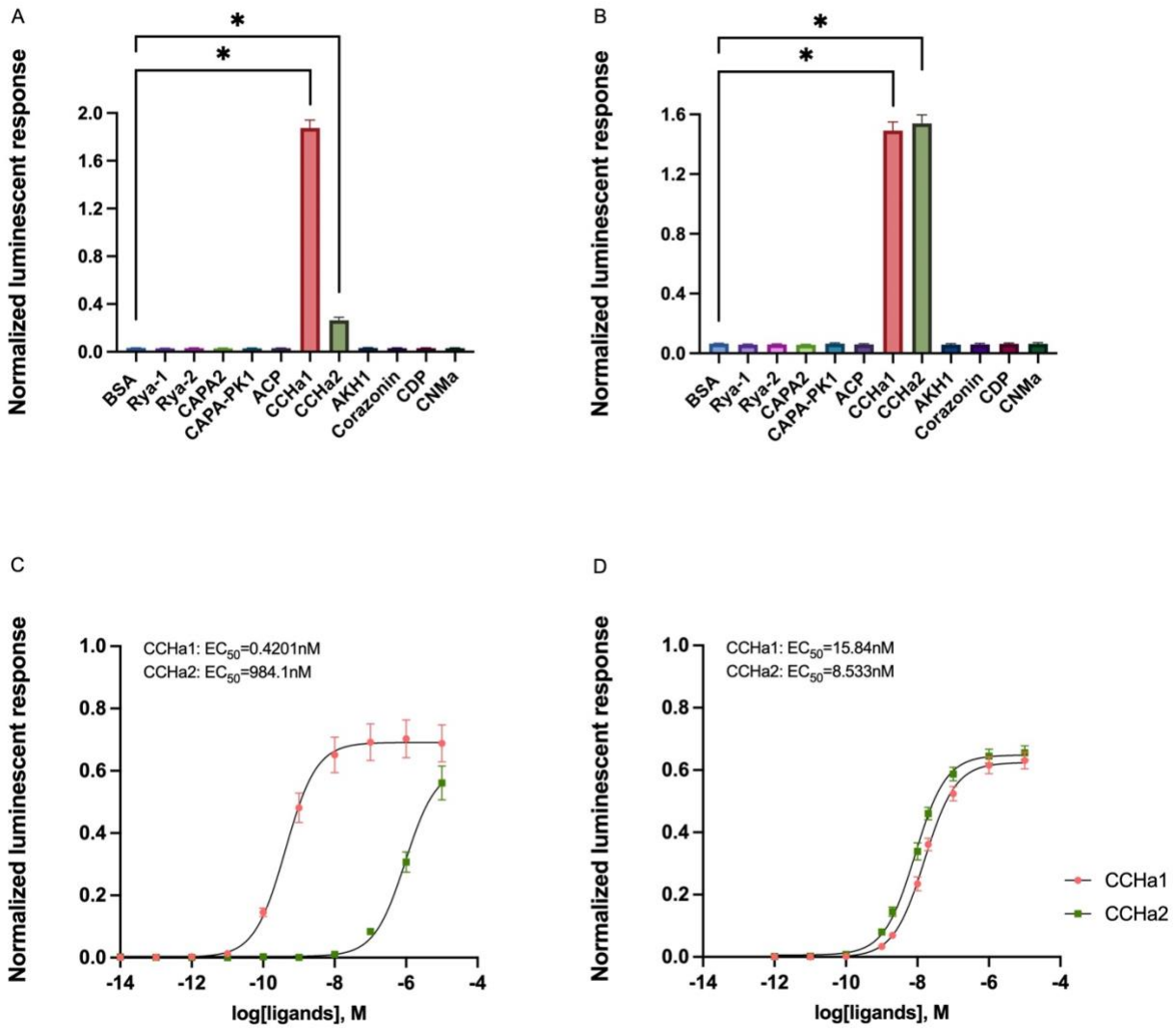

Figure 2.9 Luminescent response of CHO-K1 cells expressing *Ae. aegypti* CCHa1R (A, C) and CCHa2R (B, D). Among all examined ligands tested at saturating concentrations (10^{-6}M), CCHa1 and CCHa2 elicited a significant luminescent response activating CCHa1R (A) and CCHa2R (B). Statistical significances are denoted by asterisk, as determined by a one-way ANOVA and Dunnett's multiple comparison test ($p<0.05$). Dose-response curves of CCHa1 and CCHa2 against CCHa1R (C) and CCHa2R (D) confirmed that these neuropeptides were able to bind and activate both receptors but with different binding affinities. Statistical analysis was done following a sigmoidal dose-response model. All luminescent responses were normalized to responses generated by assay media and ATP activating an endogenous receptor in CHO-K1 cells (n=3).

