FUNCTIONAL CHARACTERIZATION OF PYROKININS AND THEIR RECEPTORS IN THE ADULT MOSQUITO, *AEDES AEGYPTI*

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ABSTRACT

The mosquito *Aedes aegypti* is a common disease vector that continues to cause devastating harm to human populations. Mosquitoes owe much of their success to their excretory system, comprised of the Malpighian tubules and hindgut, which play important roles when faced with environmental and dietary challenges. Pyrokinins are a family of neuropeptides initially identified based on their ability to regulate hindgut physiology of other insects. In light of this knowledge, this thesis aimed to examine whether pyrokinins have similar roles in *A. aegypti*. Pyrokinin receptors were functionally characterized and receptor transcript expression profiles were determined to confirm the hindgut as a target organ for these neuropeptides. A pyrokinin-2 family member was found to reduce contractile activity of the anterior hindgut, which represents the first role established for pyrokinins in any blood-feeding arthropod. These findings suggest the importance of this neuropeptide family in regulating critical processes in this disease vector.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine
ADF	anti-diuretic factor
*Ae*KR	*Aedes aegypti* kinin receptor
AQP	aquaporin
ASET	Automated Scanning Electrode Technique
AST	allatostatin
AT	allatotropin
BSA	bovine serum albumin
C	carboxyl
CA	corpora allata
cAMP	cyclic adenosine-3',5'-monophosphate
capa	*capability*
CC	corpora cardiaca
cDNA	complimentary cDNA
CHO	chinese hamster ovary
CTSH	chloride transport stimulating hormone
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DH	diapause hormone
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
GPA2	glycoprotein alpha 2
GPB5	glycoprotein beta 5
GPCR	G protein-coupled receptor
HRP	horseradish peroxidase
Ile	ileum
IP_3	inositol trisphosphate
ITP	ion transport peptide
MG	midgut
MIP	myoinhibiting peptide
MTs	Malpighian tubules
N	amino

NMU	neuromedin U
NSC	neurosecretory cell
NSS	normal sheep serum
OL	optic lobe
PBAN	pheromone biosynthesis activating neuropeptide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pGlu	pyroglutamate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PK	pyrokinin
PK-R	pyrokinin receptor
PK1	pyrokinin-1
PK1-R/DH-R	PK1 receptor
PK2	pyrokinin-2
PK2-R/PBAN-R	PK2 receptor
PKC	protein kinase C
PLC	phospholipase C
PV	pyloric valve
PVK	periviscerokinin
PVO	perivisceral organ
Rec	rectum
RNA	ribonucleic acid
rp49	ribosomal protein 49
Rt	reproductive tissues
RT-PCR	reverse transcription PCR
RT-qPCR	quantitative reverse transcription PCR
SEG	subesophageal ganglion
SEM	standard error of the mean
SIET	Scanning Ion-selective Electrode Technique
TMD	transmembrane domain
TPK	tryptopyrokinin
VNC	ventral nerve cord
WB	whole body

STATEMENT OF CONTRIBUTIONS

Chapter One

This chapter was written by A. Lajevardi with guidance and editorial support from Dr. J.-P. Paluzzi.

Chapter Two

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

CHAPTER I:
GENERAL INTRODUCTION – THE INSECT ALIMENTARY CANAL AND PYROKININ NEUROPEPTIDES

1.1 *Aedes aegypti* mosquitoes

Mosquito-borne diseases account for a significant number of human illnesses and deaths annually (Omodior et al., 2018). Disease transmission occurs when the mosquito ingests pathogens from an infected host during a bloodmeal and transmits these into its subsequent host (Kuno and Chang, 2005). *Aedes aegypti* mosquitoes are well-known vectors that spread viruses causing yellow fever, dengue, Zika and chikungunya (Kotsakiozi et al., 2017). Dengue being the most prominent of these diseases, is known to affect approximately 50-100 million people annually (Price et al., 2015). Spreading from its origin in Africa to most tropical and subtropical regions of the world, over half of the world's population currently lives in areas where these mosquitoes are present (Brown et al., 2014; Tam et al., 2016). Population growth, and the rising risk of importation through increased international trade, travel and urbanization all contribute to the global spread and persistence of these vectors (Neiderud, 2015). Since they are highly anthropophilic and thrive in close proximity to humans, *A. aegypti* has become extremely efficient in transmitting arthropod-borne viruses (Powell and Tabachnick, 2013). With these diseases on the rise worldwide and the little progress made in vaccine development to date (Manning et al., 2018), effective control mechanisms are required to reduce the risk of local and global disease transmission by this important vector. Implementing these measures, however, requires a thorough understanding of mosquito biology. The draft *A. aegypti* genome has been available for over a decade (Nene et al., 2007), serving as an invaluable resource to elucidate the interactions between mosquitoes and the pathogens they transmit at the molecular level, and facilitating the identification of various signaling pathways that are critical for mosquito survival

and reproduction. Characterizing these pathways has therefore become increasingly important to develop effective strategies that help control mosquito populations.

1.2 Excretory system

The insect excretory system plays a vital role in maintaining the hydromineral balance of the haemolymph, circulatory fluid analogous to the blood of vertebrates (Christophers, 1960). It includes the Malpighian tubules (MTs) and the hindgut. In mosquitoes, each of the five MTs are composed of a closed distal region floating freely in the haemolymph, and a proximal segment that opens into the alimentary canal at the midgut-hindgut junction, known as the pyloric valve region (Bradley, 1987). The hindgut is the final segment of the alimentary canal. It is composed of a narrow anterior tube, known as the ileum, leading posteriorly into the bulbous rectum. One of its primary roles, together with the help of the MTs, is to maintain haemolymph homeostasis through osmo- and ionoregulatory processes, which is important under a range of different conditions. Unfed adult mosquitoes, for instance, must conserve water to maintain a stable haemolymph volume and ion levels in order to avoid desiccation (Beyenbach, 2003). Ingesting a sugar meal poses a different challenge where mosquitoes must secrete the excess water load from their haemolymph. Similarly, female mosquitoes engorging a bloodmeal must rapidly eliminate the excess water and salt that is absorbed by the midgut into the haemolymph (Williams et al., 1983; Bradley, 1987; Clements, 2000). This load must be secreted by the MTs and transported to the hindgut for waste elimination (Bradley, 1987).

The movement of water and ions between the haemolymph and tubule lumen is facilitated through channels and transporters located along MT cell membranes, as well as septate junctions between these cells that permit paracellular transport (Beyenbach, 2003). At the distal region, primary urine is produced through the active transport of Na^+ and K^+ into the

tubule lumen, which establishes a lumen-positive transepithelial potential that drives passive Cl^- secretion (Williams and Beyenbach, 1984). The entry of NaCl and KCl creates an osmotic gradient, driving water into the lumen through selective aquaporin (AQP) channels (Klowden, 2013). The fluid and salts secreted by the MTs make up the primary urine that is then delivered to the hindgut for continued reabsorption (Phillips et al., 1996; Spring and Albarwani, 1993).

1.3 Mosquito hindgut

1.3.1 Ion and water transport

The hindgut serves as the final site for modifying urine before excretion (Coast, 2007). Although specific mechanisms for ion and fluid transport have been reported in the MTs, there are limited studies examining these processes in the hindgut of most insects. In the locust, the ileum has been identified as an important organ involved in active Na^+ and Cl^-, and passive fluid reabsorption (Phillips et al., 1988; Phillips et al., 1994). This organ is also involved in maintaining acid-base balance by reabsorbing $HCO3^-$ and secreting H^+ into the lumen (Phillips et al., 1994). Some ion transporters have also been immunolocalized along the hindgut of *A. aegypti*. The ileum expresses V-type and P-type ATPases on their apical and basolateral membranes, respectively, important for ion transport during excretion (Patrick et al., 2006). The expression of AQPs 1, 2 and 5 have also been detected in this region, supporting the involvement of this organ in osmoregulation. During post-prandial diuresis, excess water must be secreted and eliminated from the mosquito. To limit water reabsorption during this time, expression of these AQPs must be downregulated, as demonstrated by Drake et al. (2015) in mosquitoes 24-hours following a bloodmeal. They also examined that knocking down these three AQPs enhances desiccation resistance, further supporting their role in regulating water homeostasis (Drake et al., 2015).

The mosquito rectum serves as the final site for the reabsorption of ions, water and metabolites, ultimately determining the composition of excreted matter (Brusca and Brusca 2003; Hopkins, 1967). It consists of a thin-walled rectal sac in which six rectal pads in females and four in males, composed of simple columnar cells, protrude from the epithelium wall into the rectal lumen, serving as a site for absorption (Clements 2000; Hopkins, 1967; Tongu et al., 1969; Patrick et al., 2006). The rectal pad membranes undergo significant structural changes following a blood meal, indicative of enhanced ion and water absorption (Hopkins, 1967; Hanrahan and Phillips, 1983). Their involvement in these processes was further supported through ion transporter localization, revealing Na^+/K^+-ATPase expression on the basolateral infolding of the rectal pads, which could drive ion and ultimately water absorption (Patrick et al., 2006). Although the exact mechanisms of transcellular and paracellular transport along the rectal pads have not yet been characterized in the mosquito, similar structures have been identified in other insects and shown to play critical roles in these transport processes. For example, in the locust rectum, cation reabsorption is coupled to electrogenic Cl^- transport, which drives fluid absorption through osmosis (Phillips, 1964; Phillips, 1981; Hanrahan and Phillips, 1983). The majority of fluid and solute reabsorption is thought to occur in this organ, creating large ionic and osmotic concentration differences between urine and haemolymph composition. Finally, urine is eliminated through the anal canal, which is achieved by the ability of the hindgut to produce spontaneous and hormonally-regulated contractions (Coast, 2007; Drake et al., 2010).

1.3.2 Motility

Gut motility enables the movement of food and secreted waste through the alimentary canal (Day and Powning, 1949; Day, 1954). This is regulated by a network of visceral muscles along the length of the canal arranged as bands of circular and longitudinal muscle surrounding

the gut epithelium (Cameron, 1912). Insect visceral muscles are striated, resembling the skeletal muscles of vertebrates (Davey, 1964). They provide the force for peristaltic movements, propelling gut contents posteriorly along the foregut and midgut, which secretes digestive enzymes to aid in food breakdown. Finally, undigested foodstuff and non-absorbable products of digestion from the midgut, along with primary urine secreted by the MTs, are passed through the pyloric valve and delivered to the hindgut (Beyenbach, 2003).

Spontaneous hindgut contractions occur as peristaltic waves initiating at the pyloric valve. These waves are passed posteriorly over the ileum through the coordinated contraction of circular and longitudinal muscles that surround the basal surface of epithelial cells (Odland and Jones, 1975). Similarly, the rectum is composed of an extensive musculature network (Rocco et al., 2017), important to propel waste through the anal canal during excretion. Although hindgut motility is myogenic, requiring extracellular Ca^{2+} to produce spontaneous contractions, these processes can also be regulated neuronally (Cook and Holman, 1985; Robertson et al., 2012). This becomes extremely important after feeding, as the animal relies on these contractions to expel contents of the alimentary canal and restore haemolymph balance (Te Brugge et al., 2008).

