Characterization of RYamide neuropeptides and their receptor in the disease vector mosquito, *Aedes aegypti*

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A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO

January 2023

Abstract

Insects utilize neuropeptides to fine-tune their physiological systems, including the tissues and organs involved in excretion, respiration, reproduction, and metabolism. The focus of this study is on the recently identified RYamide insect neuropeptides, named for the arginine-tyrosine amidated C-terminus. Here, the molecular basis will be investigated to propose physiological relevance in the disease vector, *Aedes aegypti*. Utilizing a cell-based functional assay, the endogenous RYamide receptor (RYa-R) was deorphanized and shown to have high specificity to the two mosquito RYamide peptides, with little to no activation by structurally-distinct neuropeptides. RYa-1 immunolocalization was found in the central nervous system, midgut, pyloric valve, ileum, rectal papillae, and surprisingly the seminal vesicles. RYamide and its receptor transcript analysis revealed potential regulatory effects of the digestive, reproductive, and excretory systems, along with a neuromodulatory role within the central nervous system. Further research is needed to confirm the physiological relevance of this novel neuropeptide family.

Acknowledgments

There are many defining moments in one’s life and my experience of being a part of Jean-Paul’s lab will always be one of them. The completion of my master’s research paper could have not been accomplished without the help of many important people.

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Jean-Paul Paluzzi, for their guidance, support, and encouragement throughout the entire research process. Their invaluable insights and constructive feedback have been instrumental in shaping the direction and quality of my work, and beyond.

I would also like to thank all the members of the Paluzzi lab: Farwa, Salwa, Jinghan, Marishia, and Zeynab for their contributions to the project and all the other members who have helped me. Their expertise and collaboration have been invaluable in bringing this research to fruition.

Finally, I would like to extend my heartfelt thanks to my family, especially my brother, Kevin for their support throughout this journey. I would also like to mention Bea for her constant support and encouragement throughout this process. And lastly, a thank you to my friends not in science for their interest and support in my research.

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List of Abbreviations

ABDG	Abdominal ganglia
AG	Accessory glands
ATP	Adenosine triphosphate
BP	Base pair
BR	Brain
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-3’, 5’-monophosphate
CC	Central canal
cDNA	Complimentary DNA
CHO	Chinese hamster ovarian cells
CT/DH	Calcitonin-like diuretic hormone
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DH31	Diuretic hormone 31
DNA	Deoxyribonucleic acid
EE	Enteroendocrine
eGFP	Enhanced green fluorescent protein
FR	Female reproductive system
GPA2	Glycoprotein alpha 2
GPB5	Glycoprotein beta 5
GPCR	G protein-coupled receptor
HG	Hindgut
IHC	Immunohistochemistry

IP3	Inositol triphosphate
ITP	Ion transport peptide
LGR	Leucine-rich repeat-containing
MNSC	Medial neurosecretory cell
MT	Malpighian tubules
MR	Male reproductive system
NHA1/2	Sodium hydrogen antiporter
NKCC	Sodium potassium chloride cotransporter
NPF	Neuropeptide F
NPY	Neuropeptide Y
NSS	Normal sheep serum
ORF	Open reading frame
OV	Ovaries
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Pars intercerebralis
PK-2	Pyrokinin-2
PM	Posterior midgut
PV	Pyloric valve
qPCR	Quantitative PCR
RF	Arginine phenylalanine
RNA	Ribonucleic acid
RNAi	RNA interference

*rp*49	Ribosomal protein 49
rps18	Ribosomal protein s18
RP	Rectal papillae
RT-qPCR	Reverse transcription PCR
RY	Arginine tyrosine
SEG	Suboesophageal ganglia
SEM	Standard error of the mean
SIF	Cysteine isoleucine phenylalanine
SV	Seminal vesicle
TG	Thoracic ganglia
VNC	Ventral nerve cord

Introduction

In this research project, I will be investigating the novel RYamide insect neuropeptide family in the disease vector, *Aedes aegypti*. I will be utilizing various molecular techniques to expand the knowledge of insect RYamides in this mosquito, which can provide a better understanding of their physiological roles in insect tissues and organ systems. The relatively recent discovery of the novel RYamides, which is one of many neuropeptide families present in insects, provides an opportunity to explore their roles in regulating vital physiological systems, and potentially, to utilize this knowledge in the design of novel more effective pest control strategies in the future.

The *Aedes aegypti* Mosquito

Insects constitute the largest group of animals in the world and are comprised of a great range of species diversity. Additionally, insects are distributed throughout diverse global landscapes, where they complete their lifecycle in a broad range of climates. A family of insects that is no exception to this is the Culicidae, better known as mosquitoes, which have an extensive distribution throughout tropical and temperate regions. Seen as pests, mosquitoes are a great bane to human populations. In particular, blood-feeding mosquitos can carry pathogens leading to disease in humans. The *Aedes aegypti* mosquito, which has both aquatic and terrestrial life stages (Figure 1), is one such blood-feeding species that can carry disease-causing pathogens including dengue fever, Zika fever, Rift Valley fever, and yellow fever viruses. One distribution model of this insect disease vector suggests a predominant presence throughout regions of Central and South America, Central Africa, and Southeast Asia (Ding et al., 2018). These disease vectors carry and spread viruses through the action of blood feeding by females (Figure 2). In addition, the act of blood feeding aids in the propagation of these insect disease vectors, as the blood meal provides the proteins and other nutrients needed for proper egg development in female mosquitoes (Lea et al., 1956). Vector-borne diseases pose a global impact, for example, Dengue fever poses an $8.9 billion-dollar global economic burden (Shepard et al., 2016). As a result, various pest control strategies have been developed to limit the disease-spreading ability of *A. aegypti* which involves chemical and physical measures (Santos and Pereira, 2020; Weeratunga et al., 2017). Pest control strategies involving chemical interference exploit the endogenous biological and chemical processes of the mosquito. Thus, understanding the inner mechanisms that contribute to a mosquito's survival is vital in the development and deployment of pest control strategies.

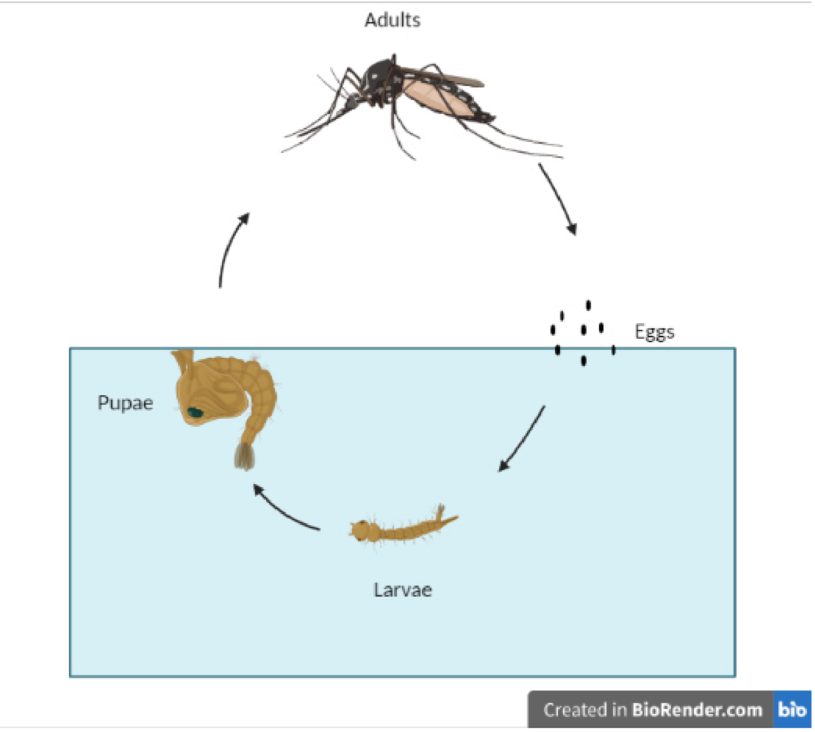

Figure 1. **The *A. aegypti* lifecycle.** Eggs are deposited above but near the water line of any natural water source such as ponds, but also artificial, human-influenced water sources such as rainwater deposited in flowerpots. The subsequent larvae and pupae stages reside in an aquatic environment (blue), while the adult stage occurs within a terrestrial environment.

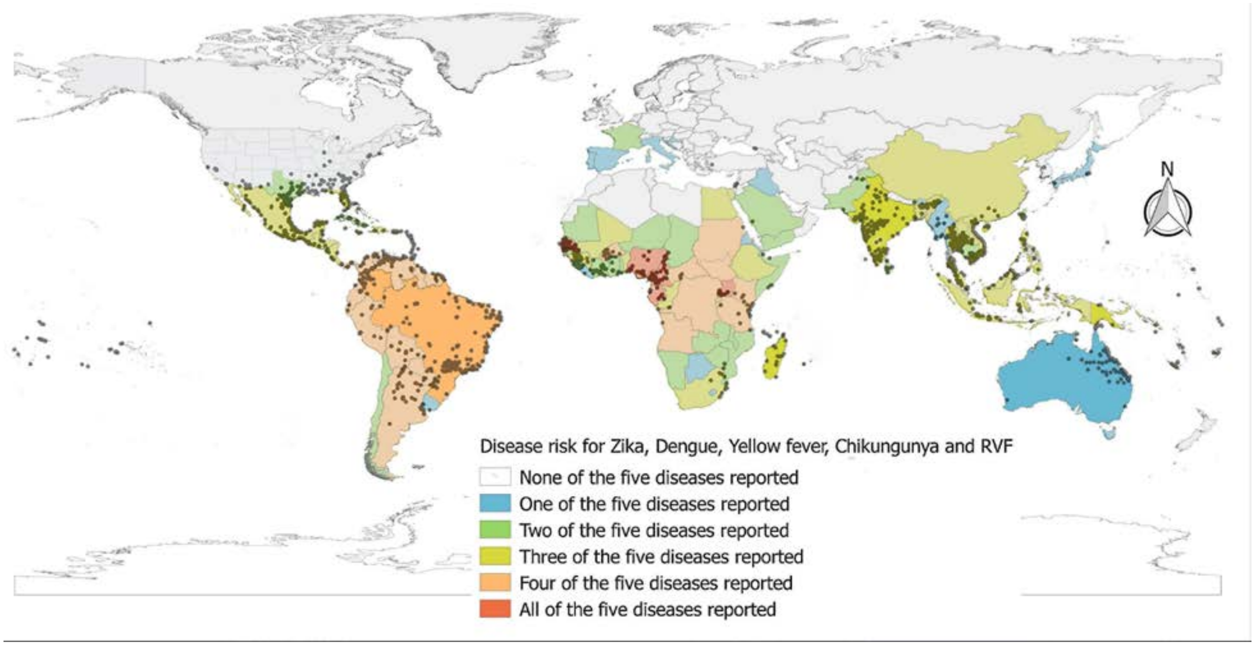

Figure 2. Reported occurrences of *A. aegypti* (black dot) and distribution of diseases that can be transmitted by this disease vector. These mosquitoes are found in temperate and tropical regions. The image is adapted from Leta et al. 2018, and Kamal et al. 2018.

The Nervous System of *A. aegypti*, Insect Neuropeptides, and their Receptors

The wide range of habitats that insects exploit can be attributed to their ability to maintain homeostasis. Various physiological processes must work in concert to counter any stress to their internal balance that is imposed by the external environment. Such physiological processes include ion and water balance, digestion, reproduction, and development to name a few. An important method of regulating these physiological processes under various conditions involves the use of neuropeptides. Neuropeptides are synthesized and released from the nervous system. In the brain of insects, several neurosecretory cells are responsible for the synthesis of neuropeptides (Predel et al., 2010). The protocerebrum is the dorsal region of the brain and is known to be involved in the insect senses such as vision and olfactory signaling pathways (Li et al., 2022b). Within the protocerebrum are the optic lobes, medulla, and pars intercerebralis (PI). The PI contains neurosecretory cells, where removal and implantation studies have indicated roles in reproduction, sugar metabolism, and diuresis (Shiga, 2003). Neuropeptides have been identified from the PI, including SIFamide and myosuppresin (Predel et al., 2010). Positioned below the brain is the subesophageal ganglia, which further contains various neurosecretory cells. The subesophageal ganglia is a portion of the nervous system mainly responsible for the control of feeding and hunger (Hartenstein et al., 2018). Extending from the brain is the ventral nerve cord (VNC) which runs along the thorax and abdomen and is comprised of multiple ganglia. In adult *A. aegypti*, there are three thoracic ganglia and six abdominal ganglia (Brown and Cao, 2001). The abdominal ganglia, for example, contains neurosecretory cells that have nerve projections that extend towards perivisceral organs (neurohaemal sites) and/or terminate upon tissues (Nässel, 1996). Immunoreactivity of neuropeptides has been observed within mosquito perivisceral organs, including CAPA and leucokinin (Chen et al., 1994; Sajadi et al., 2020). Regarding the neurosecretory cell products, neuropeptides are initially synthesized as precursors named prepropeptides which are then processed post-translationally within their cell bodies. Generally, post-translational modification normally entails the removal of the signal peptide and proteolytic cleavage of the precursor at mono and di-basic cleavage sites (Veenstra, 2000). Aside from proteolytic modifications, additional chemical groups can be added and formed after translation. The C-terminus of the neuropeptide precursor is often subject to amidation, which can be responsible for the complete biological activity of proteins, and the stabilization of protein structure (Dennison and Phoenix, 2011; Merkler, 1994). Additionally, N-terminal glutamate can be converted into pyroglutamic acid, which improves protein stability and biological activity (Lai et al., 2015). After processing, neuropeptides are transported via axons that terminate at neurohaemal organs, or peripheral nerves for storage and release (Predel and Wegener, 2006). Upon release, neuropeptides can function as neuromodulators within the insect nervous system, or upon peripheral tissues to alter physiology (Nässel, 2002). These bioactive molecules exert their effects by binding to target receptors that are often members of the G protein-coupled receptor superfamily. Conserved in all G-protein coupled receptors (GPCRs) are seven transmembrane α-helical domains. In other words, GPCRs contain a single protein sequence that loops through the cell membrane seven times, with three intracellular and three extracellular loops (Menzaghi et al., 2002). Furthermore, there are three subfamilies of GPCRs ranging from A to C, with class A making up 89% of all known GPCRs in insects (Menzaghi et al., 2002). These subfamilies can be distinguished with sequence and structural differences (Katritch et al. 2013). Binding and activation of GPCRs leads to the deployment of various signaling pathways such as inositol triphosphate (IP_3) mediated calcium release, or cyclic adenosine monophosphate (cAMP) synthesis (Caers et al., 2012). The effects of these signaling pathways can result in the modulation of other cellular signaling pathways, which can stimulate or inhibit the expression of various genes and proteins (Liu et al., 2021). Such changes in cellular processes may ultimately lead to the widespread, physiological regulation of the insect.