2.2.4 Post-blood meal CCHamide expression

Since the blood meal is essential for egg maturation of female mosquitoes, and it acts as a stressor challenging water and ion homeostasis, the potential influence of a blood meal on transcript abundance of CCHamides in 5 to 6-day old female mosquitoes was examined. Results suggested that regulation of *CCHa1* and *CCHa2* expression responded to the blood meal differently in the 24h post-blood feeding period (Fig. 2.10). Compared to sugar-fed control females, *CCHa*1 expression was significantly down-regulated 1h after blood feeding, and then recovered to the normal level of expression at 6h post-blood meal (PBM) and maintained the same transcript level afterword. Comparatively, *CCHa2* transcript level was found to have no change at 1h PBM, however, there was a significant increase observed at 6h PBM. At 12h PBM, *CCHa2* abundance returned to the control level and did not change over the subsequent time points of the experiment.

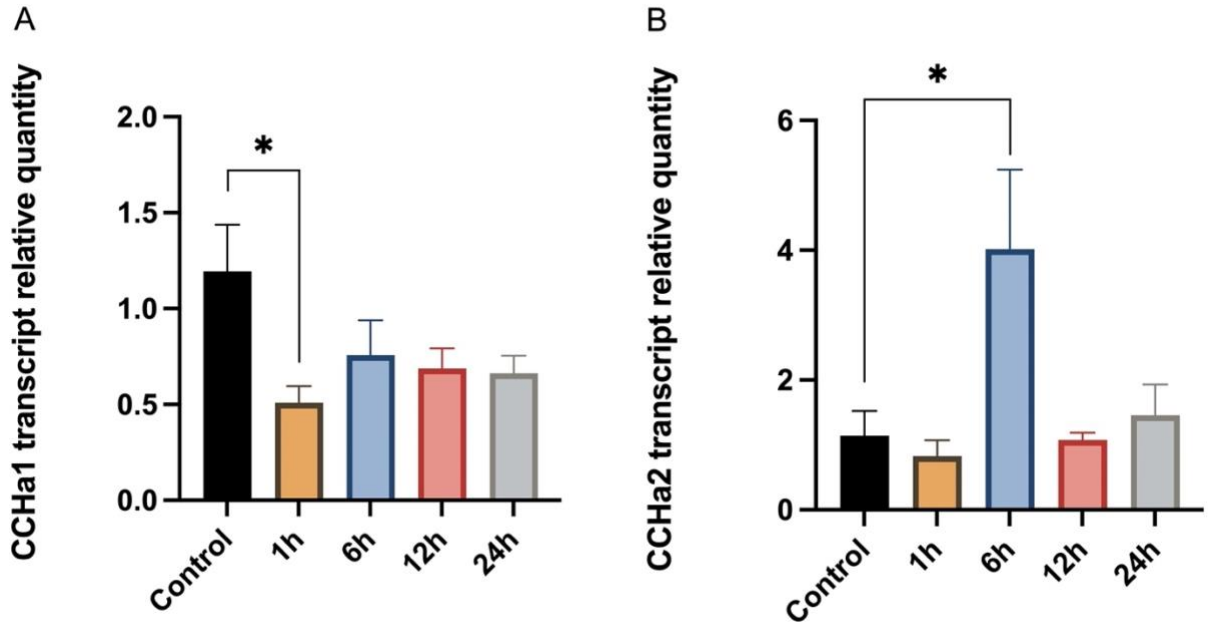

Figure 2.10 The effect of blood-feeding on the expression of *CCHa1* (A) and *CCHa2* (B) in female *Ae. aegypti*. Transcript relative quantity was normalized to the quantity of reference genes rp49 and rpS18 (for details see materials and methods section). Statistical differences are denoted with asterisk (*), as determined by a one-way ANOVA on log-transformed data ($p<0.05$). Data represent the mean ± SEM (n=5).