1.4 Neuroendocrine regulation of the hindgut

The regulation of insect gut physiology is coordinated by the nervous and endocrine systems. The neuroendocrine system synthesizes signaling molecules within neurosecretory cells (NSCs), which are sent to neurohaemal organs where they are released to exert their effects at either local or distal sites (Hartenstein, 2006). One important group of signaling molecules are neuropeptides. These oligopeptides, characterized to normally contain between 5-50 amino acid residues, are the most structurally and functionally diverse class of neuroactive substances (Hoyer and Bartfai, 2012; Nässel, 2002). Synthesized as larger precursors (prepropeptides), they

become processed, stored and released from the nervous system and bind to their cognate receptors to exert their effects, acting as neurotransmitters, neurohormones, and/or neuromodulators (Hoyer and Bartfai, 2012; Nässel, 2002). Neurotransmitters relay signals between axons by binding to receptors on a postsynaptic neuronal, muscle or other effector cell to produce a rapid and transient response (Nässel, 2002; Mercier et al., 2007). Instead, neurohormonal signal transmission is slower, as hormones are secreted into the haemolymph to mediate their effects at peripheral targets (Nässel, 2002). Lastly, neuromodulators may act either synaptically or non-synaptically to regulate extrinsic signals (Katz and Frost, 1996).

The insect neurosecretory system consists of NSCs within the brain and subesophageal ganglion (SEG), which are fused together in mosquitoes, the ventral nerve cord (VNC) housing abdominal ganglia, and the ganglia of the stomatogastric nervous system (Hartenstein, 2006). NSCs project their axons to innervate neurohaemal organs, such as the corpora cardiaca (CC), corpora allata (CA) and abdominal perivisceral organs (PVOs) to release neuroactive substances into the haemolymph (Bräunig, 1987; Nässel, 1996; Hartenstein, 2006).

1.4.1 Regulation of hindgut motility

Neuronal control of hindgut myoactivity was initially reported in the cockroach, *Periplaneta americana*. Cameron (1953) found that homogenates of the CC stimulate the hindgut. The hindgut of these insects is composed of an ileum and rectum, as described above, in addition to a region separating the two, known as the colon (Davey, 1962). Preliminary observations proposed that the CC acts on argentaffin cells within the colon, not found in the ileum or rectum, that secrete amines to modulate these effects (Davey, 1962). Koller (1954; cited in Davey, 1964) also demonstrated the ability of homogenates of the CA to stimulate hindgut contractions, whereas extracts of the SEG only increased motility at low doses but inhibited

these actions at high doses. Motor innervation of muscle fibres along the hindgut further supported the regulation of these processes by the nervous system (Brown and Nagai, 1969).

Neuroendocrine regulation of gut motility has been more recently examined in *Locusta migratoria*. Contractile activity of the hindgut is modulated through innervation from the terminal abdominal ganglion of the VNC (Huddart and Oldfield, 1982; Donini et al., 2002; Robertson et al., 2014). As a result of these findings, further studies were conducted to elucidate insect neuropeptides exhibiting myotropic activity (Schoofs et al., 1991a, b), whereby these factors can either stimulate or inhibit the activity of the hindgut.

Various neuropeptides have already been identified to elicit myotropic actions on the hindgut, with early studies examining large model species, such as the locust and cockroach. The allatostatin (AST) family (previously known as A-type ASTs) was initially isolated from the cockroach *Diploptera punctata* and characterized to inhibit juvenile hormone biosynthesis (Weaver et al., 1995). It has since been recognized as having other physiological roles. AST-like processes detected along the VNC innervate the hindgut, and AST-related peptides inhibit both spontaneous and proctolin-induced hindgut contractions in the larval midge, *Chironomus riparius* (Lange et al., 1995; Robertson et al., 2014). The myoinhibitory peptide (MIP) family has also been implicated in similar inhibitory actions along the hindgut of *L. migratoria*, the kissing bug, *Rhodnius prolixus* and the deer tick, *Ixodes scapularis* (Schoofs et al., 1991a; Lange et al., 2012; Šimo and Park, 2014), which may be important during fasting states to slow down gut transit.

Another neuropeptide from the allatoregulatory family, allatotropin (AT), was found to exert opposite effects. Initially isolated from *Manduca sexta*, AT is secreted by both the nervous system as well as endocrine epithelial cells for myoregulation and cardioacceleration in various

insects, including *M. sexta* and *Triatoma infestans* (Kataoka et al., 1989; Riccillo and Ronderos, 2010). Myostimulatory actions of AT was also observed by Paemen et al., (1991) in the locust hindgut, and similarly, secretion of an AT-like peptide by the MTs of the haematophagous insect *T. infestans* also resulted in stimulation of peristaltic hindgut contractions (Santini and Ronderos, 2007).

A functional link between the diuretic response by the MTs and hindgut motility is further supported, as diuretic hormones released from NSCs, which are characterized by their ability to stimulate fluid secretion by the MTs, have also been found to exhibit myoactivity. For example, diuretic hormone 31, otherwise known as the mosquito natriuretic factor, not only participates in post-prandial diuresis by the MTs, but also increases contraction frequency in isolated *A. aegypti* hindguts to aid in urine expulsion (Kwon and Pietrantonio, 2013). Serotonin (5-hydroxytryptamine, 5-HT), an endogoneous *A. aegypti* neurohormone, neurotransmitter and neuromodulator that increases fluid secretion across the MTs (Sajadi et al., 2018), was also found to mediate similar actions. Specifically, all regions of the gut in *A. aegypti* receive extensive serotonergic input from central neurons that project through axons onto the gut (Moffett and Moffett, 2005). Messer and Brown (1995) observed more rapid hindgut contractions in response to 5-HT, which helps to prevent backflow of fluid into the midgut. These results support the importance of functional coordination between the MTs and hindgut during events such as post-prandial diuresis in insects requiring rapid waste elimination.

1.4.2 Regulation of hindgut ion and water transport

Primary urine produced by the MTs is modified by reabsorptive processes in the hindgut. The regulation of these mechanisms by peptide hormones is important when insects face environmental challenges. The influence of anti-diuretic factors (ADFs) on the hindgut was

initially examined in the desert locust, *Schistocerca gregaria* (Phillips 1964). The ion transport peptide (ITP) and Cl^- transport stimulating hormone (CTSH) are two main ADFs known to regulate hindgut reabsorption in the locust (Phillips and Audsley, 1995). With their receptors situated on the basolateral membrane of epithelial cells, ITP mediates ileal reabsorption by increasing intracellular cyclic adenosine-3',5'-monophosphate (cAMP) levels. This in turn stimulates Na^+, K^+ and Cl^- entry across the apical membrane. The apical Cl^- pump is electrically coupled to K^+ to elicit passive K^+ reabsorption into the haemolymph (Phillips et al., 1986; Audsley et al., 2013). This in turn creates an osmotic gradient, which promotes water reabsorption (Phillips, 1981). Similar reabsorptive processes along the locust rectum have been shown to be regulated by CTSH. Binding to receptors on the rectal pad epithelia, CTSH also elevates cAMP levels to stimulate K^+, Cl^-, and ultimately fluid reabsorption (Hanrahan and Phillips, 1983; Phillips and Audsley, 1995; Phillips et al., 1988).

Recently, a relatively novel glycoprotein hormone (GPA2/GPB5) was proposed to be involved in iono- and osmoregulatory processes along the hindgut of the fruit fly, *Drosophila melanogaster* and the mosquito, *A. aegypti* (Sellami et al., 2011; Paluzzi et al., 2014). Receptor transcript expression revealed the hindgut as a potential target for its peptidergic ligand (Paluzzi et al., 2014), which was localized to hindgut epithelia (Sellami et al., 2011). Similar to the actions of ITP and CTSH in the locust, GPA2/GPB5 has also been suggested to exert anti-diuretic effects in *D. melanogaster* and *A. aegypti* (Sellami et al., 2011; Paluzzi et al., 2014). Paluzzi et al. (2014) demonstrated that recombinant *A. aegypti* GPA2/GPB5 reduced lumen-directed Na^+ flux (secretion) by the ileum, promoted haemolymph-directed Na^+ flux (absorption) by the rectum, and decreased K^+ absorption across the entire hindgut. These findings suggest that GPA2/GPB5 inhibits natriuresis (Na^+ secretion), and promotes kaliuresis (K^+ secretion),

revealing this glycoprotein hormone as the first apparent anti-diuretic factor of the mosquito hindgut (Paluzzi et al., 2014).

Investigating these regulatory mechanisms provides insight into how mosquitoes adapt upon facing challenges, such as desiccation stress, engorging a bloodmeal, or emerging as adults, all of which are important for understanding how mosquitoes restore haemolymph balance and maintain organismal functioning. Unfortunately, research examining how these processes are regulated by neuroendocrine factors in the mosquito hindgut remains limited. Therefore, it is important to study how neuroendocrine signaling regulates hindgut physiology, including both ion and water transport as well as motility, as these processes typically work in concert with one another.

1.5 Pyrokinin neuropeptides

One group of insect neuropeptides known to influence hindgut physiology is the pyrokinin (PK) family. Members of this family have been identified in various insects based on their FXPRL-NH_2 carboxyl (C) terminus. They include myotropins, pheromone biosynthesis activating neuropeptides (PBANs), diapause hormones (DHs), and beta- and gamma-subesophageal neuropeptides. The first pyrokinin neuropeptide was characterized as having myostimulatory actions on the hindgut of the cockroach, *Leucophaea maderae* (Holman et al., 1986). It was termed leucopyrokinin due to the pyroglutamate (pGlu) residue blocking the amino (N) terminus (Holman et al., 1986). However, it was soon shown that the pGlu is in fact not critical for eliciting these myotropic roles (Nachman et al., 1986). Other peptides sharing this C-terminus demonstrated similar roles in stimulating hindgut motility in the migratory locust, *L. migratoria*, and were originally identified as myotropins (Schoofs et al., 1993b), although falling within the large PK family. Soon after the discovery of leucopyrokinin, another peptide with a

similar carboxyl motif was shown to induce pheromone biosynthesis in the corn earworm, *Helicoverpa zea*. This led to the characterization of pheromone biosynthesis activating neuropeptide (PBAN; Raina and Klun, 1984; Raina et al., 1989). Although originally characterized as myotropic and pheromonotropic, these neuropeptides were soon found to mediate a plethora of physiological actions, summarized in Table 1-1.

Table 1-1: Overview of identified roles for members of the pyrokinin neuropeptide family.