The Insect midgut

The midgut is the middle region of the alimentary canal and functions as a part of the digestive system. It is functionally similar to the mammalian stomach and small intestine, where food is digested, and nutrients are absorbed. The insect midgut can be divided into anterior and posterior regions, which contain distinct morphology and functions. Such regions can be distinguished by differences in cell morphology, cell types, gene expression of various proteins and enzymes, and pH (Caccia et al., 2019). Regarding cell types, there are considered two main types that are found in the midgut epithelium (Caccia et al., 2019). First are the columnar cells that predominantly make up the midgut epithelium, and second are the enteroendocrine (EE) cells that synthesize and release hormones for the regulation of digestion. EE cells are capable of releasing neuropeptides, and these neuropeptides are aptly named brain-gut peptides (Wegener and Veenstra, 2015). An example of a brain-gut peptide is calcitonin-like diuretic hormone 31 (DH_{31}), which has been identified in the *Drosophila* gut through gene expression analysis and mass spectrometry (Veenstra et al., 2008). DH_{31} function was initially determined to increase fluid secretion of the Malpighian tubules, and selectively increase sodium absorption into the tubule lumen (Coast et al., 2005). Regarding the midgut, DH_{31} released from EE cells led to increased contraction frequency of the midgut visceral muscle after ingestion of exogenous bacteria in *D. melanogaster* (Benguettat et al., 2018). The increased contractility of the midgut is thought to aid in the rapid expulsion of pathogenic bacteria. This brain-gut peptide also increased the contraction frequency of the anterior midgut in *D. melanogaster* larvae (Vanderveken and

O'Donnell, 2014). The midgut itself is considered poorly innervated by the nervous system, where the nerves from the hypocerebral ganglion of the brain have been shown to innervate the anterior midgut and the pyloric valve separating the midgut and hindgut (Billingsley and Lehane, 1996; Veenstra et al., 1995a). With neuropeptides being released from the nervous system and EE cells, different physiological systems can be regulated by these bioactive molecules.

The Excretory System of *A. aegypti*

Concerning the requirements of survival in various terrestrial and aquatic environments, the insect excretory system maintains ion and water homeostasis to stabilize its internal environment. In blood-feeding insects, the ingestion of a blood meal challenges ion and water homeostasis by introducing additional salts and liquids of increasing osmotic pressure. Normally, the excretory system in insects is comprised of the Malpighian tubules and hindgut. During a blood meal, the female mosquito undergoes immediate diuresis which is facilitated by the excretory system (Piermarini et al., 2017). The Malpighian tubules mark the beginnings of urine formation. In adult *A. aegypti*, five blind-ended Malpighian tubules reside in the insect haemolymph and are attached to the gut at the junction of the midgut and hindgut. Studies on the transport mechanisms of the tubule epithelium highlight the ability of Na^+, K^+, and Cl^- ions to move from the haemolymph into the tubule lumen (Cabrero et al., 2014; Maddrell and O'Donnell, 1992). Two main cell types comprise the insect Malpighian tubules. The larger, more abundant principal cells are primarily responsible for the transport of Na^+ and K^+ and contain the V-type H^+-ATPase in their apical membrane, while the smaller and less abundant stellate cells are responsible for Cl^- transport (Weng et al., 2003). The V-type H^+-ATPase is the main driver of transepithelial transport that occurs in both a transcellular and paracellular manner. Specifically, apical V-type H^+-ATPases drive proton transport into the tubule lumen, thus establishing the electrochemical gradient required to drive the movement of ions across the tubule epithelium (Beyenbach et al., 2010). The movement of different ions including Na^+, K^+, and Cl^- requires numerous apical and basolateral transporters working in concert. In the principal cells, basolateral sodium-potassium-2-chloride (NKCC) cotransporters shuttle Na^+, K^+, and Cl^- from the hemolymph into the cells (Ianowski and O'Donnell, 2004). Furthermore, apically localized antiporters, Nha1, and Nha2 facilitate the transport of Na^+ and Cl^- ions, respectively, into the tubule lumen in exchange for a proton (Chintapalli et al., 2015). The movement of ions into the hemolymph can also occur with the Malpighian tubules. Such movement is accomplished by a basolateral Na^+/K^+-ATPase for example, which transports three Na^+ ions into the hemolymph in exchange for two K^+ ions that enter the principal cell (Rodan et al., 2012). This exchange drives K^+ movement into the principal cells by returning the Na^+ introduced by the basolateral NKCC channel back into the hemolymph (Rodan et al., 2012). Simultaneously, the stellate cells possess chloride ion channels, such as the apical and basolateral CLC chloride channel and recently identified apical secCL (Cabrero et al., 2014; Feingold et al., 2019), which together allow for the transcellular movement of Cl^- ions into the tubule lumen. Chloride ion channels are not exclusive to the stellate cells, however, as the principal cells possess apical pHCl-2 ion channels that transport Cl^- ions into the tubule lumen (Feingold et al., 2016). Much of the discussed ion movement across the tubule epithelium occurs transcellularly, that is through the cell, however paracellular movement of Cl^- ions have been reported (Beyenbach, 2003). With ions and fluids entering the Malpighian tubules, it marks the beginning of urine formation. The ion-containing fluid is then secreted into the hindgut which is the next component of the excretory system to make the final modifications of the urine before excretion. The anterior region of the hindgut contains the ileum where ion reabsorption is suggested to take place. Ions transporters have been identified in the desert locust, including an apical Cl- pump (Phillips et al., 1996). In adult female *A. aegypti*, the V-type H^+ ATPase and P-type Na^+/K^+-ATPase immunolocalization was identified in the apical and basolateral membrane, respectively, of the ileum epithelium (Patrick et al., 2006). Finally, the last site of ion and water regulation occurs in the rectum, the posterior region of the hindgut. Here, apparent invaginations of the dorsal surface of the rectum, named rectal papillae, are the main sites of ion and water reabsorption as they project into and subsequently filter the rectal lumen (Gupta and Berridge, 1966). There are six rectal papillae in adult female *A. aegypti*, while there are only four in adult males (Hopkins, 1967). These structures each contain a central canal, named the medulla, that opens into the haemolymph. Here, a large tracheal branch enters the canal and branches out within the papillae. In addition, a nerve is associated with the trachea, where its axons terminate throughout the medulla (Gupta and Berridge, 1966). Regarding ion and water reabsorption, sinuses containing abundant infoldings were suggested to be the sites where ions and fluids are collected before moving into the haemolymph (Berridge and Gupta, 1967). In fact, immunolocalization revealed the abundance of P-type Na^+/K^+ ATPases on these basolateral infoldings, while V-type H^+-ATPases were present on the apical membrane of the rectal papillae (Patrick et al., 2006). In addition, cells found in the apical region of the rectal papillae epithelium contain a large number of mitochondria, which is characteristic of cells that are involved in ion transport (Bradley, 1984; Cianficconi et al., 1985). Altogether, the hindgut acts as the final site for regulating ion and water balance before the urine is excreted.

Regulation of Insect Hindgut Physiology

The hindgut is the final site for ion and water regulation, as it modifies the contents of the primary urine formed by the Malpighian tubules before excretion. With the external environment and/or diet challenging the ion and water balance of the insect, regulation of the excretory system by various hormones is crucial for survival. For example, biogenic amines including serotonin, dopamine, and octopamine are synthesized in the nervous system, and like neuropeptides, can act as a neuromodulator within the nervous system, or neurohormones when targeting peripheral organs (Monastirioti, 1999). Biogenic amines have been shown to stimulate fluid secretion of the Malpighian tubules, where serotonin is the most potent in influencing fluid secretion (Veenstra, 1988). In *A. aegypti,* serotonin and octopamine stimulate the contraction of muscles associated with the hindgut (Messer and Brown, 1995). Furthermore, the glycoprotein GPA2/GPB5 was suggested to promote kaliuresis (K^+ excretion) and inhibit natriuresis (Na^+ excretion) in adult *A. aegypti* to deal with ionoregulatory stress during adult emergence and/or after receiving an ion-rich bloodmeal (Paluzzi et al., 2014). It was shown that the addition of this hormone to the adult hindgut ex vivo leads to the stimulation of Na^+ absorption in the ileum, while stimulating K^+ reabsorption primarily in the anterior rectum (Paluzzi et al., 2014). Likewise, neuropeptides can regulate the excretory system to meet the homeostatic needs of the insect. Control of the hindgut has been shown to involve neuropeptides in the regulation of the ion and water transport that occurs across the ileum and rectum. More than twenty years ago, the ion transport peptide (ITP) was shown to significantly increase the transepithelial movement of ions across the ileum in the desert locust, *Schistocerca gregaria* (King et al., 1999). Desert locust ITP exhibited increased Cl^- transport and K^+ permeability in the ileum. Thus, increased ion reabsorption at the hindgut suggests ITP has an antidiuretic effect on the excretory system. The *Drosophila* homolog of the desert locust ITP was suggested to be involved in antidiuresis, sensitivity to desiccation stress, water intake, and excretion upon RNAi-mediated knockdown of ITP (Gáliková et al., 2018). More specifically, the knockdown of the ITP transcript leads to an increase in water secretion and body water content, highlighting the neuropeptide’s anti-diuretic function within the excretory system. In further suggesting a role within the hindgut, candidate ITP receptor transcripts were identified in the hindgut of the silkworm *Bombyx mori* larvae (Nagai et al., 2014). Furthermore, in the desert locust, the neuroparsin neuropeptide family has been shown to alter ion absorption through the inositol phosphate pathway; however, conflicting results question the suggested effect of these neuropeptides on the hindgut (Coast et al., 2002). Aside from their control over ion and water secretion/absorption, neuropeptides can also influence the muscles associated with the gut, including those responsible for the contraction of the hindgut. Although not a direct control of transepithelial transport across the hindgut, the modulation of muscle contraction aids in expelling urine and faeces, and potentially in mixing the contents within and around the hindgut to regulate ion and water balance in the haemolymph. The leucokinin family of neuropeptides, for example, has been shown to stimulate the contraction of the hindgut in insects including the fruit fly, desert locust, cockroach, and kissing bug (Holman et al., 1991). Additionally, PK-2 of the pyrokinin family has been found to inhibit ileum myoactivity (Lajevardi and Paluzzi, 2020). Importantly, the organ is innervated by the terminal abdominal ganglia (Abou El Asrar et al., 2020) which contains a wide diversity of neuropeptides and is even regarded as the “posterior brain” (Predel et al., 2010). Thus, many more neuropeptides can potentially elicit control over the insect hindgut.

Luqin Neuropeptides and their Receptors

About a decade ago, a new group of insect neuropeptides, termed RYamides, was discovered and named based on their amidated C-terminal arginine-tyrosine motif (Hauser et al., 2010). The insect RYamides are a part of the luqin family of neuropeptides (see Table 1), which was first discovered in the crab *Cancer borealis* (Li et al., 2003). In particular, the insect RYamides are orthologous to the luqin neuropeptides found in other invertebrates including the crustaceans, mollusks, and annelids, which may possess the RYamide motif, and thus share a common ancestral gene (homologous) but due to speciation events, the gene has evolutionarily diverged (Yañez-Guerra & Elphick, 2020). Luqin physiology has indicated a potential role in feeding by controlling the longitudinal muscle of the digestive tract in the sea cucumber, *Apostichopus japonicus* (Li et al., 2022). In addition, luqin-type neuropeptides have shown an influence on the buccal muscle contraction of the African giant snail, *Achatina fulica* (Fujimoto et al., 1990). Overall, it appears that luqin-type neuropeptide physiology involves muscular contractions of various physiological systems including feeding, digestion, and reproduction. Luqin neuropeptides are absent in chordates, yet the putative *Drosophila* RYamide receptor (CG5811) was identified twenty years ago for its ability to bind to the human NPY (Li et al., 1991). However, phylogenetic analysis indicates that NPY and luqin are not evolutionarily related; instead, the insect neuropeptide F (NPF) and NPY are regarded as homologs (Caers et al., 2012) Therefore, NPY and NPF share a common ancestral gene but have diverged due to a gene duplication event. Deorphanization of the RYamide receptor has indicated CG5811 as a genuine receptor for the RYamides (Collin et al., 2011; Ida et al., 2011). In addition, orthologs of the putative *Drosophila* RYamide receptor have been identified in the silkworm *Bombyx mori* and red flour beetle *Tribolium castaneum* (Collin et al., 2011; Matsumoto et al., 2019). The CG5811 RYamide receptor is a G protein-coupled receptor and is considered part of the class A or rhodopsin-like subfamily (Hanlon and Andrew, 2015). The downstream, physiological effects of RYamides and their receptor was initially investigated in insect feeding behavior as they share a similar C-terminus with the mammalian neuropeptide-Y (NPY), which primarily mediates the sensation of hunger in humans (Beck, 2006).

Table 1. **Peptide sequences of luqin and luqin-like neuropeptides found in the spiralia and ecdysozoa phyla and the evolutionarily unrelated mammalian neuropeptide** Y. Highlighted letters indicate the conservation of residues at the same position across different phyla. The human NPY has been shortened to show eleven out of thirty-six amino acids to highlight the C-terminus sequence.