2.2.5 CCHamide transcript expression during starvation stress

Based on studies in *D. melanogaster* that indicated that the function of CCHa2 and CCHa2R was tightly associated with food consumption and feeding behaviours, the effect of 24h starvation stress on expression of *CCHa2* and *CCHa2R* in 1-day old male and female mosquitoes was examined. The one-day duration was selected since preliminary experiments (data not shown) indicated that a longer duration (48 hours) of starvation appeared to exert an extremely high stress, resulting in the death of the vast majority of experimental mosquitoes. Starvation had no effect on the expression of *CCHa2* and *CCHa2R* (Fig. 2.11). Immunohistochemistry was also conducted on 24h-48h starved and desiccated mosquitoes. A similar number of CCHamide immunoreactive endocrine cells were found in the same location of the posterior midgut as observed in mosquitoes under non-stressed conditions (Fig. 2.12). Based on qualitative observations, the intensity of CCHamide immunoreactive staining of endocrine cells was similar between sugar-fed control and starvation-stressed experimental groups.

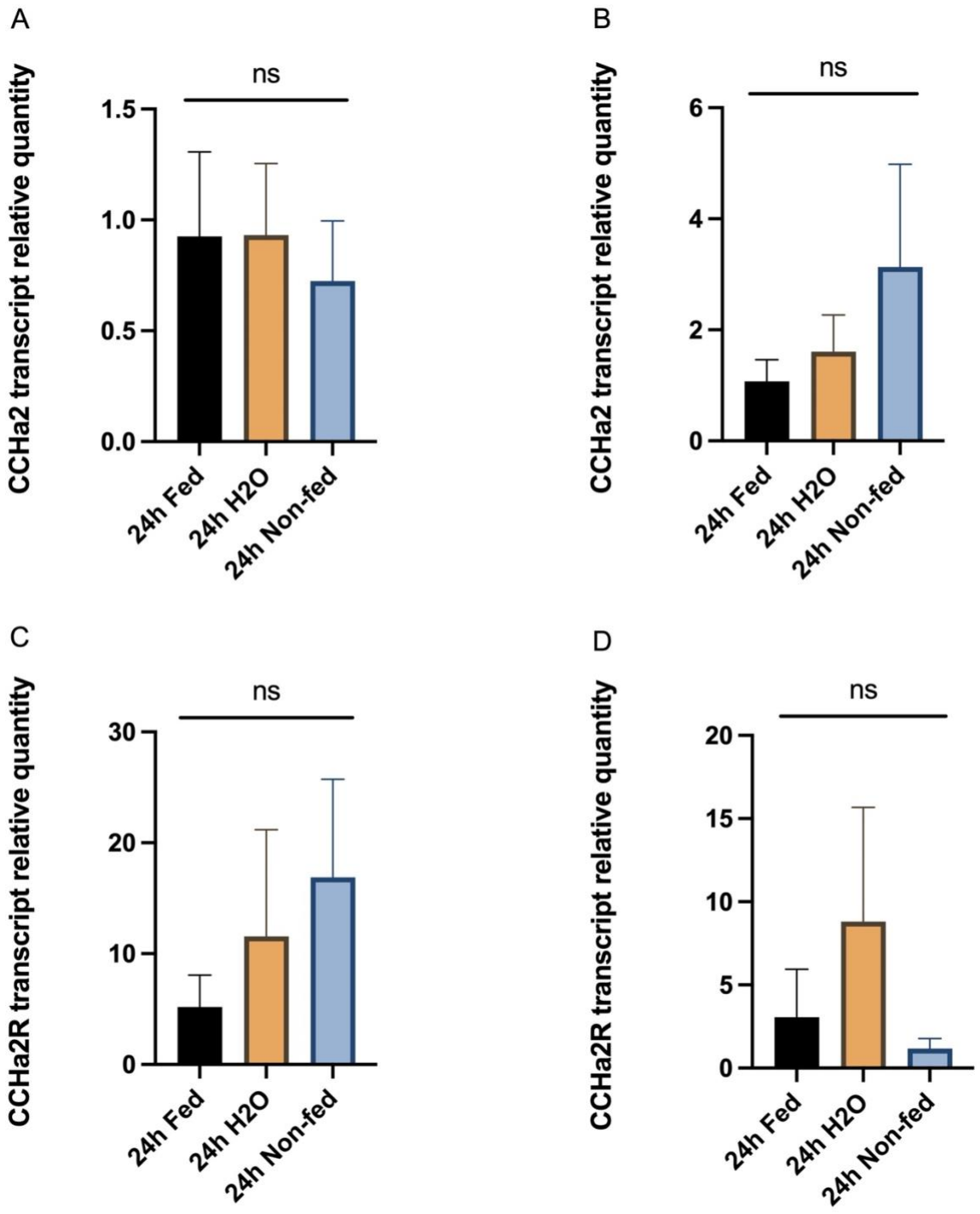

Figure 2.11 The effect of 24h starvation on the expression of *CCHa2* in male (A) and female (B) mosquitoes, and *CCHa2R* in male (C) and female (D) mosquitoes. Transcript relative quantity was normalized to the quantity of reference genes rp49 and rpS18 (for details see materials and methods section). Statistical differences are denoted with an asterisk (*), as determined by a one-way ANOVA on log-transformed data ($p<0.05$). Data represent the mean ± SEM (n=3).