Subgroup	Role	Organism(s)	Source
PK2/PBAN[a] FXPRL-NH_2	Stimulates hindgut motility	*Leucophaea maderae* *Periplaneta americana* *Zophobas atratus* *Tenebrio molitor*	Holman et al., 1986; Predel and Nachman, 2001; Marciniak et al., 2012
	Stimulates oviduct motility	*Locusta migratoria* *Periplaneta americana* *Zophobas atratus* *Tenebrio molitor*	Schoofs et al., 1993a; Predel and Nachman, 2001; Marciniak et al., 2012
	Influences heartbeat rhythm	*Zophobas atratus*	Marciniak et al., 2012
	Activates sex pheromone biosynthesis	*Helicoverpa zea* *Helicoverpa armigera*	Raina et al., 1989 Rafaeli et al., 1990
	Induces embryonic diapause	*Bombyx mori* *Orgyia thyellina*	Imai et al., 1991; Uehara et al., 2011
	Terminates pupal diapause	*Helicoverpa armigera*	Sun et al., 2003; Zhang et al., 2004
	Stimulates larval cuticle melanization	*Leucania separata* *Spodoptera littoralis*	Matsumoto et al., 1990; Altstein et al., 1996
	Accelerates pupariation	*Sarcophaga bullata*	Zdarek et al., 1997; Verleyen et al., 2004
PK1/DH[a]/TPK[a] WFGPRL-NH_2	Induces embryonic diapause	*Bombyx mori* *Orgyia thyellina* *Locusta migratoria*	Yamashita, 1996; Uehara et al., 2011; Hao et al., 2019
	Terminates pupal diapause	*Heliothis virescens* *Helicoverpa zea*	Xu and Denlinger, 2003; Zhang et al., 2008
	Promotes ovarian development	*Orgyia thyellina*	Uehara et al., 2011

[a] abbreviations: (PBAN) pheromone biosynthesis activating neuropeptide, (DH) diapause hormone, (TPK) tryptopyrokinin

Another PK type was later characterized by its highly-conserved C-terminal WFGPRL-NH_2 motif. Its role in eliciting embryonic diapause in the silkworm *Bombyx mori* led to its identification and nomenclature as the diapause hormone (DH; Yamashita, 1996). Their involvement in regulating diapause extends to other moths (Xu and Denlinger, 2003; Zhang et al., 2008), as well as *L. migratoria* (Hao et al., 2019). To date, peptides possessing this conserved motif have been identified in various insects, including Dipterans (Predel et al., 2004; Predel et al., 2010). However, studies examining biological roles of this peptide subclass remain limited. Functions of PKs have been primarily studied in lepidopterans over the past several decades. However, more recent studies have shifted towards understanding their roles in other insects (Table 1-1). These pleiotropic neuropeptides regulate an array of physiological processes, with some functional overlap between the two subtypes (Table 1-1).

The DH-related neuropeptides possessing a WFGPRL-NH_2 carboxyl terminus, also commonly referred to as tryptopyrokinins (TPK; Veenstra, 2014), are classified as the pyrokinin-1 (PK1) type, while PBAN-related neuropeptides, characterized by their pentapeptide core motif, are considered to be the pyrokinin-2 (PK2) type (Jurenka, 2015; Ahn et al., 2018). These peptides are processed from one of two genes in insects: *pban* (or *hugin*) and *capability* (*capa*). The *pban* gene was first isolated from *H. zea* (Raina et al., 1989), characterized in *B. mori* (Kawano et al., 1992; Sato et al., 1993), and encodes both PK1 and PK2 peptides. This gene has been identified in several insects, however the processing of the peptides slightly differs between insects. In *D. melanogaster*, a homologous gene to *pban*, called *hugin* (Meng et. al., 2002), only encodes two PK2 peptides (Nässel and Winther, 2010).

The *capa* gene was first characterized in *D. melanogaster* (Kean et al., 2002; Baggerman et al., 2002). Homologous *capa* genes were subsequently identified across various insect groups (Loi and Tublitz, 2004; Paluzzi et al., 2008). They encode two CAPA or periviscerokinins (PVK-1 and PVK-2), which have been extensively studied in several insects, playing a role in regulating fluid secretion across the MTs (Kean et al., 2002; Pollock et al., 2004; Terhzaz et al., 2012; Sajadi et al., 2018). Although derived from a common precursor, PVKs do not belong to the PK family, as they are distinguished by their PRV-NH_2 motif (Predel et al., 2010). In addition to PVKs, the *capa* gene also typically gives rise to one PK1 peptide. One such exception is found within the European honey bee, *Apis mellifera*, whereby PK1 is encoded only by *hugin* (Jurenka and Nusawardani, 2011). In contrast, some insects, including *D. melanogaster* and *R. prolixus*, use *capa* as the sole source of PK1, as *hugin* strictly encodes PK2 in these organisms (Paluzzi et al., 2008; Paluzzi and Orchard, 2010; Jurenka and Nusawardani, 2011; Nässel and Winther, 2010). Although *hugin*-derived PK1 (also known as DH-2) has been shown to regulate diapause in some insects (Yamashita, 1996; Xu and Denlinger, 2003; Hao et al., 2019), involvement of *capa*-derived PK1 (also known as DH-1) in these processes remains unknown. Recently, the *A. aegypti capa* (*AAEL005444*) and *hugin* (*AAEL012060*) genes were identified (Jurenka and Nusawardani, 2011; Strand et al., 2016). Processing of these peptide precursors is demonstrated in Fig. 1-1, based on sequences identified using peptidomic analyses (Predel et al., 2010). In *A. aegypti*, the *hugin* gene encodes three PK2 isoforms, one of which lacks the typical FXPRL-NH_2 sequence and is rendered non-functional (Choi et al., 2013).

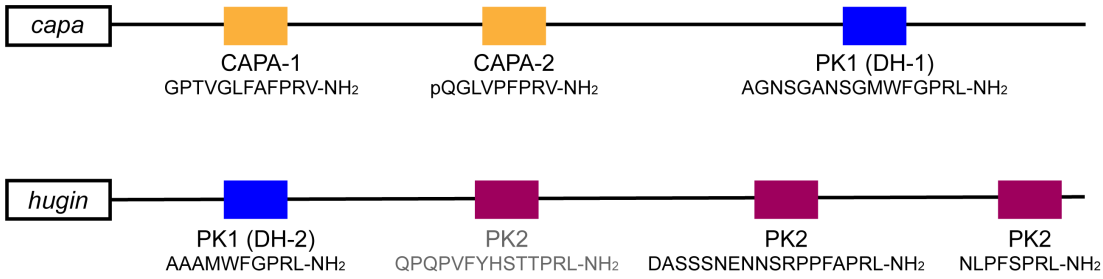

Figure 1-1: Organization of PK neuropeptides encoded by *A. aegypti capa* (*AAEL005444*) and *hugin* (*AAEL012060*) genes (Strand et al., 2016). Amino acid sequences of mature CAPA (yellow), PK1 (DH-like; blue) and PK2 (PBAN-like; purple) neuropeptides were identified by Predel et al. (2010). The non-functional PK2 isoform, lacking the conserved FXPRL-NH_2 sequence, is denoted in light grey text.

Mapping the distribution of *capa* and *hugin* gene products is useful in understanding neuropeptide functioning within and deriving from the nervous system. Recently, Schlegel et al. (2016) identified acetylcholine as a co-transmitter in *hugin* neurons, which are both required to regulate feeding behaviour in *D. melanogaster*. Here, PK2 is produced by neurons in the subesophageal zone that project axons into other neuroendocrine organs for release (Melcher and Pankratz, 2005). In moths, *hugin* mRNA is expressed predominantly in the SEG (Sato et al., 1994; Ma et al., 1998). Axons originating from these sites, containing PK2-like immunoreactivity, travel the length of the VNC (Kingan et al., 1992; Ma et al., 1996). Similar distribution mapping has been identified in other insects, where immunoreactivity extends to thoracic and abdominal ganglia, projecting to neurohaemal PVOs where peptides are released into the haemolymph (Predel and Eckert, 2000; Choi et al., 2001; Choi et al., 2011). Comparatively, *capa* gene products are typically highly abundant in the ventral abdominal ganglia, where they are released into the haemolymph through PVOs to exert their actions at target sites (Predel and Wegener, 2006; Hellmich et al., 2014; Kean et al., 2002; Loi and Tublitz, 2004). Similarly, in mosquitoes, *hugin* transcript is expressed primarily within the SEG, whereas the *capa* gene is expressed predominantly in a pair of neurosecretory cells in the abdominal ganglia and a subset of neurons of the SEG (Predel and Wegener, 2006; Hellmich et al., 2014). PK-producing neurons localized in the abdominal ganglia (Fig. 1-2) have axons extending to PVOs via the unpaired median nerve, where peptides either act on the nervous system or are released into the haemolymph to exert their actions at peripheral targets (Choi et al., 2001; Predel et al., 2010; Hellmich et al., 2014).

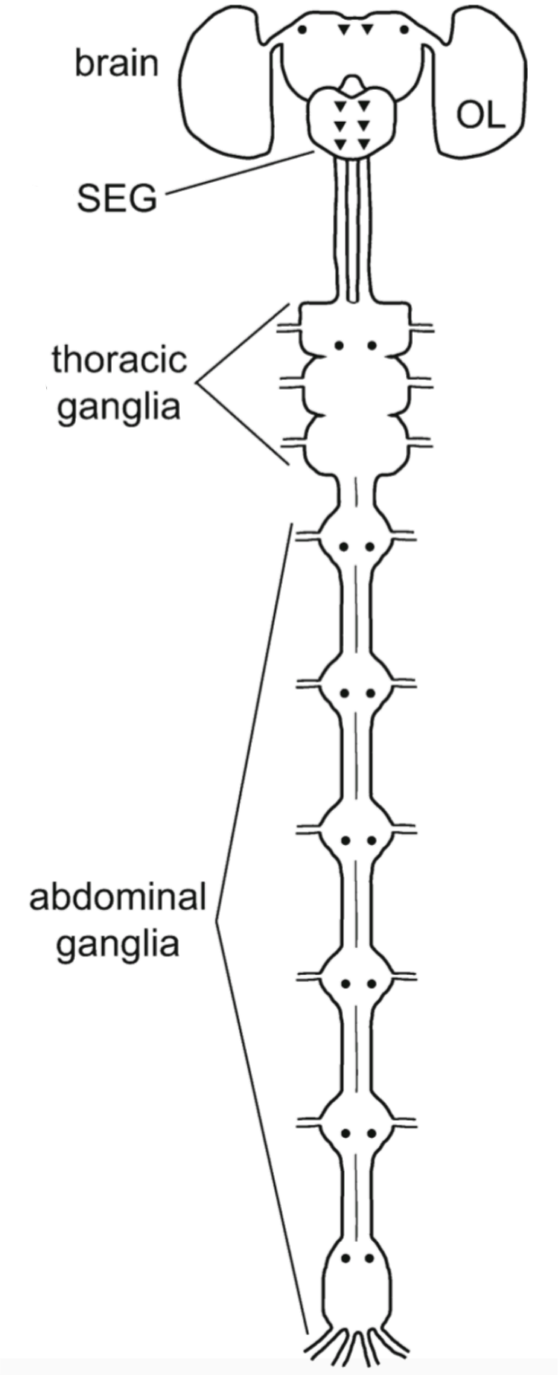

Figure 1-2. Schematic representation of adult mosquito central nervous system. Sites containing PK-like activity in multiple cell bodies are denoted by triangles, and single cell bodies are represented by circles. Subesophageal ganglion (SEG), optic lobe (OL). Reproduced from Hellmich et al. (2014).

1.6 Pyrokinin receptors

Similar to most other neuropeptides, PKs mediate their physiological actions by binding to membrane-bound G protein-coupled receptors (GPCRs), characterized by an extracellular N-terminus, seven transmembrane domains (TMDs) and an intracellular carboxyl tail (Caers et al., 2012). PK receptors (PK-Rs) belong to the rhodopsin family of GPCRs, and are homologs to the vertebrate neuromedin U (NMU) receptor that bind NMU (FXPRN-NH_2) peptides (Park et al., 2002; Melcher et al., 2006; Fujii et al., 2000). PK-Rs are predicted to couple to a Gq protein, which interacts with phospholipase C (PLC) upon ligand binding. PLC breaks down phosphatidylinositol-4,5-bisphosphate (PIP_2) to synthesize inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Caers et al., 2012). IP_3 elicits Ca^{2+} release from the endoplasmic reticulum, which stimulates plasma membrane-bound Ca^{2+} channel activation to further increase cation influx while DAG activates protein kinase C (PKC). The importance of cytosolic Ca^{2+} mobilization through these channels for relaying cellular signals upon PK-R activation has been extensively studied in *B. mori* (Matsumoto et al., 2009; Hull et al., 2009, 2010). Knocking down genes encoding these membrane-bound channel proteins, for instance, reduced sex pheromone production in moths, *in vivo* (Hull et al., 2009). In *Spodoptera littoralis*, PKC-dependent matrix-associated protein kinase activation is triggered by PK2 receptor binding to stimulate cuticle melanization (Zheng et al., 2007). Additionally, PK signaling in some moth species involves the second messenger, cAMP (Rafaeli et al., 2003). This has been shown to regulate fatty acid biosynthesis required for pheromone production (Jurenka et al., 1991).