Phylum	Species	Peptide name	Sequence
Echinodermata	*Apostichopus japonicus* (sea cucumber)	Luqin-type	KPYKFMRWamide
	Strongylocentrotus purpuratus (sea urchin)		EIRSPGGKPHKFMRWamide
Mollusca	*Aplysia californica* (sea slug)	Luqin	APSWRPQGRFamide
	Lineus longissimus (ribbon worm)		EAQWRPQGRFamide
Arthropoda	*Cancer borealis* (crab)	RYamide	EGFYSQRYamide
	Drosophila melanogaster	*d*RYamide-1	PVFFVASRYamide
		dRYamide-2	NEHFFLGSRYamide
	Aedes aegypti	*Aedes* RYamide-1	PFFVGSRYamide
		Aedes RYamide-2	NDRFFLGSRYamide
	Triboleum castaneum	*Triboleum* RYamide-1	VQNLATFKTMMRYamide
		Triboleum RYamide-2	ADAFFLGPRYamide
Chordata	*Homo sapiens*	Neuropeptide Y	-HYINLITRQRYamide

Insect RYamides and their Physiological Roles

Generally, the insect RYamide gene encodes a precursor peptide that produces two RYamides (examples of which are provided in Table 1). The physiological relevance of the insect RYamides has been investigated in multiple insects. Initially, these neuropeptides were tested concerning their ability to control feeding behavior. It was shown that, after injection with *D. melanogaster* RYamide-1 (*d*RYamide-1), adult blowfly *Phormia regina* were less motivated to feed as quantified by a decrease in the frequency of extending their proboscis in the presence of a sugar meal (Ida et al., 2011). Another study showed that *d*RYamide-2 also suppresses the proboscis extension reflex (albeit partially) similar to the activity of *d*RYamide-1 (Maeda et al., 2015). The extension of the proboscis can occur when the gustatory sensilla (sensory organs) of the labella detects sugar above a threshold concentration. Within each sensillum are different neurons that can detect various substances, including a sugar-detecting neuron. After noticing that there was a decrease in feeding initiation in the presence of a sugar meal, the changes in the neuronal activity of the sugar receptor were observed after the RYamide injection of the blowfly. The neuronal activity was determined by measuring the frequency of electrical impulses between 0.15 and 0.35 seconds after stimulation of the sugar receptor neuron. *d*RYamide-1 injection led to a gradual desensitization of the sugar receptor neuron, but no effect was observed after dRYamide-2 injection (Maeda et al., 2015). It was suggested that dRYamide-1 acts indirectly on the sugar receptor neuron, as the gradual, incomplete desensitization of the sugar receptor neuron occurred over sixty minutes, while the putative RYamide receptor became completely desensitized after five seconds when expressed in cultured mammalian cells (Collin et al., 2011). Thus, a downstream effect on the sugar receptor by the RYamide likely occurred. In continuation of investigating the role of these neuropeptides in feeding behavior, a study using the larval silkworm *Bombyx mori* first attempted to identify compounds that inhibited feeding behavior (Matsumoto et al., 2019). Purified peptide extracts from homogenized midguts from *ad libitum* fed 2-day-old fifth-instar *B. mori larvae* were injected into larval *B. mori*, and peptide extracts that were active in delaying larval feeding were isolated and sequenced. Two peptides, designated as GSRYa-1 and GSRYa-2, were identified as inhibitors of feeding behavior and orthologous to the *Drosophila* RYamides. In addition, administration of these RYamides onto isolated larval gut preparations revealed inhibition of foregut and hindgut contractions in both fed and non-fed larvae except for contractions of the pharynx (anterior foregut), which was not affected in non-fed larvae (Matsumoto et al., 2019). Thus far, based on these various physiological studies, the RYamide neuropeptides appear to be involved in the inhibition of feeding behavior. Studies have also identified RYamide and the putative receptor transcripts in tissues related to feeding, which includes the foregut and midgut (Guo et al., 2021; Matsumoto et al., 2019; Veenstra and Khammassi, 2017), which supports the involvement of these neuropeptides in feeding. Excitingly, RYamides and their putative receptor transcripts have been identified in the hindgut, testes, and ovaries, which suggests additional functionality (Leader et al., 2018; Matsumoto et al., 2019; Veenstra and Khammassi, 2017). For example, hindgut expression of the RYamide receptor provides support for a role in regulating ion and water reabsorption, and/or digestion while enrichment in the testes and ovaries supports that the RYamides regulate the reproductive system.

Transcript Expression and Immunolocalization

Tissue and developmental transcript expression can provide further insight into the function of a neuropeptide. RYamide transcript expression and immunolocalization are found throughout the central and peripheral nervous system, including the brain and abdominal ganglia (Roller et al., 2016; Veenstra & Khammassi, 2017). Immunohistochemistry can also indicate where a neuropeptide may exert its actions. For instance, RYamide immunolocalization within axonal processes was found associated with the rectal papillae in the hindgut, a site for ion and water reabsorption in adult *D. melanogaster* (Veenstra and Khammassi, 2017). Additionally, transcriptome data mining has identified an ortholog to the *Drosophila* RYamide receptor (CG5811) transcript in *A. aegypti*, where transcript expression is present in the male testes and female ovaries of adult mosquitoes (Matthews et al., 2016). Thus, investigating transcript expression more extensively can provide insight that can be used to study the putative RYamide receptor in other insects, enabling the investigation of the potential roles of these neuropeptides.

Objectives and Hypotheses

The relatively recent discovery of RYamides within insects has provided researchers with new insight and directions in the study of neuropeptides. With the RYamides being identified in various insects, including for example *D. melanogaster, A. aegypti,* and *B. mori* (Collin et al., 2011; Roller et al., 2016; Veenstra and Khammassi, 2017), the initial groundwork of these neuropeptides can be used to assist in future physiological studies and experimentation. In my thesis, I focus on establishing the molecular basis of RYamides, including mRNA expression and peptide distribution in the mosquito, *A. aegypti.* This mosquito is a globally important disease vector and a better understanding of the mechanisms by which insect physiological processes are regulated by neuropeptides can allow for their exploitation in the future development of novel control strategies targeting insect pests.

My first objective was to determine the immunolocalization of *A. aegypti* RYamide-1 (Rya-1) using immunohistochemistry (IHC) with a custom antibody specific to the RYamide-1 peptide sequence. Since the RYamides are neuropeptides, that is, peptides synthesized and released primarily from the nervous system, I hypothesized that RYamide-1 immunolocalization would be distributed in the central and peripheral nervous system. Further, based on prior studies involving *Drosophila* and *Bombyx* (Roller et al., 2016; Veenstra and Khammassi, 2017), I also predicted that RYamide-1 immunolocalization would be present along the digestive tract, including the midgut and hindgut. Immunolocalization of the RYamide-1 neuropeptide would help elucidate the target tissues of RYamide-1 signaling, where further investigations into the function of this neuropeptide within *A. aegypti* can be made.

In support of the potential functionality of RYamide established using IHC, transcript expression of the RYamide gene will be determined with qPCR. The developmental and tissue-specific expression profiles in adult stage *A. aegypti* will be characterized and used to further determine and support potential RYamide functionality in this important disease vector species.

Additionally, the *A. aegypti* RYamide receptor gene (AAEL017005) was identified to be encoded from a gene homologous to the *Drosophila* RYamide receptor, CG5811 (Li et al., 2003). To determine this receptor as a *bona fide* RYamide receptor in the mosquito, a heterologous assay was used to deorphanize this receptor by testing it against different neuropeptides, including RYamides. It was hypothesized that the receptor would be activated only by the two endogenous mosquito RYamides, *Aedae*RYamide-1 and *Aedae*RYamide-2, as this receptor is related to the *Drosophila, Tribolium,* and *Bombyx* RYamide receptors that are specifically activated by RYamides (Collin et al., 2011; Guo et al., 2021; Ida et al., 2011). Likewise, the RYamide receptor transcript expression profiles were examined. Given that the homologous *Drosophila* RYamide receptor transcript was detected in the hindgut of adult *Drosophila* (Leader et al., 2018; Veenstra and Khammassi, 2017), it was hypothesized that the *A.*

aegypti RYamide receptor transcript would be expressed in the hindgut of adult stage mosquitoes.

Materials and Methods

Animal rearing

Adult mosquitoes of the Liverpool strain were maintained in colony cages at room temperature (~21 °C, 20% RH) under a 12:12 hour light:dark cycle. Mosquitoes were fed *ad libitum* with 10% sucrose (w/v) and provided with a container containing ddH_2O as a water source. Female mosquitoes were provided with sheep's blood in Alsever's solution (Cedarlane Laboratories, Burlington, ON). Furthermore, a container lined with Whatman filter papers (GE Bioscience) was provided in the cages to collect oviposited eggs. Filter paper containing oviposited eggs was dried and stored for future use. Once needed, the filter paper was submerged in ddH_2O which was contained in a 26°C incubator. Hatched larvae were provided with a solution containing equal parts 2% brewers yeast and beef liver powder by weight. Pupae were collected and transferred to unsealed glass jars in ddH_2O and adult mosquitoes were further isolated for experiments or colony upkeep. Isolated adult mosquitoes were also kept in unsealed glass jars and provided with 10% sucrose (w/v).

Receptor Cloning and Expression

The gene AAEL017005 encoding a predicted *A. aegypti* neuropeptide receptor was identified as an ortholog of the *Drosophila* RYamide receptor (CG5811) gene using VectorBase. Various forward and reverse primers were designed specifically for the AAEL017005 gene using Geneious software (see Table 2). To clone the full receptor sequence, including portions of the 5' and 3' untranslated regions, amplification utilized Q5 High-Fidelity DNA polymerase (New England Biolabs, Whitby, ON) with adult *A. aegypti* hindgut cDNA as a template, along with the primers which produce a 1901 bp amplicon. Thermocycling conditions for the Q5 polymerase-mediated amplification were as follows: initial denaturation at 98°C for 30 seconds, then 35 repeated cycles of (1) denaturation at 98°C for 10 seconds, (2) annealing at 64-66°C for 30 seconds (see Table 2 for primer-specific temperature), (3) extension at 72°C for 30 seconds, and a final extension at 72°C for 2 minutes. Afterward, an aliquot of the amplified products was verified using gel electrophoresis, and the expected products were purified using the Monarch PCR purification kit (New England Biolabs, Whitby, ON). The resulting products were A-tailed with Taq polymerase, then ligated into the pGEM-T vector (ThermoFisher Scientific, Burlington, ON) and cloned using DH5-alpha competent *E. coli* cells (New England Biolabs, Whitby, ON). White colonies resulting from the disrupted lacZ gene grown upon LB-medium plates containing XGAL (80µg/mL) and ampicillin (100µg/mL) were selected and inoculated into liquid culture. An additional colony PCR was performed using OneTaq polymerase (New England Biolabs, Whitby, ON) to verify the presence of the correct inserts belonging to the predicted receptor's open reading frame and cDNA. Plasmid purification from the resulting overnight cultures was done using a Monarch Plasmid DNA purification kit (New England Biolabs, Whitby, ON). Amplified products pertaining to various regions of the receptor cDNA were confirmed by Sanger sequencing (Centre for Applied Genomics, SickKids, Toronto, ON). Two of the primer pairs were utilized for RT-qPCR which encompass different exon-exon boundaries producing PCR product sizes of 191 and 231 base pairs (bp) (Table 2). Another primer pair amplifies a large portion of the receptor cDNA, including the entire open reading frame (ORF) along with portions of the 5' and 3' untranslated regions producing a product size of 1901 bp. Finally, a primer pair was used to amplify just the ORF of the receptor, while also incorporating a Kozak and stop sequence to produce a 1397 bp product.

Table 2. **Designed primers specific to the *A. aegypti* RYamide receptor gene, AAEL017005**. Primers are grouped into their forward-reverse primer pair, along with their nucleotide sequence, and annealing temperature for Q5 DNA polymerase amplification. Sequence length indicates the number of nucleotides of the amplified qPCR product from the associated forward and reverse primers.

Primer Name	Sequence (5'-3')	Amplicon size (base pairs)	Annealing Temperature (°C)
RYa-qPCR-F RYa-qPCR-R	CTAATCCTTCTAGTCAGTGCGG AGCGGGATCCAAGAAAGAAGC	252	65
RYaGPCR-qPCR-F1 RYaGPCR-qPCR-R1	AATCTGCAAGGAAATTTGGCC AACACTATGACGACCGTTACC	231	65
RYaGPCR-qPCR-F2 RYaGPCR-qPCR-R2	ATCTACTGCTACATGAACGCC TGTGGTGCATGTGTTAACTCG	191	65
RYaGPCR-Kozak RYaGPCR-Stop	TTCTGCCGCCACCATGAGCG GGTTGTTGTATCACCGTAGC	1397	66
RYaGPCR-cDNA-F RYaGPCR-cDNA-R	GAGAAGAGCTGTTTCTACGGG CTCCACGTGTCTTTAAGCGG	1901	64

To express the receptor heterologously, pBud and pcDNA mammalian expression vectors were used. Two different expression vectors were initially used to observe and compare receptor sensitivity with different ligands, and thus subsequent functional assays were used with the more sensitive receptor construct. For the preparation of the receptor-pBud construct, new primers were designed containing a SalI and XbaI restriction site within the respective forward and reverse primers used for amplification of the 1397 bp product (see Table 2), allowing for the double digestion of the ORF from the pGEM-T vector as well as the pBud vector to generate the expression construct within the CMV multiple cloning site. The pBud vector is a dual promoter vector, and the second multiple cloning site was previously prepared to contain the murine promiscuous G protein, Gα15 (Wahedi et al., 2019) which helps drive receptor coupling to calcium signaling. For the preparation of the second expression construct, the ORF was digested from pGEM-T using the NotI restriction enzyme. The pcDNA expression vector was also digested with NotI and subsequently dephosphorylated to prevent self-ligation of the vector and other non-recombinants. Ligation of the ORF to the pBud and pcDNA mammalian expression vectors utilized Anza restriction enzymes as per manufacture protocol (Anza™ cloning system; ThermoFisher Scientific, Whitby, ON, Canada). Digested products were verified using a 1% agarose-TAE gel containing ethidium bromide, and digested ORF products were gel-extracted and purified using a Monarch DNA Gel Extraction Kit following manufacture protocol (New England BioLabs, Massachusetts, USA). Purified products were quantified and ligated with their respective mammalian expression vector using T4 DNA Ligase. Chemically competent DH5α-strain *Escherichia coli* cells were transformed with either pBud or pcDNA containing the receptor ORF and grown on ampicillin (pcDNA) or zeocin (pBud) LB-agar plates. A Colony PCR of select colonies was used to screen for recombinant vectors using Taq DNA polymerase

(New England Biolabs, Whitby, ON). Selected colonies were grown overnight and a midiprep plasmid purification (Zymo Research, USA) was completed to prepare for Sanger sequencing and subsequent heterologous study of the receptor.