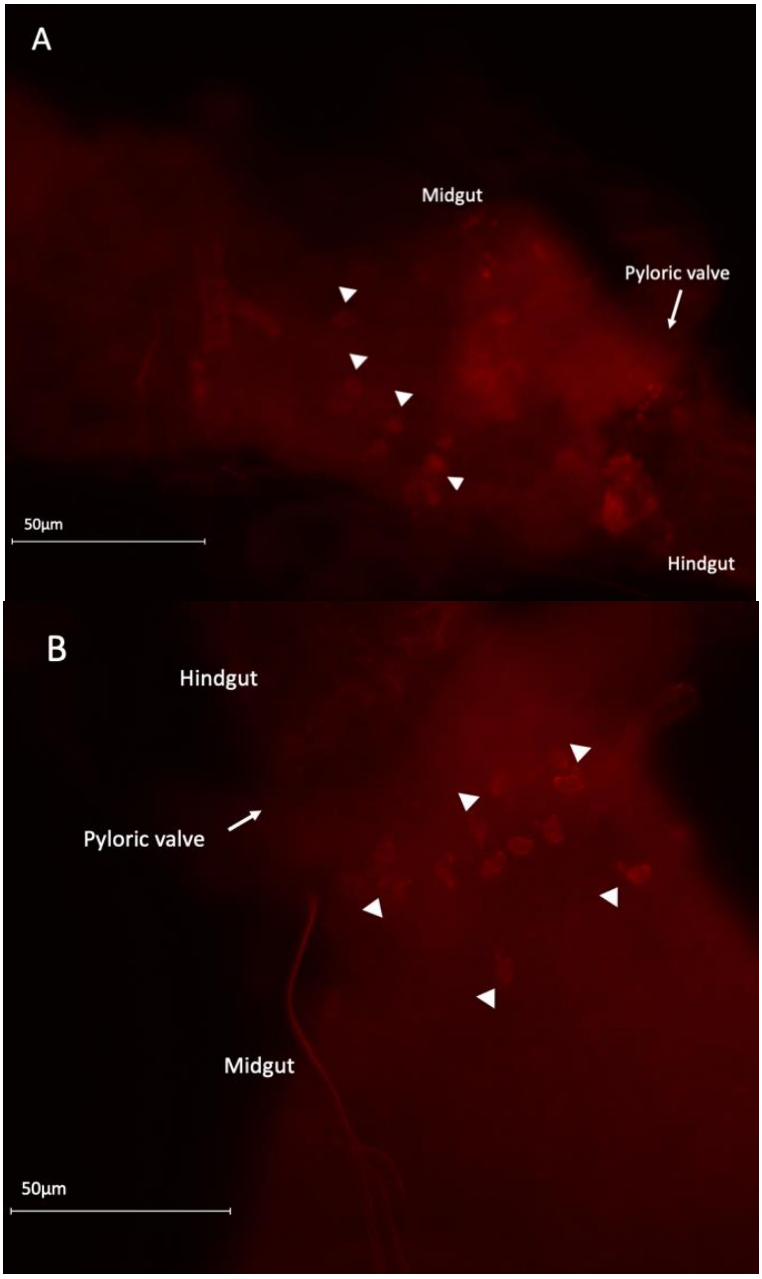

Figure 2.12 CCHamide immunoreactive endocrine cells in the posterior midgut of *Ae. aegypti* male (A) and female (B) adults after 24h-48h starvation and desiccation. Multiple immunoreactive endocrine cells (denoted by white arrowhead) with similar number and intensity of CCHamide immunoreactive staining to as observed in sucrose-fed mosquitoes.