Evidently, signal transduction pathways may differ across species. Although these mechanisms have not yet been characterized in non-Lepidopterans, all PK-Rs reported to date are dependent on Ca^{2+} to elicit a response (Rosenkilde et al., 2003; Homma et al., 2006;

Cazzamali et al., 2005; Olsen et al., 2007). As a result, studies make use of calcium-based bioluminescence heterologous assays (Prasher et al., 1987; Stables et al., 1997) to study PK-R activation and binding specificity for a subset of putative ligands. In light of completion and availability of insect genome sequences, two groups of PK-Rs have been identified: PK1-R/DH-R which exhibits high affinity (typically at low nanomolar doses) towards PK1/DH peptides, and PK2-R/PBAN-R that selectively binds PK2s/PBANs (Choi et al., 2003; Kim et al., 2008; Homma et al., 2006). These receptors have been deorphanized in various lepidopterans and dipterans through the use of heterologous expression systems (Altstein et al., 2013), the first of which was identified in *D. melanogaster* (Park et al., 2002; Rosenkilde et al., 2003). Rosenkilde et al. (2003) cloned two PK2-Rs that showed significantly higher affinity for their subfamily-specific PK2 ligands possessing the conserved FXPRL-NH_2 consensus sequence than endogenous PK1 and other related peptides (Rosenkilde et al., 2003). The *D. melanogaster* PK1-R was soon after characterized based on its selective binding for PK1 peptides (Cazzamali et al., 2005). Similar findings were observed with PK-Rs of the African malaria mosquito, *Anopheles gambiae* (Olsen et al., 2007), *A. aegypti* (Choi et al., 2013), the European corn worm, *Ostrinia nubilalis* (Nusawardani et al., 2013), and the red flour beetle, *Triboleum castaneum* (Jiang et al., 2014, 2015). Despite some evidence of cross-reactivity, each receptor type demonstrates greater selectivity for its cognate ligand.

Although PK1-Rs have been characterized by their selectivity for PK1 in several insects, as previously described (Cazzamali et al., 2005; Olsen et al., 2007; Choi et al., 2013; Jiang et al., 2014, 2015), some studies have examined differential PK1-R binding with *capa*- and *hugin*-encoded PK1, also referred to as DH-1 and DH-2, respectively. DH-1 binds more selectively to *T. castaneum* PK1-R, whereas PK2-Rs are more responsive to DH-2, suggesting functional

divergence of these TPKs (Jiang et al., 2014). Although DH-2 has been characterized to regulate diapause (Xu and Denlinger, 2003) it has not yet been reported whether DH-1 plays a similar role. Instead, this raises the question of whether receptor ligand specificity is determined based on the common precursor or mature peptide consensus sequence. It has been shown, however, that *capa*-encoded PK1 (DH-1) is typically unable to activate CAPA receptors, unless tested with high doses (Paluzzi et al., 2010; Terhzaz et al., 2012; Sajadi et al., 2019).

Ligand specificity for its receptor arises from recognition of the binding pocket of the GPCR. Therefore, to better understand how one receptor is more selectively activated by its native ligand, it becomes important to identify these binding sites. Through mutagenesis studies and generating chimera receptors, putative binding pockets within PK-Rs have been proposed in some insects. The importance of the extracellular N-terminal domain in maintaining PK-R stability was demonstrated by Choi et al. (2007). They showed that replacing the two putative N-glycosylation sites (consisting of asparagine residues) with glutamines on the PK2-R reduced ligand binding (Choi et al., 2007). They also proposed that the third extracellular loop of these receptors is critical for peptide ligand recognition (Choi et al., 2007). Other key residues important for ligand binding have also been identified within TMDs 2 and 3 (glutamates), TMD 6 (phenylalanines), and TMD 7 (tyrosines and phenylalanine) of the *B. mori* PK2-R (Kawai et al., 2014). Recently, the *A. aegypti* PK1-R and PK2-R were cloned and characterized based on activation in response to their cognate ligands (Choi et al., 2013), opening the path for further examination of PK signaling in this mosquito vector. Although exact binding sites have not yet been confirmed for these specific receptors, regions identified to be important in G protein activation and ligand recognition in other insects are indicated on the predicted *A. aegypti* PK-R structures (Fig. 1-3).

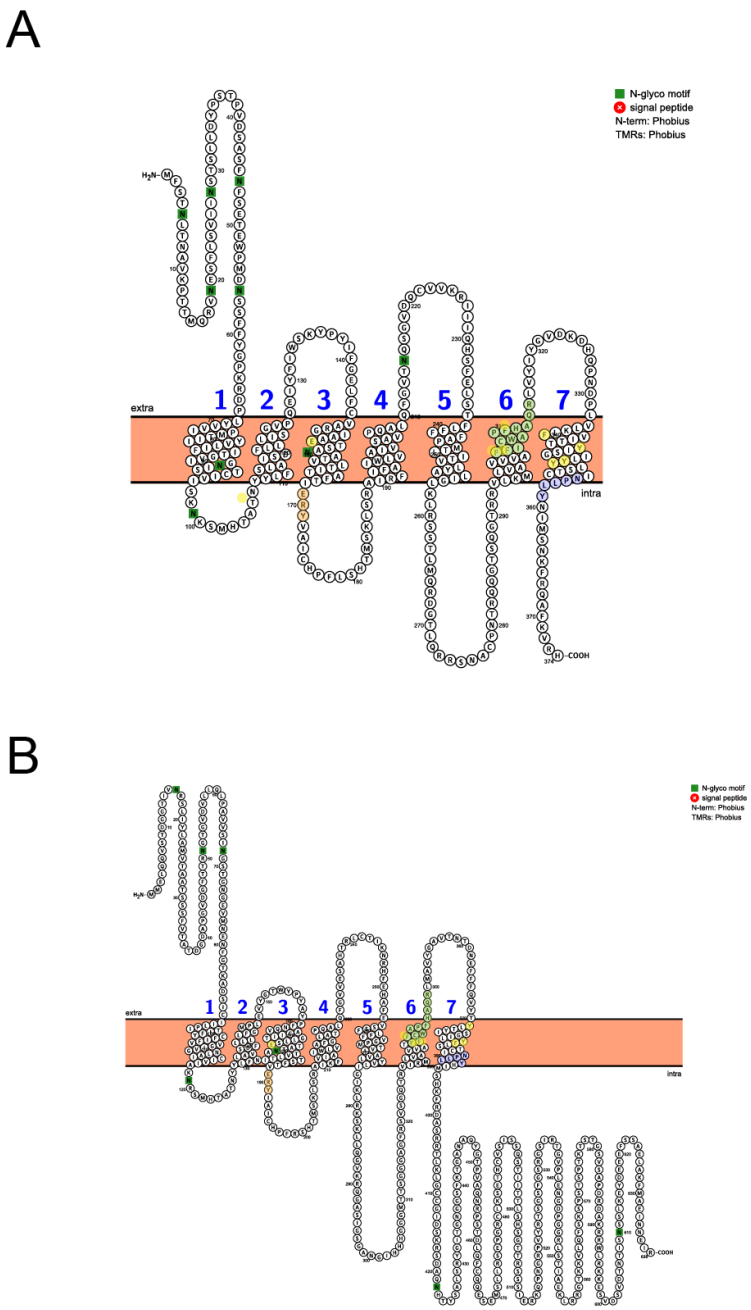

Figure 1-3. Predicted topologies of the *A. aegypti* PK1-R (A) and PK2-R (B) using Protter. Structure consists of seven transmembrane domains spanning the membrane, with an extracellular N-terminus and an intracellular C-terminus, consistent with G protein-coupled receptors. Sites proposed to be involved in ligand recognition in other insects are highlighted in yellow (Jurenka and Nusawardani, 2011), receptor activation are denoted in green, which triggers a tryptophan rotamer switch (Floquet et al., 2010), and regions proposed to undergo conformational changes to activate the Gq protein are highlighted in purple (Nygaard et al., 2009).

There are several approaches to elucidate neuropeptide target sites, which could help identify potential physiological roles. One such method is examining receptor distribution patterns. Temporal receptor expression recently revealed greater PK-R expression in immature stages than in adults (Choi et al., 2013). PK1-R enrichment throughout larval, pupal, and early adult stages led these authors to suggest that PK1 may play a role in pupal development or eclosion (Choi et al., 2013), as was reported for PK2, which mediates similar processes in the flesh fly, *Sarcophaga bullata* (Zdarek et al., 1997). Spatial expression patterns of these receptors have also been examined in other insects. Based on PK-R expression profiles, previous studies have identified several organs as putative PK targets including: pheromone glands of *H. zea*, *B. mori* and *Heliothis peltigera* moths (Choi et al., 2003; Hull et al., 2004; Altstein et al., 2003), reproductive tissues of *B. mori*, *H. zea*, *R. prolixus* and the cattle tick, *Rhipicephalus microplus* (Watanabe et al., 2007; Zhang et al., 2014; Paluzzi and O'Donnell, 2012; Yang et al., 2015), and the rectal sac, a structure analogous to the insect rectum, in *R. microplus* (Yang et al., 2015). These receptors have also been detected in neuronal tissues of *R. prolixus*, *R. microplus* and *I. scapularis* (Paluzzi and O'Donnell, 2012; Yang et al., 2015; Gondalia et al., 2016), suggesting that PK signaling may play neuromodulatory roles by acting on local neuronal sites.

1.7 Research objectives and hypotheses

The adult mosquito *A. aegypti* transmits pathogens causing many harmful diseases. A thorough understanding of mosquito biology is required to provide new avenues geared towards vector control. When faced with challenges to haemolymph homeostasis, the insect excretory system, comprised of the MTs and hindgut, functions to alleviate any risks posed to optimal physiological functioning, requiring the coordination of ion transport and myotropic activity. Unfortunately, our knowledge on the regulation of these processes in adult mosquitoes remains

limited. As mentioned above, PKs were initially characterized for their roles in regulating physiological processes along insect hindguts (Holman et al., 1986). These initial reports were therefore used herein to model possible regulatory mechanisms within the hindgut of *A. aegypti.*

My research is focussed on elucidating the function of PK neuropeptides through receptor characterization and functional activity. PKs and their receptors have been identified in many insects, however, their role remains unknown in blood-feeding arthropods. Therefore, the primary goal of my research is to investigate the distribution and physiological role of PKs in adult *A. aegypti*.