Transient Expression in CHO-K1 Cells

Chinese hamster ovary (CHO)-K1 cells stably expressing aequorin as previously described (Paluzzi et al., 2012) were maintained in Dulbecco's modified eagles medium: nutrient F12 (DMEM: F12; 1:1) media containing 200 µg/mL geneticin, and 10% heat-inactivated fetal bovine serum. The cell line was grown and maintained in a water-jacketed incubator at 37°C with 5% CO_2 to maintain pH. Transfection of the pBud and pcDNA mammalian expression vectors containing the RYamide receptor ORF was carried out at approximately 90% confluency using Lipofectamine 3000 reagent and P3000 enhancer reagent at a 3:1 and 2:1 reagent to DNA ratio, respectively (Life Technologies, Burlington, ON).

Heterologous Functional Assay of the RYamide Receptor

At 48 hours post-transfection, cells were prepared for the functional receptor assay as described previously (Wahedi & Paluzzi, 2018). Serial dilutions of various commercially synthesized peptides from 10^{-4} to 10^{-13} M were prepared in BSA assay medium were loaded onto 96-well luminescence plates. BSA medium was added alone as a negative control, while ATP at $5x10^{-5}$ M was used as a positive control, as endogenous purinergic receptors of the CHO-K1 cells are activated by ATP (Michel et al., 1998). CHO-K1 cells were added to each well by an automatic injector, and the luminescence from the cells was immediately measured for 20 seconds after the addition of test compounds using a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT). The luminescent responses in each well were normalized to the average ATP luminescent response and then analyzed using Prism 8 (GraphPad Software, USA).

Tissue and Developmental RNA Extraction and cDNA Synthesis

Adult *A. aegypti* were briefly anesthetized using CO_2 and submerged in nuclease-free PBS. After removal of the wings and legs, the following tissues and body segments were dissected, isolated, and pooled in the following order: hindgut, reproductive systems, Malpighian tubules, midgut, abdominal carcass (containing cuticle, fat body, musculature, and abdominal ganglia), thorax (containing cuticle, fat body, musculature, and thoracic ganglia), and head (containing the brain along with cuticle, fat body, musculature). The isolated male and female reproductive systems were separated, and both included the primary sex organs, namely testes, and ovaries, respectively, along with their respective accessory glands. Here, each pool of body parts was considered a biological replicate. Subsequently, the samples were stored in 1x DNA/RNA protection buffer (New England Biolabs, Whitby, ON) at -20°C until subsequent RNA purification. To lyse tissues, the protection buffer contained tissues was thawed, and an RNA lysis buffer containing 1% 2-mercaptoethanol was added following the manufacturer’s protocol. For determining the developmental expression profile of the RYamide transcript and the RYamide receptor, whole-body RNA extraction of adults, larvae, and pupae specimens were collected in 1.5mL microcentrifuge tubes and homogenized in lysis buffer using a sterile plastic pestle. Afterward, RNA was extracted using a Monarch Total RNA kit (New England Biolabs, Whitby, ON) and purified total RNA was quantified using a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT) with a Take3 micro-volume plate. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Mississauga, Canada) with 50 and 100 ng total RNA for the tissue and developmental expression profile, respectively. Synthesized cDNA was diluted 10-fold before subsequent use for qPCR analysis.

Developmental and Adult Spatial Expression Analysis of RYamide and its Receptor by Real-time Quantitative PCR (RT-qPCR)

Tissue and developmental expression of the RYamide transcript and the RYamide receptor transcript were evaluated using RT-qPCR. A portion of the RYamide and its receptor was amplified using gene-specific qPCR primers (see Table 2), based on the previously identified genes AAEL011702 & AAEL017005, respectively. The reference genes *rp49* and *rsp18* were used to normalize expression using primers described previously (Paluzzi et al., 2014). PowerUP SYBR Green Mastermix (Applied Biosystems, Carlsbad, CA, United States) was the reagent used for the amplification and detection of qPCR products. The PCR cycling conditions were as follows: 1) 50°C for 2 minutes, 2) 95°C for 20 seconds, 3) 40 cycles of 95°C for 3 seconds, and then 60°C for 30 seconds. 3-5 biological replicates were used for determining transcript expression profiles, with each including 3 technical replicates. Reactions containing no cDNA template and template from a cDNA synthesis reaction excluding reverse-transcriptase were used as a negative control. Statistical analysis utilized one-way ANOVA with Tukey’s multiple comparison test, with $p < 0.05$ being considered significant.

Immunohistochemistry

Two custom affinity-purified polyclonal primary antibodies were produced against the *A. aegypti* RYa-1 antigen sequence which originate from two different rabbits (Biomatik Corp., Kitchener, ON). Primary antibody working stocks were prepared at a concentration of 10 µg/ml in a 0.4% Triton solution containing 2% NSS (v/v), 2% BSA (w/v) in PBS, and preincubated overnight at

4°C. Adult 1-day-old male and female mosquitoes were used for this experiment. Desired tissues were dissected and fixed using 4% paraformaldehyde prepared in PBS for 1 hour at room temperature in a 1.5 ml microcentrifuge tube. Afterward, tissues were washed with PBS and incubated in a solution containing 4% Triton solution,10% NSS, and 2% BSA, in PBS for 1 hour at room temperature to permeabilize cells. The Triton solution was discarded, and tissues were subsequently washed several times with PBS. After the washes, the tissues were incubated in the primary antibody solution for 48 hours at 4°C. Following the primary antibody incubation, the tissues were washed with PBS, with the primary antibodies saved for future use. Tissues were subsequently incubated with fluorophore-tagged secondary antibody at a 1:200 dilution in a PBS solution containing 10% NSS overnight at 4°C. Tissues were subsequently washed with PBS, and the secondary antibody was saved for future use. Glycerol mounting media containing DAPI was used to mount tissue samples on glass microscope slides. Finally, mounted tissues were viewed under a fluorescent microscope. Both antibodies were tested separately for their specificity to RYa-1 by assessing immunofluorescence intensity and specificity. Biological replicates include approximately ten pooled samples per tissue examined.

Results

Distribution of RYamide Immunoreactivity in Adult *A. aegypti*

To investigate the potential role of RYamide-1 in regulating the excretory system of adult stage *A. aegypti*, whole-mount immunohistochemistry was utilized to determine the distribution of RYamide immunoreactivity in the posterior regions of the alimentary canal in adult mosquitoes. In particular, immunolocalization of RYamide-1 was observed in the hindgut, specifically the rectum (Figure 3). Here, RYamide immunofluorescence was observed in axonal processes closely associated with the haemolymph-facing side of the rectal papillae among both male and female mosquitoes. Furthermore, processes are seen converging within the central region of each papilla which contains a small number of highly immunoreactive cells. This central region contains a canal that runs the length of the papilla, where immunofluorescence can be seen along this canal (Figure 3, arrow). In addition to the rectum region of the hindgut, two axonal processes were observed over the ileum (not shown), extending over the rectum and rectal papillae (Figure 3). Overnight preincubation of the primary antibody with *Aedes* RYamide-1 peptide (10^{-6} M) abolishes all immunoreactive staining in the rectum (Figure 4). No RYamide immunoreactivity was observed associated with the ionoregulatory Malpighian tubules.

The midgut region contains many RYamide-immunoreactive cells. An abundance of circular cells approximately 20 µm in diameter appear to be spread throughout the epithelia of the posterior midgut (Figure 5A). Furthermore, there appears to be a greater density of cells in the anterior region compared to the posterior region of the posterior midgut, and no two cells are in contact with each other. The immunofluorescence within each cell is uniform, and the staining intensity is the same with all cells. At the midgut-hindgut junction, a region known as the pyloric valve, RYa-1 immunoreactive staining was observed within a ring-like process encircling this junction (Figure 5B). Similar to the nervous system and rectum, overnight preincubation of the primary antibody with the RYamide-1 peptide (10^{-6} M) resulted in the loss of all immunoreactive staining including cells stained in the posterior midgut and immunoreactive processes localized to the pyloric valve (not shown).

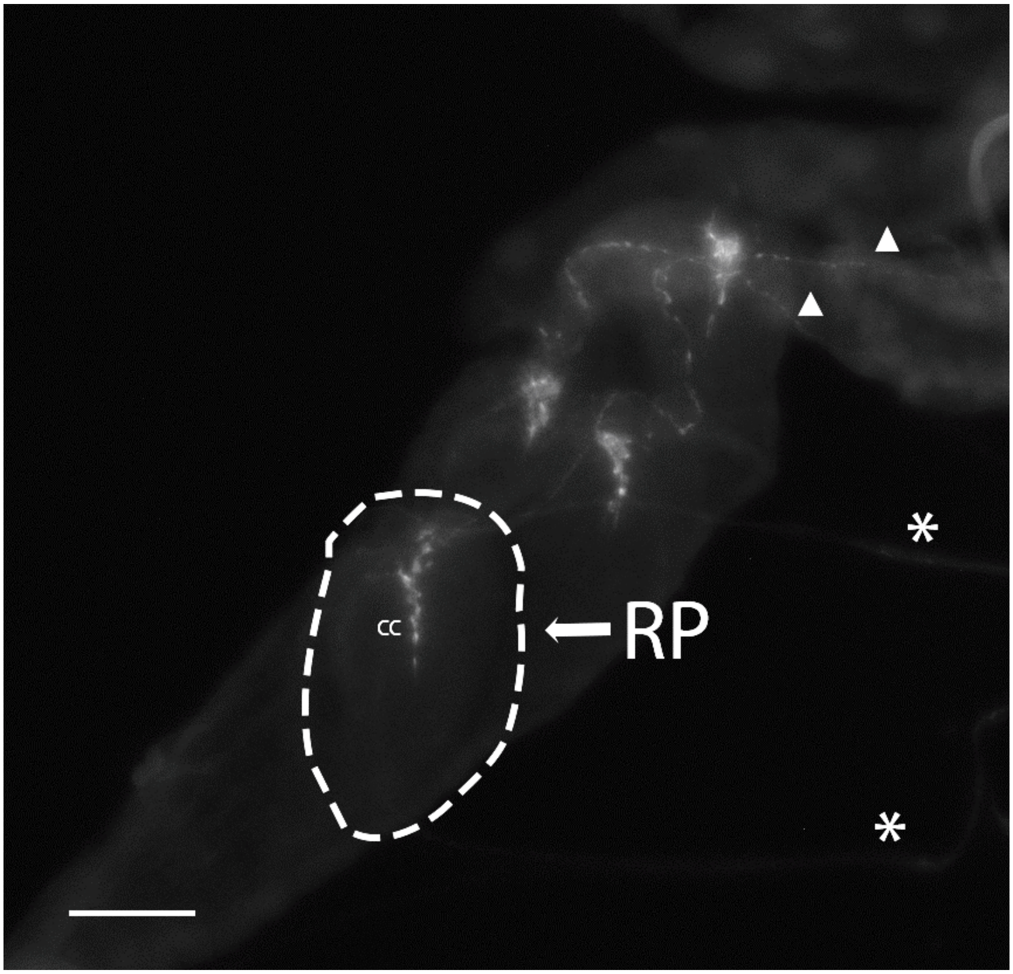

Figure 3. **RYa-1 immunoreactivity in the hindgut of 1-day-old adult *A. aegypti*.** In the hindgut, visible immunoreactive staining can be seen extending from the ileum at the upper right region as two processes into the rectum (triangles). These two processes run along the entire ileum (not shown). Immunostaining is associated with each of the rectal papillae (RP), with the dotted line encircling one rectal papilla. Staining of the rectal papillae terminates in the central canal (CC). Axonal processes within innervations from the terminal abdominal ganglia also display RYa-1 immunofluorescence (asterisks). Staining is identical between males and females. Image acquired under 20x objective. Scale bar, 100 µm.

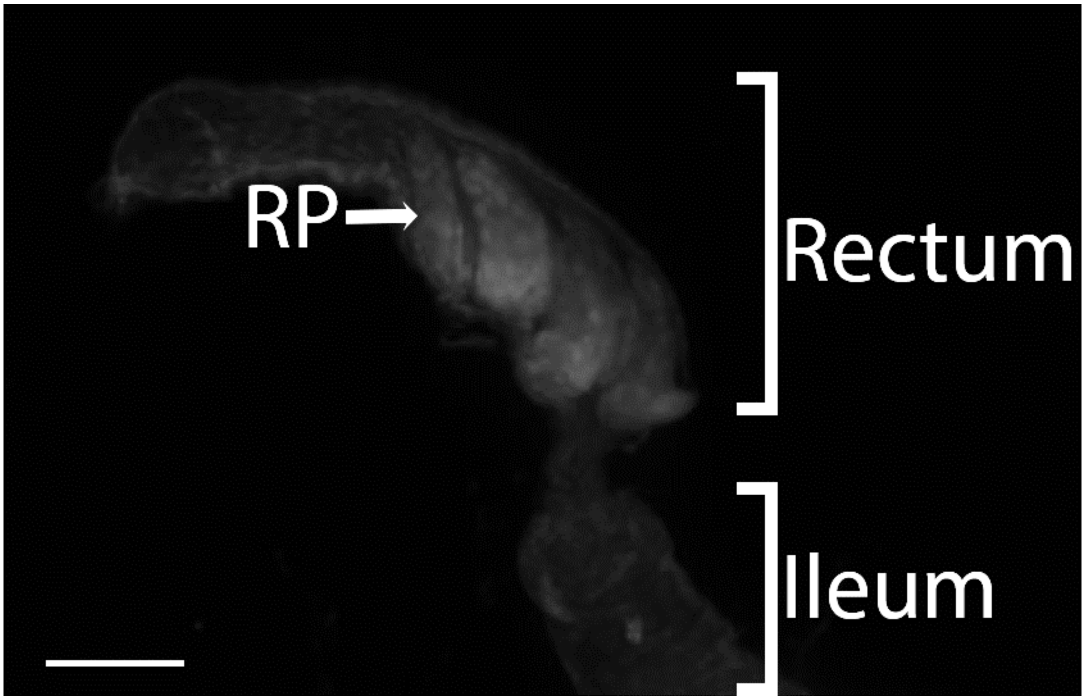

Figure 4. **Absence of RYa-1 immunoreactive staining in the hindgut after the primary antibody is preincubated with *A. aegypti* RYa-1 peptide (10^{-6} M).** The structure of each rectal papillae can be distinguished. However, there is no immunoreactive staining observed associated with the rectal papillae, and the axonal processes that extend to the ileum are also absent following preincubation of the primary antibody with its antigen. Scale bar, 100 µm.