2.3 Discussion

CCHamide neuropeptide sequences were noted over two decades ago (Zdárek et al., 2000), and biochemically isolated and sequenced a decade later and identified as endogenous ligands for orphan receptors CCHa1R and CCHa2R in *Drosophila* (Ida et al., 2012). However, beyond the studies on the fruit fly *D. melanogaster*, CCHamides research is limited. This has kept CCHamides and their associated receptors uncharacterized among many other insects, including the mosquito *Ae. aegypti*. In this study utilizing *Ae. aegypti*, I have functionally deorphanized two CCHamide receptors, examined both developmental and spatial expression profiles of transcripts encoding CCHamides and CCHa2R, and localized the distribution of CCHamide immunoreactivity in adults to help unravel their potential physiological roles in mosquitoes.

2.3.1 Developmental expression of CCHamide and CCHa2R transcripts

CCHamide transcripts were highly enriched in mosquito adults with *CCHa2* expression being more prominent in earlier adulthood while more *CCHa1* transcript was detected in older adults. Additionally, the highest expression of *CCHa2R* was found in the late pupa stage. This pattern is generally consistent with findings in *D. melanogaster* (Li et al., 2013). Noticeably, sexual dimorphism in functionality of CCHamides in adult mosquitoes is possible because both peptide and receptor transcript was observed more abundant in the males in fruit flies and mosquitoes. Even though the lineage of *Ae. aegypti* and *D. melanogaster* diverged around 185-160 million years ago, the regulatory role of CCHa2 uncovered in fly development still provides ideas for investigating its effect in mosquitoes due to similar expression patterns (Wiegmann et al., 2003; Strand et al., 2016). It is reasonable to hypothesize that CCHa2 is involved in larval growth and pupariation process, and possibly promotes or facilitates these events directly or

indirectly through other neuropeptides or signaling pathways, including for example ILPs (Ren et al., 2015; Sano et al., 2015).

CCHa2 expression reached the highest abundance in the late pupa stage just before eclosion, which might suggest CCHa2 plays a role in the process of eclosion or in the preparation for eclosion. In addition, it was observed that its corresponding receptor *CCHa2R* was also expressed at the highest levels in the late stage pupa confirming that this neuropeptide can be functional at this time and lead to downstream responses through CCHa2-CCHa2R signaling pathways. Furthermore, the expression profile of *CCHa2R* was rather ubiquitous throughout the development of mosquitoes, that may imply the physiological function carried through this pathway is regulated by changing the expression of the *CCHa2* rather than its receptor under certain conditions.

In terms of *CCHa1*, although no studies to date have shown that it is involved in insect growth, development and eclosion, since it was also expressed in the late pupa stage alongside *CCHa2* expression, this might suggest it carries similar function during these events. One possibility is that it may act as a supplement to CCHa2, because CCHa1 was able to activate CCHa2R with comparable efficacy, which will be discussed in detail later. One key difference is that a high level of *CCHa1* transcript was detected in the 4th instar larva stage, suggesting that CCHa1 peptide potentially plays a role in the pre-adult stage of mosquitoes.

2.3.2 Midgut as the major source for CCHamide production

A tissue expression profile revealed that the midgut of mosquitoes is likely the primary source producing CCHa1 and CCHa2 peptides alongside the CNS. However, unlike the CCHa2 receptor in *Drosophila* which was determined to be expressed almost exclusively in the brain (Li et al., 2013; Sano et al., 2015), a broader *CCHa2R* expression pattern across various

tissues/organs was observed in mosquitoes (Fig. 2.2 and Fig. 2.3). Even though a moderate amount of *CCHa2R* transcript was detected in the head and carcass, which also contains abdominal ganglia, slightly higher levels were observed in the hindgut and the reproductive organs of both sexes.

CCHamide immunoreactivity was evident as multiple oval-shaped endocrine cells localized in the posterior end of the midgut adjacent to the pyloric valve of both male and female adult mosquitoes. ELISA tests confirmed that although the custom antibody was designed to specifically target *Aedae*CCHa2, the binding affinity of the antibody to CCHa1 was slightly higher than to CCHa2; in other words, the antibody binds to both CCHamides. As to why the antibody is not specific to either of the two CCHamides, one hypothesis is that the two neuropeptides share a similar three-dimensional folding structure, which the antibody cannot distinguish between, due to shared characteristics including a disulfide bond formed by two cystines, a YGH motif, and a C-terminal GXH-NH_2 (Ida et al., 2012). Since both neuropeptide transcripts (*CCHa1* and *CCHa2*) were enriched in the midgut region, I could not conclude if the endocrine cells of the midgut produce both or just one of the CCHamides.