My first objective was to examine the functional activation of *A. aegypti* PK1-R and PK2-R in response to PKs and structurally-related peptidergic ligands, including CAPA peptides. PK receptors previously deorphanized in *D. melanogaster*, *A. gambiae*, *R. prolixus* and *T. castaneum* demonstrated specificity of their respective ligands (Cazzamali et al., 2005; Olsen et al., 2007; Paluzzi and O'Donnell, 2012; Jiang et al., 2014). Similar findings were shown by Choi et al. (2013) who partially deorphanized these receptors in *A. aegypti* based on their activation by pyrokinins encoded only by the *hugin* gene. Given that DH-1 and DH-2 has been previously shown to demonstrate varying binding affinities for PK-Rs (Jiang et al., 2014), it was important to elucidate whether DH-1 (*Aedae*PK1) derived from the *capa* gene is able to selectively bind to and activate its authentic receptor, PK1-R. Based on findings in *T. castaneum* (Jiang et al., 2014), I hypothesized that *capa*-encoded *Aedae*PK1 would demonstrate strict specificity for its authentic receptor, PK1-R, and to a lesser extent PK2-R, as was observed with *hugin*-derived PK1 (DH-2) in *A. aegypti* (Choi et al., 2013). Although some promiscuity may be observed, similar to *I. scapularis* PK-Rs (Gondalia et al., 2016), I hypothesized that PK1-R and PK2-R

would most strongly be activated at low nanomolar doses by their specific ligands, possessing the conserved WFGPRL-NH_2 and FXPRL-NH_2 motifs, respectively.

Next, peptide and receptor localization were used to reveal peripheral PK target sites. Transcript PK1-R and PK2-R expression patterns, and PK-like immunoreactivity were examined along organs of adult mosquitoes. Based on receptor expression profiles described in other haematophagous arthropods (Paluzzi and O'Donnell, 2012; Yang et al., 2015), as well as previously proposed roles for PKs in insect feeding behaviour and reproductive processes (Bader et al., 2007; Schoofs et al., 1993a; Yamashita, 1996; Uehara et al., 2011), I hypothesized that receptor transcripts would be enriched in the mosquito gut and reproductive organs, with PK-like immunoreactive staining found within processes innervating these sites.

Lastly, to uncover a potential function for the PK signaling pathway, contraction assays and the Scanning Ion-selective Electrode Technique were utilized to examine the potential involvement of PK-related peptides in regulating motility and ion transport in the hindgut. Due to the previously described roles of PK neuropeptides in hindgut myostimulation (Holman et al., 1986; Predel and Nachman, 2001; Marciniak et al., 2012), as well as defined receptor transcript expression along distinct regions of the hindgut examined in this study, I hypothesized that PK1 and PK2 peptides would stimulate rectal and ileal motility, respectively. In addition, PK1-like processes were detected along the rectal pads, regions previously proposed to be involved in ionoregulatory processes (Hopkins, 1967; Patrick et al., 2006). Due to extensive basolateral Na^+/K^+-ATPase localization at these sites (Patrick et al., 2006), I further hypothesized that PK1 may influence Na^+ transport across the rectal pad epithelia.

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CHAPTER II:
RECEPTOR CHARACTERIZATION AND FUNCTIONAL ACTIVITY OF PYROKININS ON THE HINDGUT IN THE ADULT MOSQUITO, *AEDES AEGYPTI*

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2.1 Summary

Pyrokinins are structurally-related insect neuropeptides, characterized by their myotropic, pheromonotropic and melanotropic roles in some insects, but their function is unknown in blood-feeding arthropods. In the present study, we functionally deorphanized the *Aedes aegypti* pyrokinin-1 and pyrokinin-2 receptors (PK1-R and PK2-R, respectively) using a heterologous cell system to characterize their selective and dose-responsive activation by members of two distinct pyrokinin subfamilies. We also assessed transcript-level expression of these receptors in adult organs and found the highest level of PK1-R transcript in the posterior hindgut (rectum) while PK2-R expression was enriched in the anterior hindgut (ileum) as well as in reproductive organs, suggesting these to be prominent target sites for their peptidergic ligands. In support of this prospective role, pyrokinin-like immunoreactivity was localized to innervation along the hindgut. Indeed, we identified a myoinhibitory role for PK2 on the ileum and, although we found that PK1 did not influence myoactivity or Na^+ transport in isolated recta, the pyrokinin-like immunolocalization terminating in close association to the rectal pads suggests this region of the rectum could be a target of PK1 signaling and may regulate the excretory system in this important disease vector species.

2.2 Introduction

Neuropeptides regulate an array of physiological processes in insects, including feeding, metamorphosis, diapause, and reproduction (Nässel and Winther, 2010). One such group of neuropeptides is the pyrokinin (PK) family. The first member to be identified, leucopyrokinin, was isolated from the cockroach, *Leucophaea maderae*, based on its stimulation of hindgut motility (Holman et al., 1986). Subsequently, a neuropeptide isolated based on its induction of sex pheromone production in female corn earworm moths (*Helicoverpa zea*; Raina et al., 1989), so named pheromone biosynthesis activating neuropeptide, was found to have the same carboxyl terminus, FXPRL-NH_2, and identification of the *pban* gene revealed additional encoded peptides with this conserved motif (Raina and Kempe, 1992; Sato et al., 1993). Related PKs have also been identified in other insects and shown to regulate cuticle melanization, embryonic diapause, pupariation, and feeding behaviour (Matsumoto et al., 1990; Imai et al., 1991; Zdarek et al., 1997; Verleyen et al., 2004; Bader et al., 2007).

The existence of another PK subfamily with the highly conserved WFGPRL-NH_2 carboxyl terminus was revealed with the characterization of the diapause hormone (DH) that regulates the onset of embryonic diapause in the silkworm *Bombyx mori* (Yamashita, 1996). The role of DH in diapause has been identified in some lepidopteran species (Xu and Denlinger, 2003), and more recently in *Locusta migratoria* (Hao et al., 2019). Other peptides with this conserved sequence have been identified in Diptera (Predel et al., 2004; Predel et al., 2010), but their physiological roles remain unclear. The DH/PKs comprise the pyrokinin-1 type (PK1) or tryptopyrokinins (Veenstra, 2014), and the PBAN/PKs, the pyrokinin-2 type (PK2) (Jurenka, 2015; Ahn and Choi, 2018). Members of the PK1 subfamily are encoded by two genes in most insects. The first was characterized for *B. mori* and called *pban* (Kawano et al., 1992; Sato et al.,

1993), which also encodes PK2 peptides. A homologous gene has been characterized in *Drosophila melanogaster*, termed *hugin*, expressed in a subgroup of neurosecretory cells within the subesophageal ganglion (SEG) (Meng et al., 2002; Bader et al., 2007), however, this gene in *D. melanogaster* only gives rise to PK2 peptides (Nässel and Winther, 2010). The second gene in insects encoding PK1 is the *capability* (*capa*) gene, which was first identified in *D. melanogaster* (Kean et al., 2002; Baggerman et al., 2002), which encodes not only a PK1 but also two additional neuropeptides known as CAPA or periviscerokinins that influence the activity of insect Malpighian tubules (Kean et al., 2002; Pollock et al., 2004; Terhzaz et al., 2012; Sajadi et al., 2018). Homologous *capa* genes were subsequently identified across insect groups and found to be expressed predominantly in a pair of neurosecretory cells in the abdominal ganglia and a subset of neurons of the SEG (Predel and Wegener, 2006; Hellmich et al., 2014). PK-producing neurons localized in these ganglia have axons extending to perisympathetic organs, where peptides either act on the nervous system or are released into the haemolymph to exert their actions at peripheral targets (Choi et al., 2001; Hellmich et al., 2014).

Efforts to define PK signaling in target cells have identified G protein-coupled receptors that selectively bind PK1 or PK2 forms found in *D. melanogaster*, the African malaria mosquito (*Anopheles gambiae*), and the kissing bug (*Rhodnius prolixus*) (Cazzamali et al., 2005; Olsen et al., 2007; Paluzzi and O'Donnell, 2012). These studies were conducted with cell systems expressing putative PK receptors cloned from the insects of interest, as was accomplished for the yellow fever mosquito, *Aedes aegypti* (Choi et al., 2013). However, in this latter study, the PK1 and PK2 receptors (PK-Rs) were only tested using pyrokinins encoded by the *AAEL012060* (*hugin*) gene, whereas activity of PK1 encoded by the *AAEL005444* (*capa*) gene was not determined. Although no functions have yet been assigned for PK neuropeptides in mosquitoes

or any other hematophagous arthropods, expression profiles of PK receptors in *R. prolixus* and the deer tick, *Ixodes scapularis*, show enrichment in the nervous system and reproductive tissues, and to a lesser extent, in the prothoracic glands and hindgut of *R. prolixus* (Paluzzi and O'Donnell, 2012; Gondalia et al., 2016).

Female *A. aegypti* are chief vectors of the chikungunya, dengue and yellow fever, and Zika viruses that are the causative agents of acute and chronic illnesses in humans globally (Kotsakiozi et al., 2017). Improving our understanding of mosquito biology and the regulation of underlying physiological processes by neuropeptides is imperative in order to develop new methods for vector control. Studying neuropeptide receptors in particular helps to unravel the neurocrine control of these uncharacterized regulatory mechanisms. The current study set out to examine the potential physiological roles of PK signaling in a vector mosquito by first examining the expression profiles of two PK receptors in different organs of adult *A. aegypti*. We then investigated whether the *A. aegypti* PK1-R and PK2-R identified previously (Choi et al., 2013) are activated by the *AAEL005444* (*capa*) gene-derived *Aedae*PK1, since this particular pyrokinin was not previously examined. Our current results along with the ability of PKs to stimulate hindgut motility in other insects (Holman et al., 1986) prompted us to further investigate the potential that these neuropeptides, acting through their cognate receptors, may influence myotropic and ionomodulatory activity in the hindgut, as these critical processes contribute towards maintenance of hydromineral balance in nectar- and blood-fed female mosquitoes.

2.3 Materials and methods

2.3.1 Animal rearing

A. aegypti eggs (Liverpool strain) oviposited onto Whatman filter papers (GE Bioscience) were collected and hatched in plastic containers with distilled water, as previously described (Rocco et al., 2017). Larvae and pupae were reared in a 26°C incubator under a 12:12 hour light:dark cycle. Larvae were fed daily with 2% brewers yeast:beef liver (1:1) powder solution (NOW foods, Bloomingdale, Illinois). All adult mosquitoes were fed 10% sucrose (w/v) *ad libitum*, and females in the colony cages were regularly fed with sheep blood in Alsever's solution (Cedarlane Laboratories, Burlington, ON) for egg production to maintain the colony. All experiments were performed on four-day old female and male mosquitoes that were isolated during the pupal stage and transferred into glass mesh-covered jars.

2.3.2 Receptor expression profiles and pyrokinin immunolocalization

2.3.2.1 Tissue dissections, RNA isolation, cDNA synthesis and RT-qPCR

Female (n = 20) mosquitoes were immobilized with brief exposure to CO_2, and submerged in nuclease-free Dulbecco's phosphate-buffered saline (DPBS; Wisent Inc., St. Bruno, Quebec, Canada). The midgut, Malpighian tubules, pyloric valve (midgut-hindgut junction), ileum, rectum, and reproductive organs (ovaries with accessory reproductive organs, including the common and lateral oviducts, and spermathecae, pooled together) were dissected and transferred into RNA lysis buffer containing 1% 2-mercaptoethanol. Whole-body total RNA samples were obtained from 7-8 females submerged in RNA lysis buffer and homogenized with a plastic microcentrifuge tube pestle and then frozen at -20°C overnight. Total RNA was subsequently extracted using the EZ-10 RNA Miniprep Kit (Bio Basic Inc., Markham, Ontario, Canada) following the manufacturer's protocol. The purified RNA was loaded onto a Take3

micro-volume plate and quantified using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). cDNA was synthesized with 25 ng total RNA as template from each sample using iSCRIPT Reverse Transcription Supermix (Bio-Rad, Mississauga, ON) following the manufacturer's instructions and diluted 10-fold for subsequent qPCR analysis.