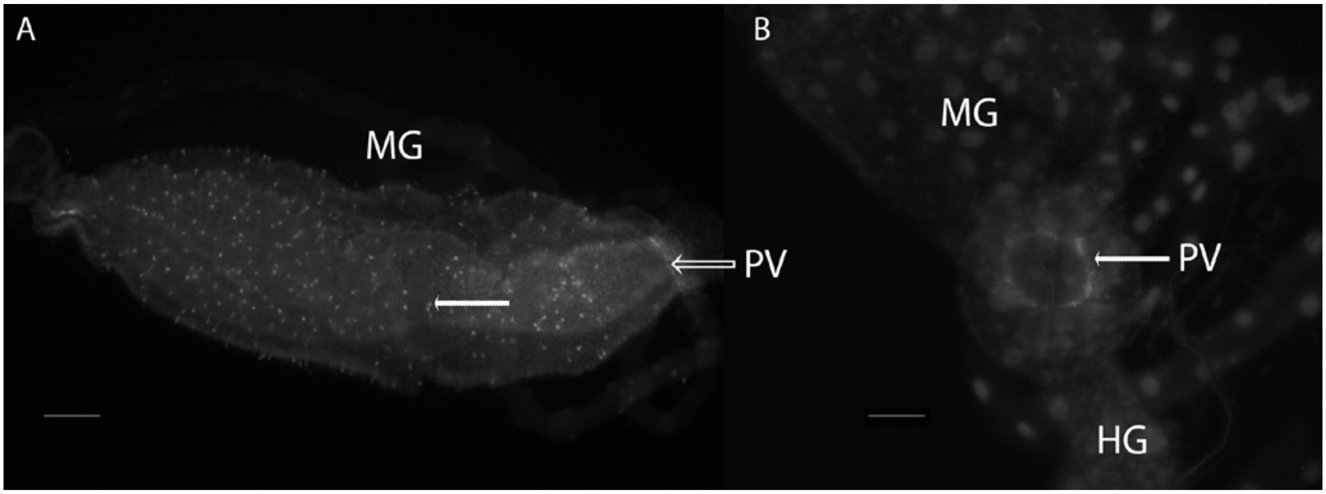

Figure 5. **RYa-1 immunoreactivity within the midgut of 1-day-old adult *A. aegypti*.** (A) The midgut (MG) shows numerous immunoreactive cells. There is an increase in immunoreactive cell density towards the anterior region of the posterior midgut, pointing in the direction of the solid arrow. Scale bar 100 µm, observed using 10x objective. (B) The midgut-hindgut junction, which also contains the pyloric valve (PV), is encircled by a ring of immunoreactivity (solid arrow). The region above and below the staining represents the midgut and hindgut, respectively. Malpighian tubules extend throughout the image. Staining is identical between males and females. Scale bars represent 50 µm at 20x magnification.

RYamide immunoreactivity was examined within the nervous system of 1-day-old adult mosquitoes. Within the brain, immunoreactive cells are clustered in the dorsal and ventral regions, corresponding to the protocerebrum and subesophageal ganglia (SEG), respectively (Figure 6A). Various regions of the protocerebrum are immunoreactive, including four-five cells in the pars intercerebralis (PI). Furthermore, there are five-six immunoreactive cells in the SEG. These cells are approximately 15 µm in diameter and generally possess a circular shape. In addition to the neurosecretory cells, immunoreactive processes and associated varicosities are found in the protocerebral region. However, the origins of the immunoreactive processes, that is, from the neurosecretory cells are untraceable. In the thorax, the associated thoracic ganglia were also found to contain RYamide-immunoreactive cells throughout the ventral region of the pro-, meso- and metathoracic ganglia (Figure 6B-C; left to right). Innervation of the adult mosquito hindgut and reproductive organs originates from the terminal abdominal ganglion (Copenhaver, 2007), whereas the abdominal ganglia 1-5 innervates tissues including various musculature, and the spiracles (Nässel, 1996). Immunostaining of the terminal abdominal ganglia revealed a contrasting number of immunoreactive cells compared to the other abdominal ganglia. Specifically, abdominal ganglia 1-5 display staining in a lateral pair of cells, as well as a singular medial cell (Figure 6D). In contrast, only a pair of lateral immunoreactive cells are present within the terminal abdominal ganglia (Figure 6E). Immunoreactive cells in the abdominal ganglia are approximately 10 µm in diameter. The nerves that innervate the rectum contain RYa-1 immunofluorescence within axonal processes, which can be traced back to the terminal abdominal ganglia (Figure 3).

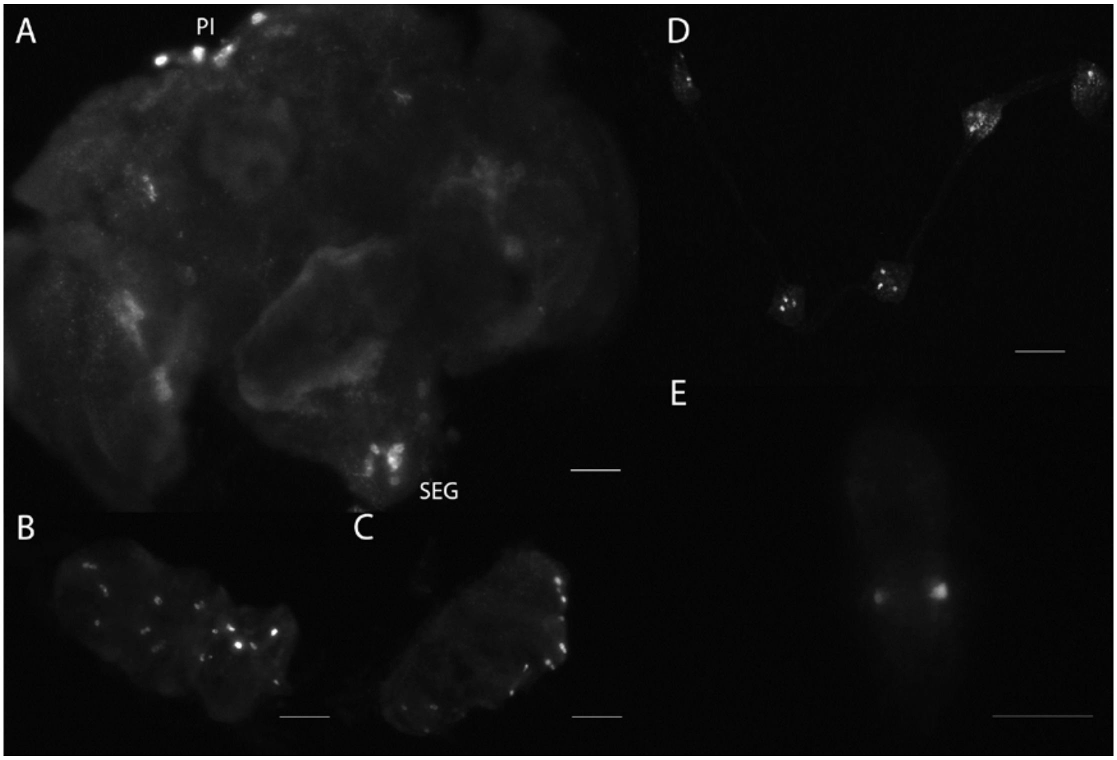

Figure 6. **RYa-1 immunoreactivity within the nervous system of 1-day-old adult *A. aegypti*.** (A) A cluster of immunoreactive cells in the dorsal region of the brain (PI) and subesophageal ganglia (SEG). Note the various processes and associated varicosities in the protocerebral region and optic lobes (OL). (B) Ventral and (C) lateral view of the thoracic ganglia showing a spread of immunoreactive cells. Some immunoreactive cells exhibit pairing with other immunoreactive cells. (D) Abdominal ganglia 1-5 showing three immunoreactive cells and (E) 40x magnification of the terminal abdominal ganglia which innervates the hindgut, possessing two cells with immunoreactivity. Each scale bar represents 50 µm.

Within the adult reproductive systems, RYa-1 immunoreactivity was absent in the testes and ovaries (Figure 7A, B). However, immunoreactive cells were identified in the adult male seminal vesicles (Figure 7C). Here, several immunoreactive cells are concentrated on the ventral side of the seminal vesicle, and some processes are visibly extending from these cells. Also, these circular immunoreactive cells are approximately 10 µm. Altogether, the pattern of immunoreactivity observed in the nervous system, midgut, and hindgut is identical between male and female 1-day-old adult *A. aegypti* mosquitoes (Figure 8).

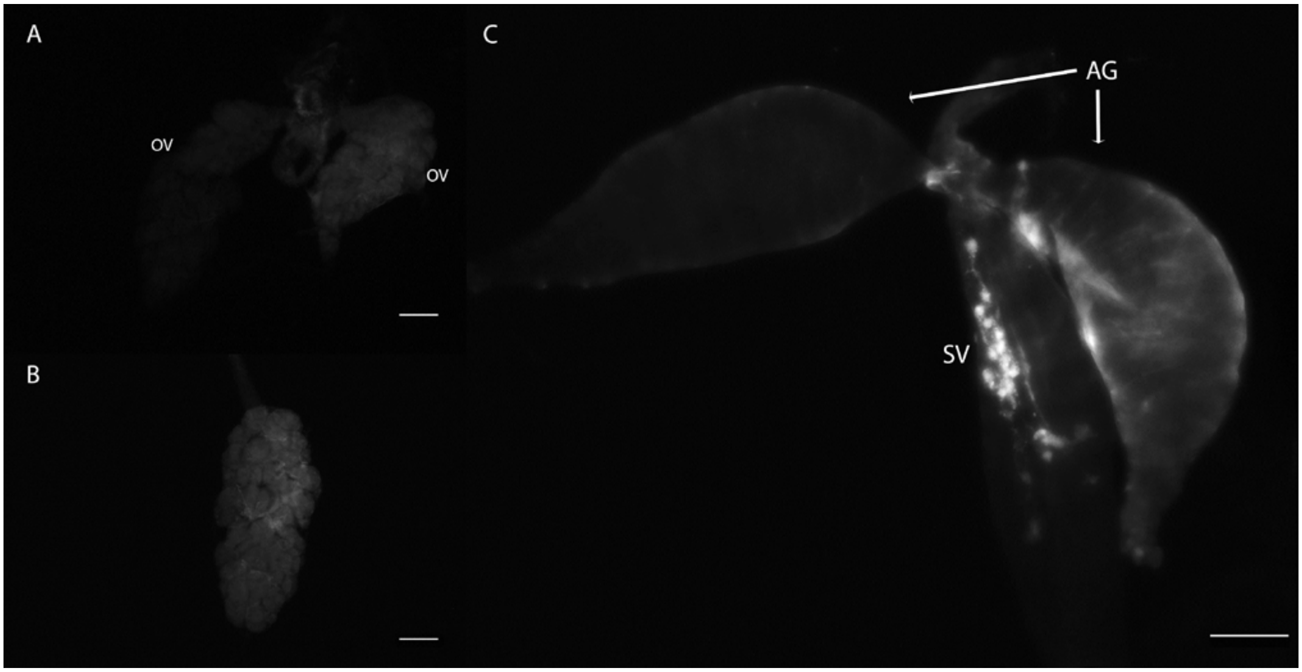

Figure 7. **RYamide-1 immunoreactivity of the adult *A. aegypti* reproductive system.** (A) The female ovaries (OV) and (B) male testes do not exhibit any RYa-1 associated immunoreactivity. (C) RYamide-1 immunoreactivity was observed in the male seminal vesicle (SV). There are immunoreactive cells, along with associated processes localized to the ventral region of the seminal vesicle. Immunofluorescence is also visible in the accessory glands (AG). Each scale bar represents 100 µm.

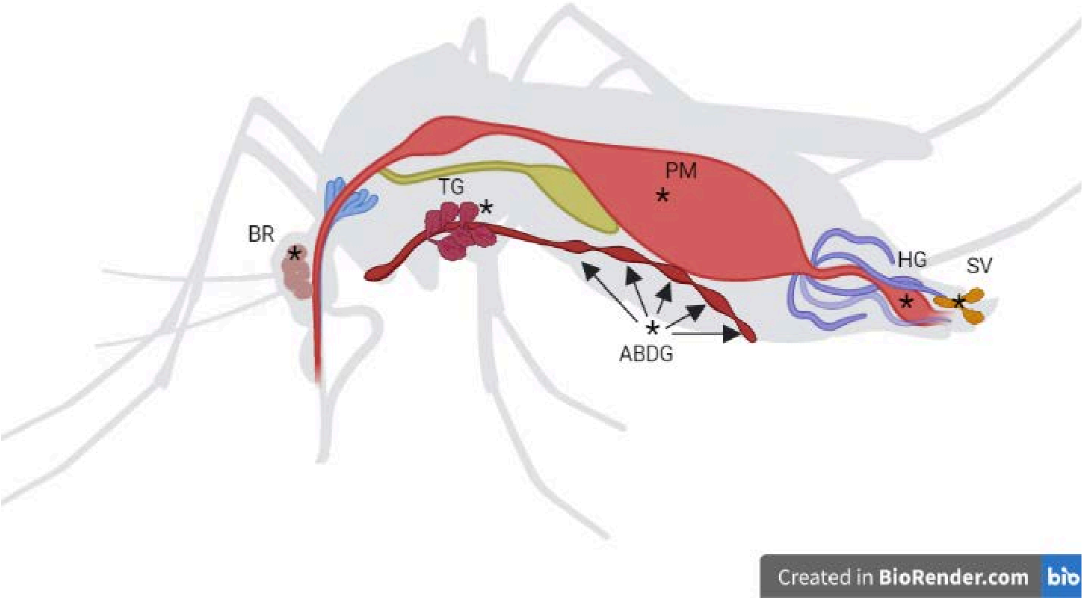

Figure 8**. Schematic overview of RYa-1 immunoreactivity in 1-day-old adult *A. aegypti*.** Organs, where RYamide immunoreactivity was observed, are marked by an asterisk. Starting in the most anterior region of the mosquito and moving posteriorly, staining is seen in the: brain (BR), thoracic ganglion (TG), posterior midgut (PM), abdominal ganglia (ABDG), hindgut (HG), and the seminal vesicles of males (SV).