Insect midgut is an important organ responsible for food digestion, nutrient absorption and water and ion homeostasis (Caccia et al., 2019; Cui and Franz, 2020). Multiple neuropeptides that play regulatory roles in various vital physiological processes are localized within intestinal endocrine cells in the insect midgut (Wu et al., 2020). Studies have revealed that at least 10 kinds of neuropeptide were observed in the midgut region of *Drosophila* (Chen et al., 2016). Physiological events that these gut-derived neuropeptides are involved in can be roughly divided into three categories. The first category is insect development and ecdysis. For example, allatotropin expressed in the digestive tract was suggested to have an inhibitory role for *in vitro*

synthesis of juvenile hormone (JH) in the lubber grasshopper, *Romalea microptera* and the fall armyworm, *Spodoptera frugiperda* (Abdel-latief et al., 2004; Li et al., 2005). Biosynthesis of JH was also blocked by another closely associated neuropeptide, allatostatin-C which inhibits citrate transport in mitochondria of mosquitoes (Nouzova et al., 2015). Ecdysis, one of the most critical events that insects undergo during their development, is mediated through gut-produced orcokinin and prothoracicotropic hormones (PTTH) (Gelman et al., 1991; Wulff et al., 2017). Another subset of gut-derived neuropeptides play functional roles related to feeding, feeding-related activities and nutritional status. Midgut endocrine cells that secrete the head peptide in *Ae. aegypti* inhibit the host-seeking behaviour of females (Stracker et al., 2002). Insulin-like peptides (including ILP2, 4, 5) and tachykinin-related peptides expressed in the midgut of *Drosophila* and other insects are associated with the growth, development, lifespan and response to nutritional conditions (Brogiolo et al., 2001; Grönke et al., 2010; Van Loy et al., 2010; Yamagishi et al., 2018). A third category of gut-derived neuropeptides relate to nutrient and ion absorption and transport, and intestinal muscle contraction. For instance, allatostatin A enriched specifically in the endocrine cells in the posterior midgut of larval *D. melanogaster* affects K^+ absorption and gut contraction (Spit et al., 2012; Vanderveken and O'Donnell, 2014). The neuropeptide myosuppressin was localized in the same area of the midgut and was shown to reduce the frequency and amplitude of anterior midgut and hindgut muscle contractions in *Rhodius prolixus* (Lee et al., 2012). Given that CCHamides are localized in the same area in adult mosquitoes, this suggests they may also participate in some of these physiological processes.

Titos and Rogulja indicated that *CCHa1* expression responded to ingestion of protein-rich food but not to other nutritional substances like sugars or fats due to an observed significantly increase in *CCHa1* transcript level in the midgut of *Drosophila* 24h post amino acid-rich meal.

(Titos and Rogulja, 2020). They suggested that up-regulated CCHa1 transcript levels reduced the arousability and improved fragmentation of sleep of *Drosophila*. Interestingly, other than female mosquitoes consuming a protein-rich blood meal for egg maturation during the gonotrophic cycle, female and male mosquitoes feed on nectars. The question is what function does CCHa1 possess in females under nectar-fed condition and in males, which never consume protein-rich meals as adults? Although the expression profile of *CCHa1* generally overlapped with the pattern observed in *Drosophila*, it suggests this peptide may play a conserved function in terms of regulating arousability and fragmentation of sleep in *Ae. aegypti* (Titos and Rogulja, 2020). Moreover, *CCHa1* might be modulated through factors other than protein ingestion; or it might lose this feeding-related role altogether and possess different functions. Based on our current knowledge, the options are infinite because neuropeptides are versatile and multi-functional (Nässel, 2002). Farhan *et al.* revealed that the expression *CCHa1R* was directly proportional to the starvation stress in flies, which suggests that *CCHa1* could regulate foraging behavior of mosquitoes (Farhan et al., 2013). Through a single-nucleus RNA sequencing analysis in female *Ae. aegypti*, a recent study reported that *Tachykinin*, *Neuropeptide F*, *short Neuropeptide F*, and *CCHa1*were only found in enteroendocrine (EE) clusters; moreover, most EE (58.9%) only secrete one type of neuropeptides, which is typical, and a small fraction of EE within the cluster was able to co-express *Neuropeptide F*, *short Neuropeptide F*, and *CCHa1* (Cui and Franz, 2020).