The synthesized cDNA from mosquito organs was used to assess PK1-R and PK2-R transcript expression by amplifying a 249-bp and 203-bp fragment, respectively, with the forward (5'-TGTACGCTCTGATTGGCCTGAA-3'; PK1-R) or (5'-TATTGTACTTTCTGTCGACGTGC-3'; PK2-R) and reverse (5'- GCACTAATGGATCGTTCGGCTG-3'; PK1-R) or (5'-ATTTGCACCCGTTTTGAAGGAG-3'; PK2-R) primer sets based on a previously identified sequence (Choi et al., 2013; GenBank accession: EAT35008.1, PK1-R; KC155994.1, PK2-R) using PowerUP SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, United States). A 214-bp fragment of the *rp49* (GenBank accession: AY539746) gene was also amplified as a reference control using primers described previously (Paluzzi et al., 2014) with all cycling conditions as follows: 1) 50°C for 2 min, 2) 95°C for 20 seconds, and 3) 40 cycles of i) 95°C for 3 seconds and ii) 62°C for 30 seconds. Expression profiles were determined using 3 biological replicates (consisting of 20 organs per replicate), each of which included 4 technical replicates.

2.3.2.2 Enzyme-linked immunosorbent assay (ELISA) and whole mount immunohistochemistry

A custom synthesized rabbit polyclonal antibody (*Rhopr*CAPA-2 antigen sequence: EGGFISFPRV-NH_2, generously provided by Prof. Ian Orchard, University of Toronto Mississauga, ON, Canada) was used to visualize PK immunolocalization along mosquito tissues. To confirm that the antibody recognizes PKs, a competitive ELISA was performed. In brief, 96-well plates were coated with anti-*Rhopr*CAPA-2 primary antiserum diluted to 1:1000 in carbonate buffer (15 mM Na_2CO_3-H_2O and 35 mM $NaHCO_3$ in water; pH 9.4) and incubated

overnight at 4 °C. Plate contents were discarded, blotted, and rinsed three times with wash buffer (350 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 5.15 mM Na_2HPO_4-H_2O, and 0.05% Tween-20 (v/v) in water). Wells were then incubated for 1.5 h on a rocker with block buffer (0.5% skim milk powder (w/v) and 0.5% BSA (w/v) in PBS). The block solution was discarded, and standard solutions were added (100 µL/well). Standards consisted of *Aedae*CAPA-1 and *Aedae*CAPA-2 diluted in block buffer to achieve concentrations ranging from 250 pm to 250 nM, or *Aedae*PK1 and *Rhopr*PK2 from 4.8 nM to 75 uM). After a 1.5 h incubation on a rocker, 1 nM biotinylated-*Drome*CAPA (ASGLVAFPRV-NH_2, diluted in block buffer) was added (100 µL/well) to compete with the standards for antibody binding. Following overnight incubation at 4°C, the wells were washed four times with wash buffer, and incubated at 4°C for 1.5 h with Avidin-HRP (1:2000; Bio-Rad, Mississauga, ON, CA). The wells were washed three times, and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (100µL/well; Sigma-Aldrich, Oakville, ON) for 15 minutes at RT for colour development. Reactions were stopped with 100µL/well 2N HCl and absorbance at 450 nm was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

In light of PK receptor transcript expression and antibody validation confirming pyrokinin cross-reactivity, the pyloric valve region separating the midgut and hindgut organs, as well as the hindgut, including both the ileum and rectum of four-day old adult female mosquitoes were used to examine PK1-like immunoreactivity, following a procedure described previously (Rocco et al., 2017). Tissues were incubated in primary antibody solution (diluted 1:1000) made up in 0.4% Triton X-100, 2% normal sheep serum (NSS) (v/v) and 2% BSA (w/v) in PBS. Negative controls involved primary antibody solution pre-incubated with 5 µM *Aedae*PK1 overnight at 4°C. Both experimental and control antibody solutions were prepared and left at 4°C

overnight prior to incubating with tissues. Following a 48-hour primary antibody incubation at 4°C with gentle agitation, tissues underwent three 10-minute washes with PBS, and were then incubated with Alexa Fluor 568-conjugated AffiniPure goat anti-rabbit secondary antibody (1:200 dilution; Life Technologies) and 0.165 μM Alexa Fluor 488-conjugated phalloidin (Life Technologies) in 10% NSS (v/v) made up in PBS overnight at 4°C with gentle agitation. Tissues were then rinsed three times with PBS and mounted on slides with mounting media containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize cell nuclei in tissue preparations. Images were analyzed using a Lumen Dynamics X-Cite™ 120Q Nikon fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) and a Zeiss Cell Observer Spinning Disk Confocal Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.3.3 Heterologous functional receptor assay

2.3.3.1 Preparation of mammalian expression constructs with *A. aegypti* PK1-R and PK2-R

The complete open reading frame was amplified based on the partial (Nene et al., 2007) and complete sequences (Choi et al., 2013) reported earlier for the *A. aegypti* PK1-R (Genbank accession: EAT35008.1) and PK2-R (Genbank accession: KC155994.1). Forward 5'-ATGTTCAGTACAAACCTAAC-3'(PK1-R) or 5'-ATGATGGAGCTGCAGCAGGTGTCA-3' (PK2-R) and reverse 5'-TTAATGACGTACCTTGAAAGCTTG-3' (PK1-R) or 5'-TCAGCGAATCTCATTGTTGATTTCGGCC-3' (PK2-R) primers were designed over the start and stop codon, respectively, and used to amplify the complete coding sequence using Q5 high-fidelity DNA polymerase following manufacturer recommendations (New England Biolabs, Whitby, ON). The 1125 bp (PK1-R) and 1917 bp (PK2-R) PCR products were purified using a Monarch PCR purification kit (New England Biolabs, Whitby, ON) and reamplified using the identical reverse primers but forward primers possessing the consensus Kozak translation

initiation sequence (Kozak, 1984; Kozak, 1986), 5'-GCCACCATGTTCAGTACAAACCTAAC-3' (PK1-R) or 5'-GCCACCATGATGGAGCTGCAGCAGGTGTCA -3' (PK2-R). The resulting products were cloned into pGEM-T Easy sequencing vector and miniprep samples were sequenced to verify base accuracy. The receptor constructs were excised using standard restriction enzyme digestion and subcloned into the mammalian expression vector, pcDNA 3.1^{+} (Life Technologies, Burlington, ON). Transfection quality plasmid DNA was purified from an overnight bacterial culture using the PureLink MidiPrep Kit (Invitrogen, Burlington, ON) following manufacturer guidelines.

2.3.3.2 Cell culture, transfections and bioluminescence assay

Chinese hamster ovary cells (CHO-K1) described previously (Paluzzi et al., 2012; Gondalia et al., 2016; Wahedi and Paluzzi, 2018) were grown in Dulbecco's modified eagles medium: nutrient F12 (DMEM:F12) media containing 10% heat-inactivated fetal bovine serum (FBS; Wisent, St. Bruno, QC), 200µg/mL geneticin, and antimycotic-antibiotic mixture as described previously (Wahedi and Paluzzi, 2018). Cells were grown to approximately 80% confluency and were co-transfected with mammalian codon-optimized aequorin using Lipofectamine 3000 transfection reagent following recommended guidelines (Invitrogen, Burlington, ON) to transiently express either the *A. aegypti* PK1-R, PK2-R or pcDNA3.1^{+} mammalian expression construct containing mCherry, which was utilized as a transfection control. Cells were then prepared for the functional assay 48 hours post-transfection following a protocol described previously (Wahedi and Paluzzi, 2018), at which point mCherry-expressing cells showed a transfection efficiency of about 90%. Various concentrations of synthesized PK peptides and other peptides (purity >90%; Genscript, Piscataway, NJ) were prepared in BSA media and loaded in quadruplicate into white 96-well luminescence plates (Greiner Bio-One,

Germany).

Luminescence responses to *A. aegypti* CAPA-1 (GPTVGLFAFPRV-NH_2), CAPA-2 (pQGLVPFPRV-NH_2) and PK1 (AGNSGANSGMWFGPRL-NH_2) peptides, along with the *R. prolixus* PK2 orthologs (Paluzzi and O'Donnell, 2012), PK2a (NTVNFSPRL-NH_2) and PK2b (SPPFAPRL-NH_2) were examined. Cells prepared for the functional assay were loaded into each well of the plate using an automated injector unit and luminescent response was measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Negative controls were carried out using BSA media alone whereas 50 μM ATP, which activates endogenously expressed purinoceptors (Iredale and Hill, 1993; Michel et al., 1998), was used as a positive control. Luminescence responses were normalized to ATP responses and analyzed in GraphPad Prism 7.02 (GraphPad Software, San Diego, USA). EC_{50} values were determined using dose-response curves from multiple biological replicates.

2.3.4 Hindgut contraction assays

2.3.4.1 Preparation of hindgut tissues

Hindgut contraction assays were conducted on isolated ilea and recta of female *A. aegypti*. Mosquitoes were anesthetized with CO_2 and dissected under saline, containing 150 mM NaCl, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfuronic acid (HEPES), 3.4 mM KCl, 7.5 mM NaOH, 1.8 mM $NaHCO_3$, 1 mM $MgSO_4$, 1.7 mM $CaCl_2$-$2H_2O$ and 5 mM glucose, titrated to pH 7.1. The isolated tissue was secured with a minutien pin within a Sylgard-lined petri-dish.

2.3.4.2 Peptide and neurotransmitter dosages

Given its high sequence similarity to endogenous *A. aegypti* PK2 peptides, we used the *R. prolixus* PK2-2 (PK2b) isoform (Paluzzi and O'Donnell, 2012), herein named *Rhopr*PK2 for

motility bioassays. Commercially synthesized peptides (Genscript, Piscataway, NJ, USA), *Aedae*PK1 (AGNSGANSGMWFGPRL-NH_2) and *Rhopr*PK2 (SPPFAPRL-NH_2), were used to examine myomodulatory activity. Serotonin (5-hydroxytryptamine, 5-HT; Sigma-Aldrich, Oakville, Ontario, Canada), previously shown to stimulate *A. aegypti* hindgut contractions (Messer and Brown, 1995), was used as a stimulatory control. Given the inhibitory properties of myoinhibitory peptides (having the consensus W(X_6)W-NH_2 carboxyl terminus) on insect hindgut motility (Lange et al., 2012), *Rhopr*MIP-7 (AWNSLHGGW-NH_2; Genscript, Piscataway, NJ, USA; Paluzzi et al., 2015) was used as an inhibitory control. All hormones were diluted in saline to achieve a final concentration of 1μM.

2.3.4.3 Electrophysiological measurements of recta

To assess whether PK1 plays a role in regulating rectal motility, contractions were monitored in saline to obtain baseline contraction rates and subsequently following *Aedae*PK1 or 5-HT treatments, using probes connected to an impedance converter (UFI model 2991, Morro Bay, California, USA) connected to a Powerlab 4/30 and laptop computer running LabChart Pro 6.0 software (AD Instruments, Colorado Springs, CO, USA). The isolated rectum was contained in a small circular ridge of a Sylgard coated dish which was bathed in saline. Probes were positioned on either side of the rectum by observation with a dissecting microscope (Olympus SZ61), and contractile responses were recorded on LabChart Pro 6.0 software (AD Instruments, Colorado Springs, CO, USA). Contractions were monitored for two minutes prior to, and after the addition of either *Aedae*PK1 or 5-HT.