Deorphanization and Functional Characterization of the RYamide Receptor in *A. aegypti*

The specificity of this predicted RYamide receptor (AAEL017005) was determined by utilizing a heterologous assay involving cultured mammalian cells. In order to functionally characterize the RYamide receptor in the mosquito *A. aegypti*, two RYamide receptor constructs were initially prepared to compare their activities. Specifically, pBUD and pcDNA receptor constructs were tested for their sensitivity to the two endogenous *A. aegypti* RYamides, RYamide-1 (PFFVGSRYamide) and RYamide-2 (NDRFFLGSRYamide) as well as the CAPA gene derived neuropeptide, PK-1 (AGNSGANSGMWFGPRLamide). The pBud construct expresses the murine G-protein, Gα15, which promiscuously couples receptors to G-proteins that allow for Ca^{2+} mediated signaling that is otherwise absent with certain receptors (Offermanns and Simon, 1995). Thus, this construct could enhance Ca^{2+} mobilization upon receptor activation which will aid in aequorin luminescence. Both the pBud and pcDNA construct showed the receptor was similarly activated by RYa-1 and RYa-2 (Figure 9). The pcDNA construct showed approximately 1.5-fold higher sensitivity than the pBud construct to the RYamides (Figure 9). In response to the negative control treatment, specifically BSA media alone, the two receptor constructs did not show any activation as indicated by the lack of a luminescence response (Figure 9-10). Following these preliminary analyses, the pcDNA construct was used to test the predicted RYamide receptor against various insect neuropeptides, including RYamide-1 and RYamide-2, in order to establish it as a genuine RYamide receptor (Figure 10). Neuropeptide specificity of the receptor was tested initially at a high concentration, 10^{-6} M, where RYa-1 and RYa-2 showed approximately 18- to 22-fold higher activation compared to the average luminescence response of BSA alone and other neuropeptides (Figure 10). These various neuropeptides included other *Aedes* neuropeptides and representatives from other peptide families conserved across insects. This included the FMRFamide neuropeptide from *Rhodnius*, which possesses a similar (yet unique) amidated C-terminal sequence to the RYamides, containing arginine and the aromatic amino acid, phenylalanine. The FMRFamide was unable to elicit any notable response from the heterologously expressed RYamide receptor, producing a background signal comparable to the receptor's response to BSA media alone (vehicle). After establishing that the putative RYamide receptor showed the greatest sensitivity to the endogenous RYamides at high concentrations, a dose-response analysis of these endogenous neuropeptides was conducted (Figure 11). RYa-1 and RYa-2 showed similar activities against the RYamide receptor since the dose-response curves for RYa-1 (EC_{50}: 1.46 nM) and RYa-2 (EC_{50}: 3.54 nM) indicated these peptides activate the receptor similarly across all concentrations tested ranging from 10^{-4} to 10^{-13} M (Figure 11). With the exception of the RYamides, all other tested neuropeptides had a similar luminescence response compared to BSA media (Figure 10), which was used as the negative (vehicle) control, supporting that this receptor is the target of the endogenous RYamide neuropeptides.

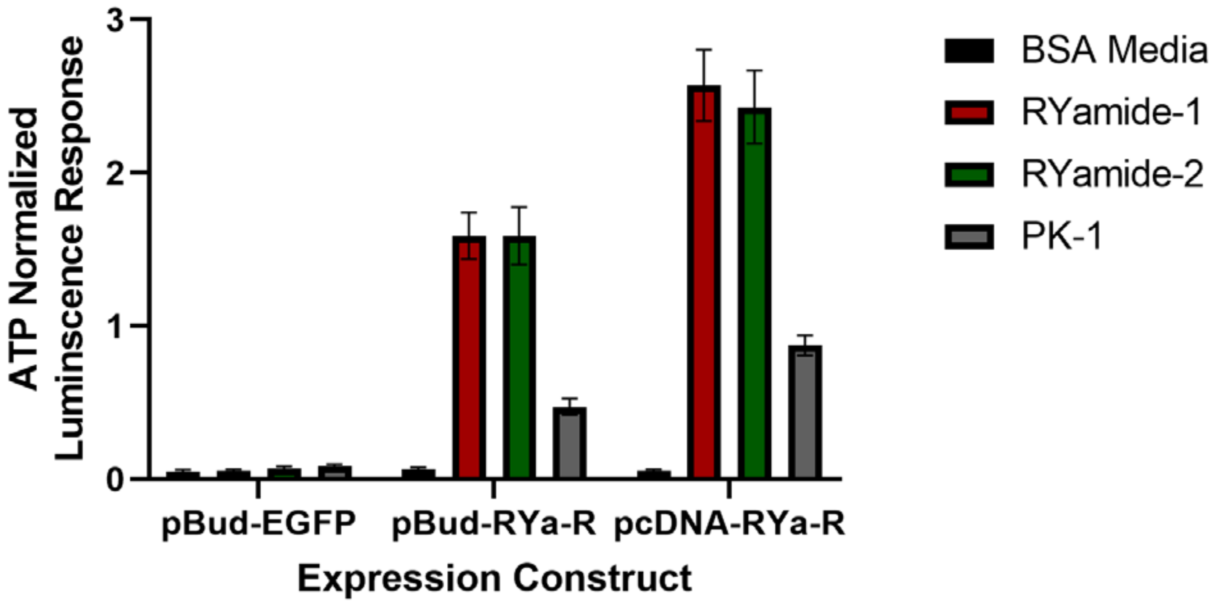

Figure 9**. Initial validation experiment displaying the differences in sensitivity of two different mammalian RYamide receptor expression constructs in response to the two *Aedes* RYamides, PK-1, or BSA media alone**. An increased sensitivity of the pcDNA mammalian expression construct compared to the pBud construct when challenged with any of the three tested neuropeptides at a concentration of 10^{-6} M. A pBud construct expressing eGFP did not respond to any of the neuropeptides. The negative control treatment, BSA media alone, did not elicit a luminescence response in cells transfected with any of the expression constructs. Data represent mean +/− standard error of 2 biological replicates.

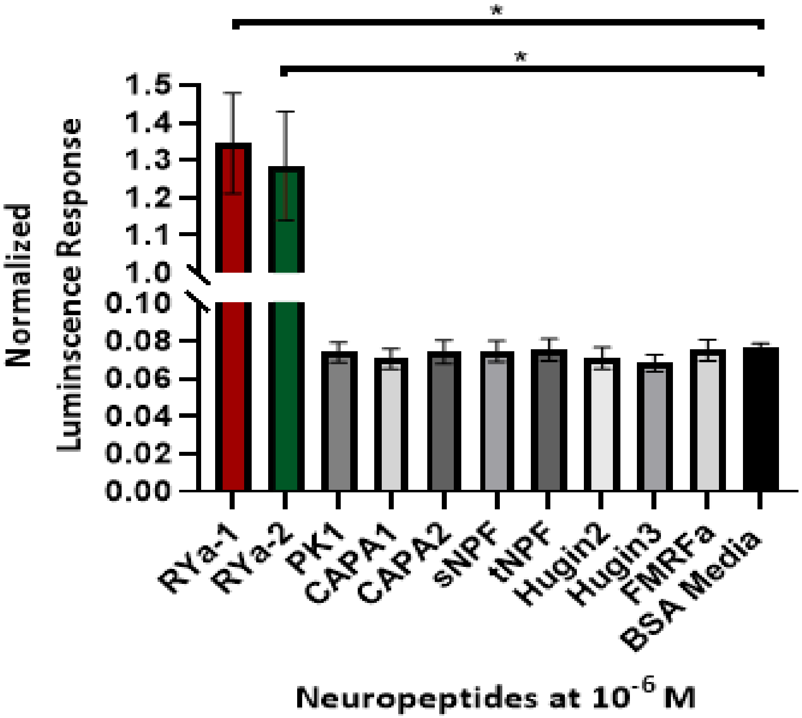

Figure 10**. pcDNA mammalian expression vector transiently expressing the RYamide receptor and its reactivity to various neuropeptides.** Note the approximate 18-fold higher activation by RYamide-1 and RYamide-2 compared to other structurally related neuropeptides, including BSA media. The luminescence responses were recorded for 20 seconds immediately following the application of test compounds and normalized to the response elicited by ATP. Responses significantly different from the control (BSA media) are denoted by an asterisk, determined by a one-way ANOVA and Dunnett's multiple comparison test ($p < 0.05$). Data represent the mean +/− standard error of three biological replicates.

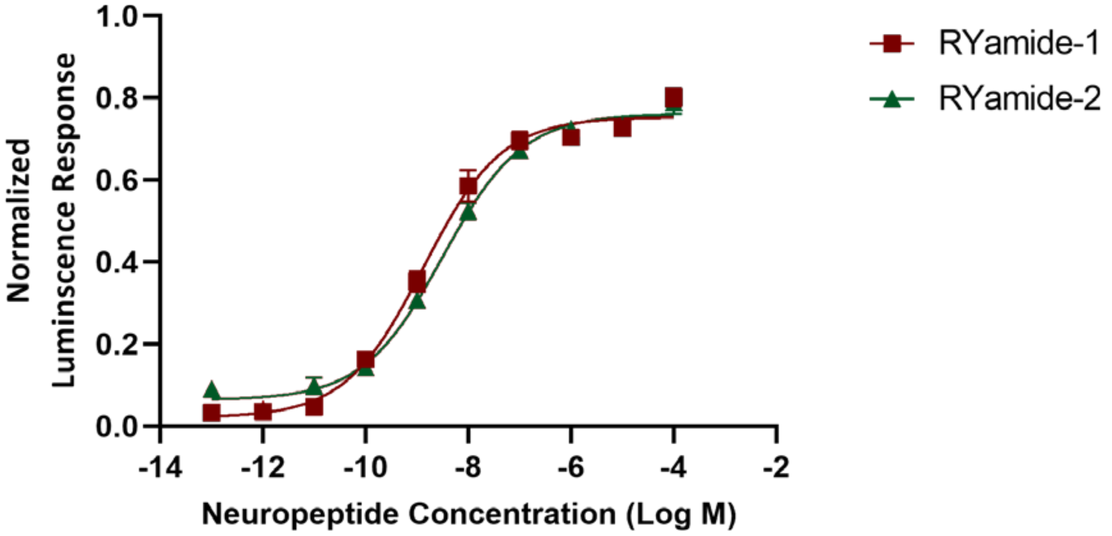

Figure 11**. Dose-dependent luminescence response (mean 0-10s) of CHOK1-aequorin cells heterologously expressing the predicted RYamide receptor in response to *A. aegypti* RYa-1 (red squares) or RYa-2 (green triangles).** Tested RYa-1 and RYa-2 dosages ranged from 10^{-4} to 10^{-13} M, and data was fitted to a non-linear sigmoidal dose-response curve. The response of the receptor to the two endogenous RYamides shows a similar degree of receptor activation. The EC_{50} of RYa-1 was 1.46 nM, while the EC_{50} of RYa-2 was 3.54 nM. These luminescent response curves represent data from one replicate; however, the listed EC_{50} values are an average of the three replicates. Data represent mean +/− standard error of 3 biological replicates.

Molecular Expression Analysis of Genes encoding RYamides and their Receptor

The developmental and adult tissue-specific expression profiles of transcripts encoding RYamide neuropeptides and the RYamide receptor (RYa-R) were determined by RT-qPCR (Figure 12). Almost all postembryonic developmental stages of *A. aegypti* showed the presence of the RYamide receptor transcript (Figure 12A). The highest RYamide receptor transcript abundance was found in 1-day-old males, albeit with a large amount of variance. All other developmental stages examined showed similar transcript abundance levels of the RYamide receptor transcript. Altogether, there were no significant differences in the developmental expression profile of the RYamide receptor transcript.

The RYamide receptor transcript expression profile in adult tissues revealed the presence in select tissues (Figure 12B). The highest enrichment of the RYamide receptor transcript was identified within the head sample, but this was not a significant difference compared to all other tissues examined. RYamide receptor transcript was also detected in the thorax and abdomen samples containing the thoracic ganglia and abdominal ganglia, respectively. Within the gut, RYamide receptor transcript expression was found within the midgut and also detected in the hindgut. A low abundance of the RYamide receptor transcript was detected in the Malpighian tubules. The male reproductive system showed expression of the RYamide receptor transcript, with similar abundance within the female reproductive system. Of note, the accessory reproductive organs were present along with the primary sex organ (i.e., testes or ovaries) within both reproductive system samples.

RYamide transcript expression was detected in all developmental stages except 4-day-old females; however, there were no significant differences detected between them (Figure 12C).

The highest levels of RYamide transcript were observed in 4-day-old males. All other tested developmental stages displayed similar, low expression levels of the RYamide transcript.

The RYamide transcript expression profile was examined in adult *A. aegypti* tissues (Figure 12D). The head sample revealed a significantly enriched abundance of the RYamide transcript, while all other tissues displayed similar low levels of expression, including the hindgut. Regarding the reproductive system, the male reproductive system showed the presence (albeit low levels) of the RYamide transcript.