Ren *et al.* and Sano *et al.* reported that both *CCHa2* and *CCHa2R* deficiency cause down-regulation of ILP2, 3, 5 in larva and adult *D. melanogaster*. Insulin-like peptides are known for their complex signaling network that is involved in regulation of growth and development. For example, the lack of ILP1-5 reduces body size and weight of *D. melanogaster* (Slaidina et al.,

2009; Semaniuk et al., 2021). Since CCHa2-CCHa2R is an up-stream signaling pathway of ILPs, this suggests an indirect pathway whereby CCHa2 regulates insect development could be through modulation of ILPs. Expression of CCHa2 was suggested to alter feeding behaviour as lack of it reduced food-seeking time and food intake (Ren et al., 2015). Further, *CCHa2* abundance can be altered by low nutritional conditions since starved larva produce significantly less CCHa2 transcript (Sano et al., 2015). Studies have indicated that CCHa2 is a sugar-responsive neuropeptide in *Drosophila*, but it may not be the case in *Ae. aegypti* as will be discussed later. Results obtained from the starvation/desiccation assay illustrated that expression of *CCHa2* and *CCHa2R* didn't appear to be affected by these particular stresses. In support of this conclusion, a similar number of CCHamide immunoreactive endocrine cells having comparable staining intensities were localized to the same location of the posterior midgut in both sucrose-fed and starved/desiccated mosquitoes.

2.3.3 The central nervous system as another source for CCHamide production

CCHamides are considered as brain-gut neuropeptides in *Drosophila* (Ren et al., 2015), and their expression profiles in *Ae. aegypti* was consistent with the brain-gut neuropeptide characteristics as well. Specifically, in addition to high expression of CCHamide transcripts in the midgut of mosquitoes, they were also detected in the CNS, including the brain and abdominal ganglia despite quantitative differences in transcript levels between male and female mosquitoes. Even though RT-qPCR results indicated the carcass was enriched with *CCHa1* and *CCHa2* transcripts, taking immunohistochemistry results into consideration, abdominal ganglia and, more specifically, the terminal ganglion, are presumed to be responsible for transcript detection found in the carcass rather than skeletal muscle tissues and fat body.

Many biological processes in insects are regulated by factors derived from the ventral nerve cord, such as development, homeostasis of water and ions, hindgut mobility, mating and oviposition (Weevers, 1985; Nässel, 1996; Raabe, 2012). Understanding neuropeptides produced and released in similar regions of the abdominal ganglia could shed light on the role of CCHamides in mosquitoes. For instance, studies have shown there is a pair of leucokinin immunoreactive neurons located in the terminal ganglia in *Ae. aegypti* and the crane fly (Cantera and Nässel, 1992; Nässel, 1996). As a multifunctional neuropeptide, leucokinin participates in the regulation of feeding (Nässel and Wu, 2021) and a leucokinin mimetic was demonstrated to suppress sugar taste neurons and feeding activity (Kwon et al., 2016). Immunoreactivity of FMRFamide-related peptides (FaRPs) was observed in the posterior region of the terminal abdominal ganglion (TAG) in the migratory locust, which project into the ventral ovipositor nerve and the receptaculum seminis nerve. Clark and Lange suggested that FaRPs affect the amplitude of neurally stimulated spermathecal contractions (Clark and Lange, 2002). Not only do FaRPs affect spermathecal muscle contraction, but FMRFamide and proctolin also have an acceleratory effect on muscle contraction of insect accessory pulsatile organs and heart of mosquito *Anopheles gambiae* (Suggs et al., 2016). Moreover, six immunoreactive proctolin neurons were observed in the anterior region of the TAG in grasshoppers and proctolin is also known to enhance intestinal muscle contraction of the midgut and hindgut of *R. prolixus* (Keshishian and O'Shea, 1985; Orchard et al., 2011).

Neuropeptides expressed in the TAG are known to mediate gut muscle contraction that may be associated with nutrient digestion, uptake of water, ion reabsorption, and mediating reproduction. Do CCHamides intervene in these physiological processes since their immunoreactive neurons have herein been detected in the mosquito TAG with axonal projections

toward the posterior of the insect where the hindgut and reproductive system are located? A moderate *CCHa2R* transcript level in the hindgut may suggest that the CCHa2 peptide plays a role in this area via CCHa2-CCHa2R signaling. Furthermore, considering the observed reduced *CCHa1* transcript level and elevated abundance of *CCHa2* in female mosquitoes at 1h and 6h post-blood meal, it is possible that these changes relate to reproductive-related activities or other processes in the gonadotropic cycle.