The number of contractions were recorded over a two-minute interval to obtain the contraction frequency (number of contractions min^{-1}). To account for variability in myoactivity between individual preparations, rectal myoactivity is expressed as a ratio in contraction

frequency upon *Aedae*PK1 or 5-HT application relative to the baseline contractile activity of the same tissue preparation in saline alone.

2.3.4.4 Video measurements of ilea

Unlike the rectum, the mosquito ileum produces weaker contractions that we were unable to measure using an impedance converter as described above. As a result, to examine the effects of the PK2 on anterior hindgut motility, video recordings of dissected ilea were obtained using an Olympus SZ microscope connected to Luminera's INFINITY1-2CB video camera. The dissected gut was pinned in the midgut and rectum to allow the ileum to freely contract in saline and following treatments. Contractions were recorded for two minutes in saline used as baseline measurements, followed by three subsequent two-minute recordings, including: i) additional saline, ii) 1 μM *Rhopr*PK2, and iii) either 1 μM 5-HT with *Rhopr*PK2 or 1 μM *Rhopr*MIP-7 with *Rhopr*PK2. The *Rhopr*PK2 was added along with the stimulatory or inhibitory hormone to maintain its 1 μM concentration in the bath.

Using the video recordings, the contraction rate (number of contractions min^{-1}), average duration of each contraction (sec), and average length of time between each contraction (sec) over the 2-minute interval were recorded individually. To account for any potential changes in tissue contractile activity upon adding solutions to the bath, each of these variables were measured relative to baseline saline recordings of the same preparation as a ratio of change, where a value above or below one indicates an increase or a decrease (respectively) in the rate, duration or length between each contraction in response to the treatment.

2.3.5 Ion transport along the rectal pad epithelia

2.3.5.1 Preparation of hindgut tissues

Female mosquitoes were anesthetized on ice for 3 minutes and dissected in Ca^{2+}-free *A. aegypti* saline to limit spontaneous hindgut contractions during ion flux measurements. Saline consisted of 20 mM NaCl, 3.4 mM KCl, 1.8 mM $NaHCO_3$, 1 mM $MgSO_4$, 25 mM HEPES, 5 mM glucose and 130 mM N-methyl-D-glucamine, adjusted to pH 7.1. The isolated rectum was then transferred to a Petri dish with saline, pre-coated with poly-L-lysine (Paluzzi et al., 2014) to allow the tissue to adhere to the bottom of the dish.

2.3.5.2 Peptide dosages and saline application

*Aedae*PK1 or *D. melanogaster* drosokinin (NSWLGKKQRFHSWG-NH_2, Genscript, Piscataway, NJ, USA; the latter provided by Prof. Dick Nässel, Stockholm University, Sweden) were solubilized in double distilled water as a 1 mM stock and then diluted in the above-mentioned Ca^{2+}-free *A. aegypti* saline to achieve a 1 μM final concentration. Control measurements were also obtained by applying equal volume of saline only, referred to as saline control treatments.

2.3.5.3 Scanning Ion-selective Electrode Technique (SIET)

To measure Na^+ flux across rectal pad epithelia, ion-selective microelectrodes and reference electrodes were used, as described previously (Paluzzi et al., 2014), with the following changes: the microelectrode was backfilled with 100 mM NaCl, front loaded with Na^+-selective ionophore (sodium ionophore II cocktail A; Fluka, Buchs, Switzerland), and calibrated before every preparation with 200 mM NaCl, and 20 mM NaCl containing 180 mM LiCl to equalize osmolarity of the standard solutions.

SIET measurements were obtained through the Automated Scanning Electrode Technique (ASET) software (Science Wares, East Falmouth, MA, USA). To obtain background recordings for every preparation, the microelectrode tip was positioned at a site located 3 mm away from the tissue. Voltage gradients were measured as the microelectrode moved perpendicularly to the tissue surface between two points separated by a distance of 100 μm. The sampling protocol used a wait time of 4 s after microelectrode movement and a recording time of 1 s after the wait period. Following background voltage readings, the ion-selective microelectrode tip was positioned at a distance of 2 μm from the rectal pad epithelia. A similar sampling protocol was used at the tissue surface. For each sample, several initial measurements were obtained at various sites across the length of one rectal pad and the site demonstrating maximal ion flow was used for all subsequent measurements. Specifically, the sampling protocol was repeated six times at this target site in saline solution, and the voltage difference between the two sites was used to calculate a voltage gradient by the ASET software. A treatment (either saline or peptide) was then directly applied to the dish to determine if this induced a change in ion flux by the rectal pad epithelia. The sampling protocol was then repeated 12 times. Following measurements at the rectal pad sites, background voltage readings were again recorded at a distance of 3 mm away from the tissue.

Calculation of ion flux used in this study has been described previously (Paluzzi et al., 2014). Approximately 70% of tissues initially exhibited Na^+ absorption in saline, whereas the remaining preparations (approximately 30%) were initially secreting Na^+ into the rectal lumen. In light of this potential source of variation, further experimental treatment was only carried out on preparations exhibiting haemolymph-directed ion transport (i.e. reabsorbing Na^+). The change in flux upon treatment application was calculated by subtracting flux values obtained during

saline measurements, in which a positive change indicates increased ion absorption, and a negative change represents a decrease in absorption.

2.3.6 Graphical representation

Data were transferred into GraphPad Prism 7.0 to create all figures and conduct statistical analyses, which are described as appropriate in the figure captions.

2.4 Results

2.4.1 Receptor expression profile and localization of PK1-like immunoreactivity

As a first step towards discovering physiological roles for pyrokinins in *A. aegypti*, prospective targets were examined. RT-qPCR was used to measure PK1 and PK2 receptor transcript levels in adult organs. Expression of PK1-R was only significantly enriched in the rectum compared to expression in the whole body (Fig. 2-1). Comparatively, PK2-R was abundant in the anterior ileum and significantly enriched in reproductive organs relative to the whole body (Fig. 2-1), and demonstrated significantly higher expression compared to PK1-R levels in these organs, which was consistent across all biological replicates.

Given this highly defined expression within the two segments of the hindgut, we next used immunohistochemistry to visualize peptide distribution along this region. Using a custom-synthesized antibody against a CAPA neuropeptide, which was shown to cross-react with and bind to PKs (Fig. 2-2), PK1-like immunostaining was observed in an axon net encircling the pyloric valve (Fig. 2-3A), which separates the midgut and hindgut. Immunolocalization was detected in axonal projections over the ileum (Fig. 2-3B) and innervating the rectal pads with immunoreactive projections terminating in close association and encircling 4-5 cells within the lumen of all six rectal pads (Fig. 2-3C; Fig. 2-4). Staining was abolished in control preparations treated with antibody pre-incubated with *Aedae*PK1 (Fig. 2-5).

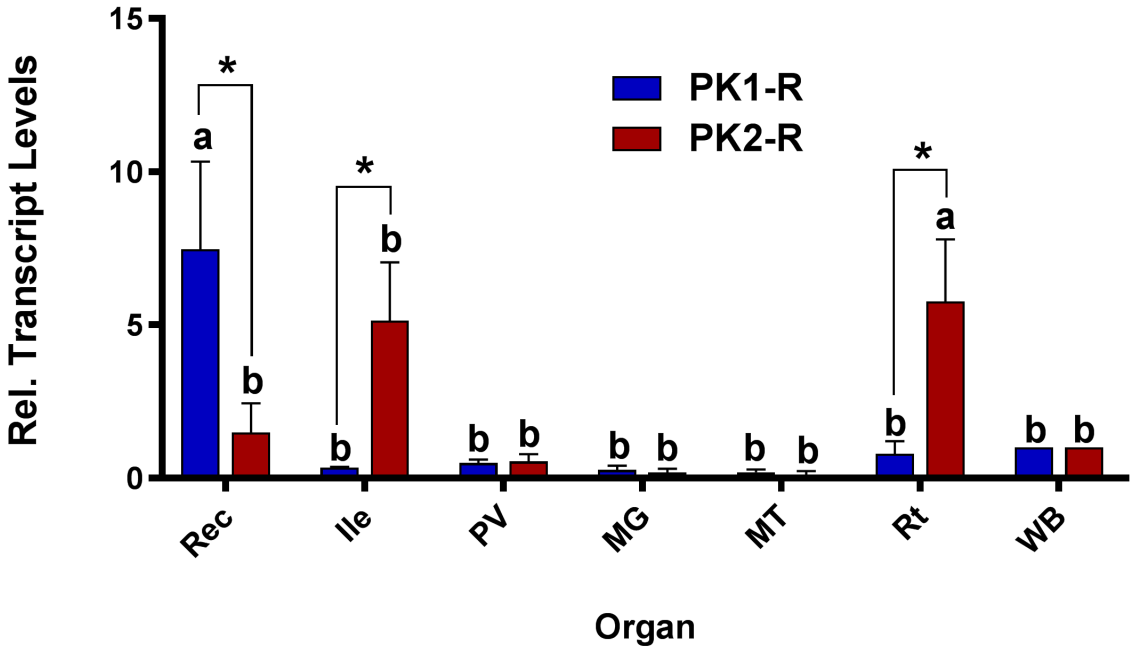

Figure 2-1: Spatial expression patterns of PK1-R and PK2-R transcript in female organs relative to the whole body (WB). normalized to the reference gene *rp49*. Expression was analyzed in the rectum (Rec), ileum (Ile), pyloric valve (PV) region, midgut (MG), Malpighian tubules (MT) and reproductive organs (Rt). Levels significantly different from the WB are denoted with different letters, and significant differences in transcript abundance between the two receptors in an individual organ are denoted by an asterisk, as determined by a two-way ANOVA and Sidak post test ($p<0.05$). Data represent the mean ± SEM ($n = 3$).

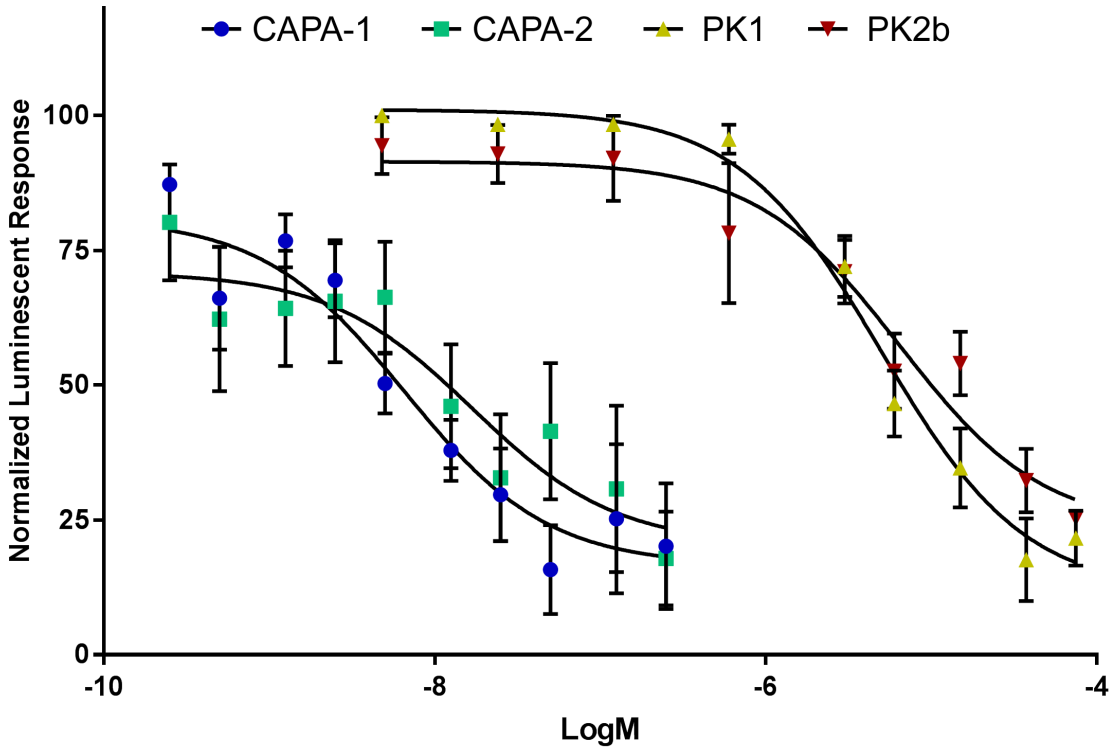

Figure 2-2: Competitive ELISA used to examine CAPA2-targeted antibody binding affinity to other structurally related peptides. The custom-synthesized antibody designed to target the antigen sequence EGGFISFPRV-NH_2 selectively targets *Aedae*CAPA-1 (IC_{50} = 6.57 nM) and *Aedae*CAPA-2 (IC_{50} = 16.93 nM), but was also able to recognize and bind to *Aedae*PK1 (IC_{50} = 4.99 µM) and *Rhopr*PK2b (IC_{50} = 6.43 µM). Normalized responses represent mean ± SEM (n = 4).