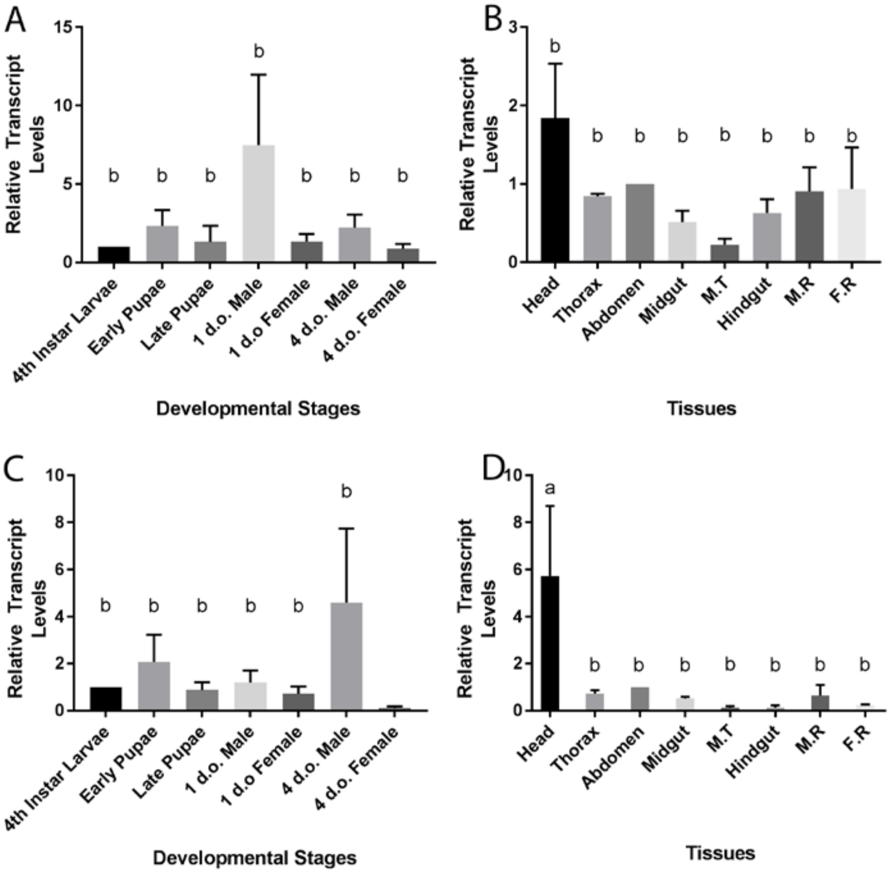

Figure 12**. Relative RYamide receptor (A & B) and RYamide (C & D) transcript expression in postembryonic developmental stages and tissues of 1-day-old adult *A. aegypti***. Developmental transcript expression levels are relative to 4th instar larvae expression, and tissue transcript expression levels are relative to abdomen expression of respective transcripts. Male and female tissues are combined for the tissue expression profiles with exception of the reproductive organs. Abbreviations: Malpighian tubules (M.T), male reproductive system with accessory glands (M.R), and female reproductive system with accessory glands (F.R). Significant differences are marked by different letters. Significance was determined by a one-way ANOVA and Tukey's test ($p < 0.05$). Gene expression is normalized to *rp49* and *rps18* transcript expression and calculated using the delta-delta Ct method. Values represent mean ± SEM (n=3).

Discussion

RYa-1 Immunolocalization within the Hindgut of Adult *A. aegypti*

The insect hindgut is responsible for the final modifications of urine content, as well as the expulsion of faeces. Various musculature controls the contractions of the hindgut, which is innervated by axonal projections from the terminal abdominal ganglia and would be regulated by neuropeptides and other neurochemicals (Abou El Asrar et al., 2020; Messer and Brown, 1995). In addition, although there has been the identification of ion transporters within the hindgut, the understanding of underlying reabsorptive transport mechanisms is not completely understood, though multiple models have been proposed (Phillips et al., 1987). In this study, RYamide immunoreactivity was examined and found to be associated with the rectal papillae of the hindgut using a specific antibody generated using the *A. aegypti* RYamide-1 peptide sequence as an antigen. The immunofluorescence showed the presence of the peptide within processes closely associated with the papillae, entering the central canal. In the following section, the different immunolocalization patterns of other neuropeptides and/or their receptors within the insect hindgut will be compared to the RYamide immunoreactivity observed in this study and begin to describe possible functions for the RYamides concerning hindgut physiology.

Regarding the pyrokinin neuropeptide family, PRXa-like immunoreactivity was identified over the hindgut, where staining was observed associated with the rectal papillae lumen of adult *A. aegypti*, suggesting a role in ion and water regulation (Lajevardi & Paluzzi, 2020). The PRXa-like immunoreactivity was almost identical to our RYamide-1 immunoreactivity, whereby axonal processes were seen terminating in the rectal papillae lumen. Furthermore, it was reported that these terminations were encircling 4-5 cells within the central canal of the rectal papillae. The association of cells with the axonal terminations within the rectal papillae suggests that these are the medullary cells of the rectal papillae (Gupta and Berridge, 1966). Additionally, PRXa-like immunoreactivity was identified in axonal projections over the ileum (Lajevardi and Paluzzi, 2020), which was observed with RYamide-1 immunoreactivity (Figure 3). The insect neuropeptide, calcitonin-like diuretic hormone (CT/DH) that is also known as DH_{31}, is involved in the control of larval midgut contractions that may regulate digestion in *D. melanogaster* (Vanderveken and O'Donnell, 2014). Regarding the excretory system, DH_{31} stimulates fluid secretion and Na^+ transport in the Malpighian tubules of adult *A. aegypti*, thus having a diuretic and natriuretic role in the insect excretory system (Coast et al., 2005). Its receptor, CT/DH-R has been identified by immunofluorescence in the principal cells of the Malpighian tubules and hindgut of adult *A. aegypti* (Kwon and Pietrantonio, 2013; Kwon et al., 2012). Immunofluorescence revealed the presence of the receptor in the inner circular muscle and outer longitudinal muscle that surround the ileum and rectum (Kwon and Pietrantonio, 2013). This contrasts with the present RYamide-1 staining, which is associated with the lumen of the rectal papillae, rather than the periphery of the rectum, thus likely ruling out a role in hindgut muscle contraction for RYamide-1. However, this is assuming that RYamide-1 immunolocalization is associated with the localization of its receptor, which is currently unknown. The aforementioned study also observed that CT/DH increases hindgut contractions in adult female *A. aegypti*. These CT/DH-induced hindgut contractions are thought to enable the circulation of diuretic hormones within the haemolymph, which maximizes their synergistic effects on fluid secretion (Kwon and Pietrantonio, 2013).

In addition to DH_{31} receptor immunolocalization in the hindgut, the *Aedes* kinin receptor was also immunolocalized in this organ (Kersch & Pietrantonio, 2011). Specifically, localization of the kinin receptor was visualized in close association with the rectal papillae in adult *A. aegypti*.

The receptor immunolocalized around the opening to the invagination of the papilla membrane, which leads into the central canal. Aedes kinin receptor immunoreactive staining can be seen surrounding the epithelia encircling the opening to the central canal, although it does not penetrate the central canal as observed for RYa-1 immunoreactivity in this study. Here, the authors suggest the location of the kinin receptor is suitable for a role in ion and water regulation, as it is where the rectal papilla opens to the haemolymph. The location of the receptor could allow for the binding of neuropeptide ligands circulating in the haemolymph. In support of a role in hydromineral balance, a recent study demonstrated that treatment of the adult *A. aegypti* female rectum with a naturally occurring analog of *Aedes* kinin, Droso-leucokinin, led to a decrease in Na^+ absorption compared to untreated baseline control preparations (Lajevardi & Paluzzi, 2020). Relatedly, the observed RYa-1 immunoreactive staining throughout the medulla may indicate that this neuropeptide acts as a neurotransmitter by its release from associated nerve terminals affecting nearby cells of the rectal papillae. With the close association of the axon and rectal papillae, it is unlikely that the RYamides are released into the hemolymph in this region, ruling out the role of endocrine-mediated signaling of the rectal papillae by these neuropeptides. Release of RYa-1 would result in modulating activity within the medulla and potentially the ion and water reabsorptive properties of the rectal papillae (Phillips, 1970).

Ion transporters that have been identified in the adult *A. aegypti* rectal papillae are the apical, lumen-facing V-type H^+-ATPases and the basolateral, haemolymph-facing P-type Na^+/K^+-ATPases. Apical V-type H^+-ATPases of the ionoregulatory Malpighian tubules act as the driving force for transepithelial ion and water transport (Beyenbach et al., 2000; Patrick *et al.*, 2006). Studies on the influence of insect rectal papillae transport mechanisms are relatively limited compared to the extensive investigations on the Malpighian tubules (Nässel and Zandawala, 2019). An apical Cl^- pump of the rectal papillae was shown to be stimulated by cAMP in the desert locust *Schistocerca gregaria* (Phillips & Audsley, 1995). On the other hand, regulation of the hindgut has identified several neuropeptides that affect contractility, including CT/DH and leucokinin (Holman et al., 1991; Kwon and Pietrantonio, 2013). In addition, leucokinin hindgut signaling has been suggested to stimulate faeces excretion (Nässel and Zandawala, 2019).

Transcript Expression of RYa-1 and its Receptor

In addition to RYa-1 peptide immunolocalization, tissue and developmental transcript expression profiles of the RYamide gene and the now-validated *Aedes* RYamide receptor were measured. Various tissues were isolated from adult *A. aegypti*, including regions of the gut, and the three body segments. Removal of the guts leaves behind a thoracic and abdominal carcass containing the ventral nerve cord, ganglia, musculature, connective tissue, and cuticle. RYamide transcript expression was found in the abdominal carcass. The presence of the transcript indicates gene expression and the synthesis of RYamide neuropeptides. Synthesis of neuropeptides occurs in the nervous system including gene expression and peptide processing (Nässel, 2002); however, some neuropeptides are considered "brain-gut" as they can be also produced by enteroendocrine cells found in the midgut (Wegener and Veenstra, 2015). Therefore, transcript expression of neuropeptides would be localized to either nervous and/or gut tissue. Of the thoracic and abdominal carcasses, the remaining nervous tissue would be where RYamide gene expression and neuropeptide synthesis occur. In addition, the transcript expression in conjunction with immunoreactive staining of the RYa-1 peptide in the regions of the central and peripheral nervous system suggests that an RYamide is being synthesized and processed. The processing of RYamide-1 can involve post-translational modifications such as amidation and proteolytic cleavage of the preprohormone within nerve cells before release, which is a fundamental characteristic of neuropeptides (Predel et al., 2010). The RYamide preprohormone is then cleaved at mono and dibasic cleavage sites to form RYa-1 and RYa-2 (Collin et al., 2011). The presence of the neuropeptide in the terminal abdominal ganglia using immunohistochemistry corresponds to its potential control of the hindgut, as the terminal abdominal ganglia innervate the hindgut (Abou El Asrar et al., 2020). The RYa-1 immunoreactivity associated with direct innervation of the rectal papillae suggests the release of RYamide as a neurotransmitter, as opposed to potential endocrine signaling by circulating RYamides within the haemolymph. In addition, RYamide receptor transcript expression in the hindgut of adult mosquitoes further supports the involvement of these neuropeptides in the rectal papillae. It has been proposed that the low RYamide receptor gene expression in *Drosophila* is sufficient for signaling as there is direct innervation of the rectal papillae, leading to a localized area of high RYamide concentrations (Veenstra & Khamassi, 2017). Overall, with RYamide and receptor transcripts having been detected in all the developmental stages of *A. aegypti* tested in this study, albeit at low levels, the insect appears to require the continuous expression of the RYamide and its receptor gene throughout its life cycle. Interestingly, in regard to the mosquito reproductive systems, RYamide transcript was detected in the male reproductive organs, and immunofluorescence revealed staining in the seminal vesicles. The insect seminal vesicle is responsible for the nourishment and storage of spermatozoa (Avila et al., 2010). Furthermore, proteins released from the seminal vesicle are transferred to the female during copulation which is required for reproductive success (Carmel, et al., 2016). Studies on the hormonal control of the insect male reproductive system are limited. A gonadotropin found in the brain of the gypsy moth *Lymantria dispar*, termed “testes ecdysiotropin”, stimulated ecdysteroid production in adult male testes (Wagner et al., 1997). More recently, the neuropeptide allatotropin has been observed in the seminal vesicles using immunohistochemistry (Čižmár et al., 2019). Allatotropin was shown to modulate seminal vesicle contractions of the silkworm *Bombyx mori* at varying concentrations ex vivo. In addition, RNAi-mediated knockdown of LGR1, a receptor of the glycoprotein GPA2/GPB5, leads to a decrease in spermatozoa quantity and reproductive success (Rocco et al., 2019). LGR1 was also shown to be immunolocalized to developing spermatids in the male testes (Rocco et al., 2019). In the female reproductive system, results revealed only the detection of the RYamide receptor transcript. Spatial expression analysis of putative RYamide receptor BNGR-A19 transcript in *B. mori* revealed slight expression in the ovaries of fifth-instar larvae (Guo et al., 2021). Furthermore, based on the developmental expression profile 1-day-old and 4-day-old males expressed higher RYamide transcript compared to their female counterparts, with slight detection of the transcript in 1-day-old females, and none in 4-day-old females. However, transcriptome data indicates that the RYamide gene is expressed in the ovaries as well (Hixson et al., 2022).

The RYamide receptor transcript expression profile determined in this study revealed discrepancies compared to other studies. Although identified in the hindgut, transcript expression of the *Drosophila* RYamide receptor, CG5811, is absent in the brain. Transcriptome analysis of the CG5811 revealed remarkably low transcript expression in the brain (Chintapalli et al., 2007). An attempt to localize CG5811 transcript expression using the GAL4/UAS system did not identify any expression in the brain of adult *D. melanogaster* (Veenstra and Khammassi, 2017). Despite these observations, this present study revealed comparably high RYamide receptor transcript expression in the head region compared to other tissues/regions. This is also supported by recent transcriptome evidence that found high enrichment of the RYamide receptor in the brain (Hixson *et al.*, 2022). RYamide receptor transcript expression is expected in the brain, as implications of RYamides as neurotransmitters or neuromodulators, and RYamide immunoreactivity has been identified in the insect brain (Maeda et al., 2015) as also determined in the current study in adult *A. aegypti* mosquitoes. Another noteworthy result was the presence of low receptor transcript expression in the Malpighian tubules. Transcriptome analysis of adult *A. aegypti* Malpighian tubules supports this result, as the slight expression of the receptor transcript was detected (Li et al., 2017). Likewise, in adult *D. melanogaster* and *B. mori*, there is very low expression of the receptor transcript in the Malpighian tubules (Chintapalli et al., 2007; Guo *et al.*, 2021). Altogether, the RYamide receptor transcript was present in all developmental stages of the mosquito, thus highlighting the potential physiological importance of RYamide signaling despite low transcript abundance. Receptor transcript expression in the male reproductive system, along with RYa-1 immunostaining in the seminal vesicles suggests a potential role in male reproduction. To reiterate, the seminal vesicles can provide storage and nourishment for developing spermatozoa. With reproductive physiology, neuropeptides can regulate processes involved in protein synthesis, behavior, and muscle contraction associated with reproductive structures (Gäde and Hoffmann, 2005). The role of RYamides in the reproductive system of *A. aegypti* remains to be investigated.