2.3.4 Deorphanization of *Ae. aegypti* CCHamide receptors

One of the main objectives of this project was addressed since *Aedae*CCHamide receptors were deorphanized with *Aedae*CCHa1 and *Aedae*CCHa2 confirmed as the ligands specifically activating *Aedae*CCHa1R and *Aedae*CCHa2R with the highest response, respectively. Compared to the negative control and various other peptidergic ligands tested, CCHa1 and CCHa2 exhibited high activity onto their receptors and were able to trigger downstream effects in the form of Ca^{2+} release. One key difference is that when activating CCHa1R, CCHa1 showed a significantly higher activity than CCHa2, while similar activities of both neuropeptides were observed when activating CCHa2R. This indicates that the *Ae. aegypti* CCHa1R is quite selective for its native ligand, CCHa1 whereas the CCHa2R is rather promiscuous and does not differentiate between the two CCHamide peptides in mosquito. Ida *et al.* indicated both disulfide bond and the amide group at the C-terminus of *Drosophila* CCHa1 and CCHa2 are essential to activate their corresponding receptors. In addition to it, both neuropeptides consist a YGH motif and a C-terminal GXH-NH_2 motif (Ida et al., 2012), so it's not surprising that *Aedae*CCHa1 and *Aedae*CCHa2 are able to bind and activate CCHa1R and CCHa2R due to their similar structure.

However, results revealed that CCHa1R was much more selective and specific in terms of differentiating CCHa1 and CCHa2 compared to CCHa2R, and subsequent dose-response

analysis confirmed this observation. The dose-response curve of CCHa1R demonstrated that CCHa1 exhibited a roughly 2000-fold higher activation efficiency than CCHa2 with EC_{50} values of 0.42nM and 984.1nM, respectively. Although CCHa2R seemed relatively unable to distinguish between high doses of CCHa1 and CCHa2 in the multiple ligand activation assay, the dose-response analysis revealed that CCHa2 still had a higher activity towards the CCHa2R with an EC_{50} value of 8.5nM, which was about 2-fold more potent than CCHa1 (EC_{50}=15.8nM). Since the EC_{50} value of CCHa1 against CCHa2R is similar to that of CCHa2 this suggests that CCHa1 released *in vivo* may under certain conditions activate CCHa2R and act as a supplement for CCHa2 in mosquitoes. Further studies can be done to explore this question by knocking out or knocking down CCHa2 expression and then applying CCHa1 to mosquitoes to examine if there is any rescue effect. On the other hand, under the circumstance of high concentrations of both neuropeptides in circulation, CCHa2 will have a hard time to compete with CCHa1 on the CCHa1R and potentially interfere the CCHa1-CCHa1R signaling pathway given CCHa1 was over three orders of magnitude more potent on the CCHa1R. Compared to CCHamide receptors in *Ae. aegypti*, similar ability in terms of differentiating CCHa1 and CCHa2 was observed in *Drosophila* CCHa1R; however, *Drosophila* CCHa2R exhibited a much higher selectivity and specificity, since CCHa2 was over four orders of magnitude more potent on the *Drosophila* CCHa2R.

2.4 Concluding remarks and future directions

This study is the first to report developmental and spatial expression profiles of *CCHa1*, *CCHa2*, and *CCHa2R* in the mosquito *Ae. aegypti*, to localize CCHamide immunoreactive endocrine cells and neurons, and to deorphanize the two CCHamide receptors, CCHa1R and CCHa2R. Regarding physiological functions of CCHamides, while this has been shown to be a nutrient-responsive peptide in *Drosophila* (Sano, 2015), the current results showed that the expression of *CCHa2* and its corresponding receptor (*CCHa2R*) does not appear to be influenced by starvation stress. With regards to any potential association between transcript expression of CCHamides and blood-meal ingestion in female mosquitoes, details are unclear and mechanisms of functional roles require further research to clarify. For example, does CCHa2 affect mosquito feeding and feeding related activities including the amount and the rate of sucrose solution they ingest? Do CCHamides influence mosquito reproduction such as the number and viability of eggs produced by females, or the development of the organism including metamorphosis, pupariation and eclosion time, or adult size/weight, *etc.*? One means of answering these questions could be using reverse genetic tools such as RNA interference (RNAi) and CRISPR-Cas9 system to knockdown or mutate expression of CCHamides or CCHamide receptors and examine any resultant abnormal phenotypes of mosquitoes. Nonetheless, this current study helps to further advance our understanding of the mosquito (neuro)endocrine system and the physiological roles that are governed by CCHamides. Since mosquitoes transmit diseases as a result of their blood feeding behaviour, studying neuropeptides that are involved in regulating foraging and feeding processes can provide insights for new strategies or specific biorational compounds useful in pest-control.

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