RYamide Immunoreactivity in Midgut Enteroendocrine Cells and Processes Encircling the Pyloric Valve, and the Nervous System

The presence of RYa-1 immunoreactive staining and RYamide transcript expression in the midgut suggests its synthesis and release in this organ, which corresponds to RYamides functioning as a brain-gut peptide originating from the enteroendocrine cells. Brain-gut peptides are synthesized from both the nervous system and enteroendocrine cells of the midgut which have pleiotropic effects involving various physiological systems such as digestion, development, and diuresis (Wu et al., 2020). In *B. mori,* RYamide expression was identified, within EE cells of the anterior and posterior midgut during the larval stage by the utilization of *in situ* hybridization (Matsumoto et al., 2019; Roller et al., 2016). Furthermore, *Bombyx* RYamides were shown to decrease the contraction frequency of the larval pharynx (foregut) and ileum (hindgut) (Matsumoto et al., 2019). However, the RYamides were shown to decrease the contraction frequency of the pharynx, in fed larvae, while the contraction frequency of the pharynx in non-fed larvae was unaffected by the experimental addition of RYamide-1 and RYamide-2. Decreased RYamide receptor expression of the pharynx was observed in unfed larvae compared to fed *ad libitum* larvae. The inhibition of the hindgut contraction frequency is thought to transition the larvae from a feeding to non-feeding state, by the influence of RYamides from enteroendocrine cells to inhibit hindgut contractions. Furthermore, insensitivity of the pharynx after exposure to RYamides in non-fed larvae suggested that the RYamide-mediated feeding state transition is solely influenced by the hindgut. The role of these neuropeptides in the inhibition of feeding has been relatively well studied. In fact, the inhibitory effects of RYamides on feeding behavior have been identified in other insects including *D. melanogaster* (Ida et al., 2011), and the blowfly *Phormia regina* (Maeda et al., 2015). The neuropeptides have been shown to increase the sugar threshold needed to initiate feeding, thus reducing feeding motivation (Ida et al., 2011; Maeda et al., 2015). Altogether, considering their established roles in other insects, the presence of RYamides in the midgut of *A. aegypti* suggests a role in feeding and digestion. In addition, RYa-1 immunoreactive staining of enteroendocrine cells was more abundant in the anterior region of the posterior midgut. In the insect midgut, neuropeptide immunoreactivity of enteroendocrine cells has been identified to be non-uniform (Veenstra et al., 1995b). For example, immunostaining of RFamides is localized to the anterior midgut and the posterior region of the posterior midgut, while immunostaining of allatostatin is localized only within the posterior midgut (Veenstra et al., 1995b). In other insects, visualization of the RYamides in the midgut has revealed transcript expression in the enteroendocrine cells of the anterior midgut among larval *Drosophila virillis* (fruit fly) and larval *B. mori* using the GAL4/UAS system and *in-situ* hybridization, respectively (Roller et al., 2016; Veenstra and Khammassi, 2017). Furthermore, immunostaining of the RYamide preprohormone also exhibited localization in the anterior midgut of larval *D. melanogaster*. The non-uniform presence of neuropeptides in enteroendocrine cells of the midgut is thought to function in spatially-restricted paracrine signaling of nearby organs, and/or aid in digestion along the midgut (Wegener and Veenstra, 2015). Therefore, the identification of RYamides in various regions of the midgut suggests that the RYamide signaling and function involves the insect midgut. In addition, EE cells of the midgut can also be morphologically distinguished as open type or closed type (Yasuhisa and Junko, 1982). Open-type EE cells have their cytoplasm projected towards the lumen of the midgut, while closed-type EE cells do not have these projections and are found at the base of the epithelium (Roller et al., 2022). Here, the RYamide-1 immunoreactive cells of the midgut appear to be the closed type of EE cells as they do not possess any projections that are associated with the midgut lumen. Finally, the RYa-1 immunoreactive ring about the pyloric valve indicates a role in excretion by potentially modulating the entry of urine from the MT to the hindgut. A DH_{31}-like peptide has been shown to immunolocalize to the pyloric region in the large milkweed bug *Oncopeltus fasciatus*, which is a small region in between the midgut and hindgut where the Malpighian tubules are connected (Te Brugge and Orchard, 2008). Furthermore, DH_{31} stimulated the contraction of the region between the pyloric valve and ileum in adult *A. aegypti*, thus possibly aiding in the expulsion of fluids into the rectum(Kwon and Pietrantonio, 2013).

Immunoreactive staining of the nervous system revealed RYa-1 peptide presence in the protocerebrum and SEG of the brain and thoracic ganglia, as shown by both immunoreactive cells and processes. These immunoreactive cells suggest RYa-1 presence within neurosecretory cells. Particularly within the brain, there are visible processes that extend from these neurosecretory cells and possess varicose structures. These structures are indicative of arborizing neurosecretory cells that are immunoreactive to insect neuropeptides including corazonin (Nässel and Homberg, 2006). The immunoreactivity of the adult *A. aegypti* brain is similar to the RYamide immunoreactivity of the *P. regina* brain, where immunoreactive cell bodies were identified in the PI and SEG (Maeda et al., 2015). Compared to *A. aegypti*, the immunoreactive cells are more numerous in the protocerebrum and PI of the *P. regina* brain, while there are fewer immunoreactive cells in the SEG. Neurosecretory cells possess axonal terminations within neurohemal organs that can release contents into the insect haemolymph, while their arborizations can have interneuronal roles (Nässel and Homberg, 2006). Together, RYa-1 presence within neurosecretory cells and their processes suggests a neuromodulatory role of neural circuits, which thus far has been proposed in the desensitization of the sugar receptor through an indirect effect by RYa-1 and RYa-2 in *P. regina* (Maeda et al., 2015). RYa-1 immunostaining of the adult male *P. regina* brain also revealed similar regions of immunoreactivity, including the protocerebrum, and SEG (Maeda et al., 2015). The neurosecretory cells of the PI are considered medial neurosecretory cells (MNSCs) where various neuropeptides have been identified by immunohistochemistry and/or peptidomics in these cells (Strand et al., 2016). The insect NPY homolog, NPF has been identified in the

MNSCs, neurosecretory cells of the SEG, and enteroendocrine cells of the midgut in *A. aegypti,* and *D. melanogaster* (Brown et al., 1999; Stanek et al., 2002). Furthermore, NPF immunolocalization was identified in a pair of neurosecretory cells within the abdominal ganglia, though only in the corn earworm *Helicoverpa zea* and blood-sucking bug *Rhodnius prolixus* (Gonzalez and Orchard, 2008; Huang et al., 2011). With a similar immunolocalization pattern as the RYamides, barring the thoracic ganglia, perhaps the functional role of NPF can help elucidate the role of RYamides. Thus far, insect NPFs have been suggested to function in feeding and reproductive behaviors, and stress (Wu *et al.*, 2003; Lee et al., 2006; Dierick and Greenspan, 2007; Xu et al., 2010). Furthermore, the FMRFamides possess immunoreactive neurosecretory cells within the same regions of the nervous system as the RYamides, including PI, SEG, thoracic ganglia, and the abdominal ganglia in adult *A. aegypti* (Brown and Lea, 1988). The biological effects of FMRFamides involve the regulation of heart, gut, and skeletal muscle contractility in *D. melanogaster* (Hewes *et al.*, 1998; Johnson et al., 2000; Kaminski *et al.*, 2002). With the immunoreactivity of RYamides found in various physiological systems of the *A. aegypti* that is comparable to other neuropeptides with pleiotropic functions, then perhaps the RYamide neuropeptides regulate a multitude of different physiological systems, including digestion, reproduction, and behavior. However, identifying the sites of action by RYamides using immunolocalization can be misleading as RYamides immunoreactivity has been shown to cross-react with similarly structured RFamides and NPF, and thus should only act as a starting point for future physiological studies (Veenstra and Khammassi, 2017; Veenstra and Schooneveld, 1984).

RYamide Receptor Deorphanization by Heterologous Functional Assay

Characterization of an orphan *Aedes* receptor revealed high specificity to the *Aedes* RYamides. Initially, the heterologous expression of the RYamide receptor using two mammalian expression constructs revealed greater receptor activation by RYamides with the pcDNA construct compared to the pBud construct. The pBud construct allows for GPCRs to elicit Ca^{2+} mobilization that does not normally utilize Ca2+, through the coupling with the promiscuous G-protein, Gα15 (Offermanns and Simon, 1995). Detection of luminescence in the utilized heterologous assay is possible following receptor activation that is linked to GPCR signal transduction involving intracellular Ca^{2+} mobilization. Specifically, the CHO-K1 cell lines used in this study stably express aequorin (Paluzzi et al., 2012), which allowed for the detection and quantification of GPCR-mediated calcium release by capturing the luminescence emitted by calcium-bound aequorin. A greater response by the pcDNA-receptor construct compared to the pBud-receptor construct suggests that the RYamide signaling normally involves intracellular Ca^{2+} mobilization. Indeed, the activation by RYamides of the putative RYamide receptor in *B. mori*, BNGR-A19 evoked Ca^{2+} mobilization when expressed in the HEK293T (human embryonic kidney) and SF9 (ovarian cells of the fall armyworm, *Spodoptera frugiperda*) without the use of the promiscuous G-protein Gα15(Guo et al., 2021). Furthermore, the utilization of calcium chelators BAPTA and EGTA to sequester intracellular Ca^{2+} led to a decrease in Ca^{2+} mobilization upon BNGR-A19 activation (Guo et al., 2021). Here, BNGR-A19 activation was shown to involve binding of the G_q protein, and subsequent ERK1/2 mediated Ca^{2+} mobilization. The RYamide receptor had higher sensitivity to RYamide-1 compared to RYamide-2 with an approximate 2.5x lower EC_{50}. The peptide sequences of the two *Aedes* RYamides contain subtle differences; RYamide-1 and RYamide-2 have eight and ten residues, respectively. They share a consensus sequence of FFXGSRYamide, where X is either a valine (RYa-1) or leucine (RYa-2). In *Aedes* RYamide-2, the RYamide consensus sequence is flanked by three additional N-terminal residues, whereas *Aedes* RYamide-1 contains only one additional N-terminal residue to this consensus sequence (Table 1). It has been shown that the two *Drosophila* RYamides activated the *Drosophila* RYamide receptor similarly based on near-identical EC_{50} values of 1 nM (Collin et al., 2011). However, the *Tribolium casteneum* (TC) RYamide receptor showed slightly higher sensitivity to *Tribolium* RYamide-2 (EC_{50}: 0.8 nM) than *Tribolium* RYamide-1 (5 nM) (Collin et al., 2011). The *Tribolium* RYamide-1 sequence is the most distinct RYamide between the three insects, as it only shares the first phenylalanine and C-terminus RYamide of the consensus sequence, FFXGSRYamide. Meanwhile, the peptide sequence of TC-RYa-2 shares the exact residues as *Aedes* and *Drosophila* RYamide-2 from position 4 to 10, although the three flanking residues are different for each species. Thus, it appears that the putative insect RYamide receptors are sensitive to the RYamide peptide FFXGSRYamide consensus sequence. Furthermore, the current data indicated that the RFamides were unable to activate the *Aedes* RYamide receptor, despite having a similar C-terminus sequence. Thus, the RYamide C-terminal motif alone may be less important in binding specificity with the receptor.

Conclusion

The physiological relevance of the recently identified RYamide neuropeptide family has thus far indicated a role in feeding among various insects. In this study, we have further established a basis for the physiological roles of these neuropeptides in the mosquito, *A. aegypti*. Immunofluorescence revealed the RYamide-1 peptide present in the brain and throughout the ventral nerve cord. Furthermore, RYa-1 was identified in the axonal projections from the terminal abdominal ganglia that innervate the hindgut and in particular, the rectal papillae.

Beyond the nervous system, RYamide immunoreactivity was also revealed in the posterior midgut, rectal papillae, and seminal vesicle of the male reproductive system. The tissue and developmental expression profiles of RYamide and its receptor transcripts were determined; however, with the exception of RYamide transcript enrichment in the brain, no significant enrichment was observed. However, trends in the expression profile indicate the RYamide transcript is more abundant in 4-day-old adult males while the RYamide receptor transcript was elevated in 1-day-old males where it appears upregulated in the brain. These results suggest a wide range of physiological functions of the RYamides as the associated transcripts are found in different tissues. The observed transcript expression of the RYamide receptor in the hindgut of adult *A. aegypti,* and immunofluorescence of the RYa-1 peptide within the rectal papillae, together suggest a role for this signaling system in ion and water reabsorption. RYamides have been involved in modulating feeding behavior among other insects including *D. melanogaster* and *P. regina*. Here, RYa-1 immunofluorescence in potential enteroendocrine cells was also observed within the anterior region of the posterior midgut and, along with the identification of RYamide and its receptor transcript expression in the midgut, infers a role in feeding and/or digestion in the mosquito, *A. aegypti*. A role in reproduction has also been suggested through transcript expression data, along with immunofluorescence in the male seminal vesicles. It appears that the RYamides have the potential to regulate multiple physiological systems as suggested by the presence of RYamide and receptor transcript along with the RYamide-1 peptide found in various tissues. In addition, I have cloned and identified an *Aedes* receptor that is specifically activated by the *Aedes* RYamide neuropeptides. Despite these results, limitations of the experimentation are present. The small sample size for the transcript expression profiles of the RYamide gene and receptor may be an inaccurate representation of the actual transcript expression within the mosquito. Additionally, the reported low expression of the *Drosophila* RYamide gene (Veenstra and Khammassi, 2017) and the observed low expression of the gene in multiple tissues and developmental stages in this study may have further led to misleading results. Another limitation of the tissue and developmental transcript profiles is the lack of separation between the male and female tissue and developmental samples, thus disregarding any differences in expression between the sexes. Regarding the heterologous functional assay, the receptor was transiently expressed rather than stably expressed. As a result, differences in luminescence responses elicited by the neuropeptides may be a result of variation in receptor expression, rather than actual receptor specificity. Therefore, future experimentation on the insect RYamides can address these limitations, and investigate these neuropeptides in greater detail. This can include studying the potential pleiotropic effects of RYamide neuropeptides, which has the potential to deepen our understanding of neuropeptide regulation on various insect physiological systems, thereby providing a foundation for designing novel pest control strategies that could target and disrupt these potentially critical regulatory mechanisms.

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