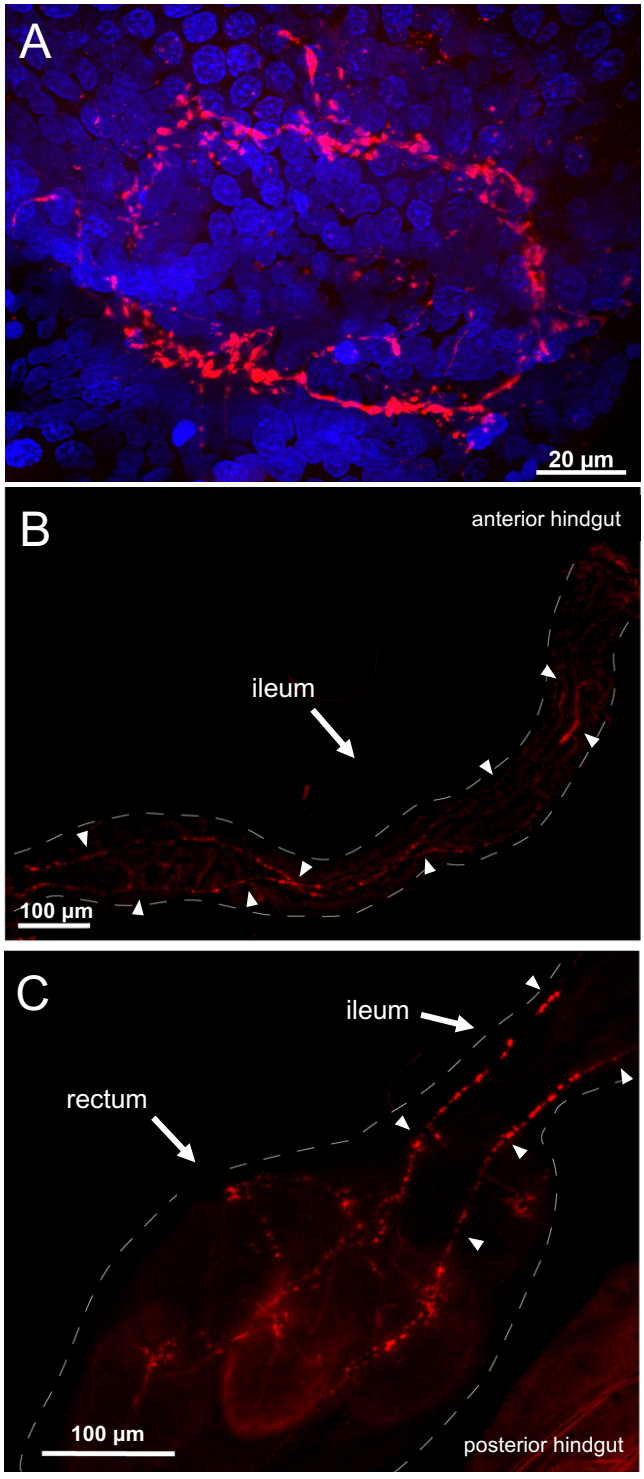

Figure 2-3: Immunolocalization of PK1-like peptides (red) with DAPI (blue) in the female gut. PK1-like immunoreactive staining was observed within an axon net encircling the pyloric valve at the junction between the midgut and hindgut **(A)**. Immunoreactive axonal projections continue over the anterior hindgut, denoted by arrowheads **(B)** and terminate within the lumen of the six rectal pads **(C)**.

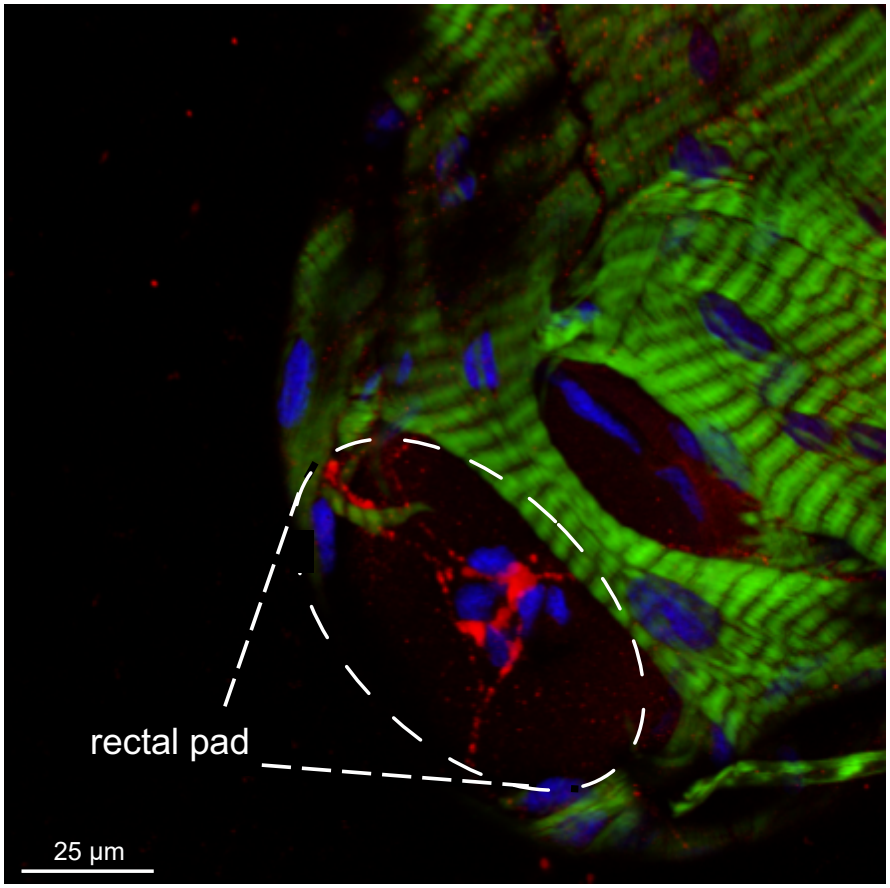

Figure 2-4: PK-like processes (red) terminating in close association to cells within the rectal pad (one of six shown) of adult female mosquitoes. No co-localization with phalloidin-stained filamentous actin (green) was detected.

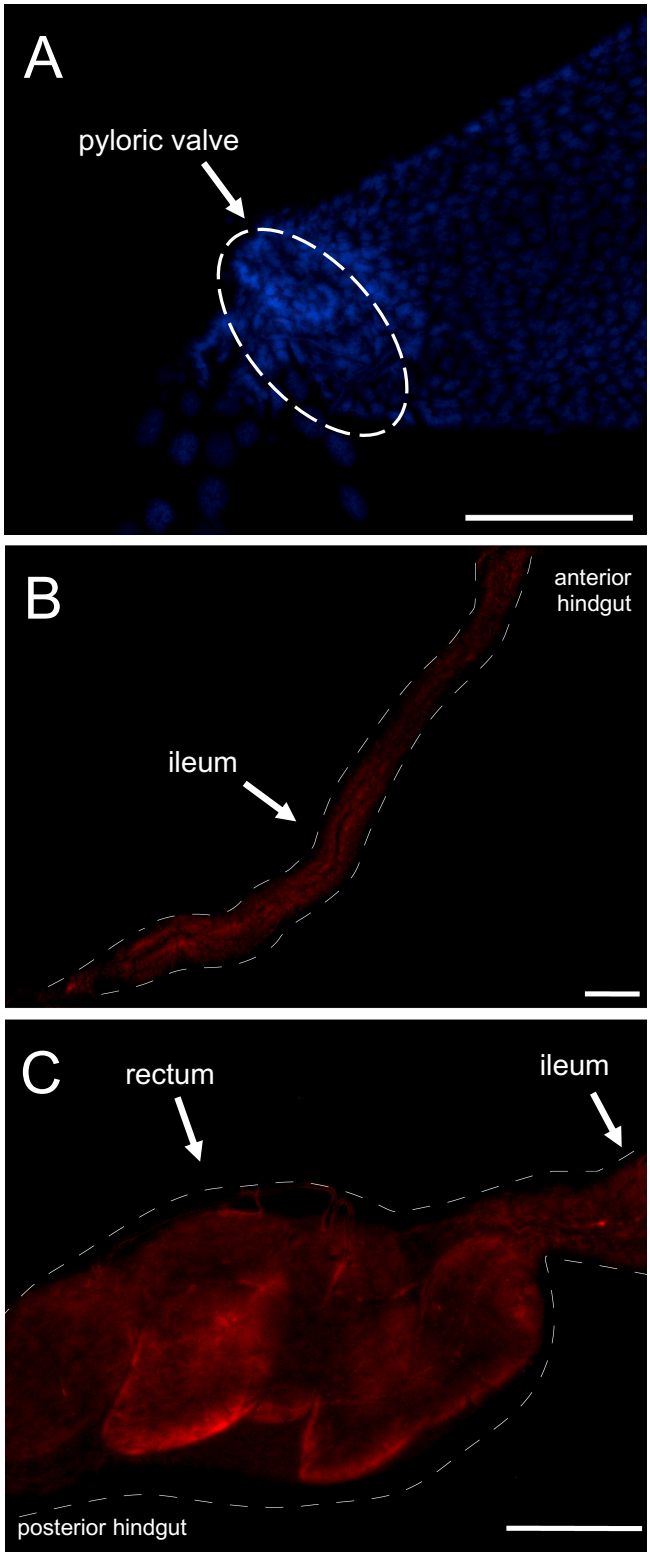

Figure 2-5: Pre-incubation of primary antibody with 5 µM *Aedae*PK1 abolishes immunoreactivity along the pyloric valve (**A**), ileum (**B**) and rectum (**C**). All exposures are equalized to settings used for experimental treatments. Scale bars, 100 µm.

2.4.2 PK1-R and PK2-R functional activation assay

Heterologous expression and functional analysis of the *A. aegypti* PK1-R revealed a robust activation by *Aedae*PK1 (EC_{50} = 37.6 nM), as demonstrated by the dose-dependent luminescent response (Fig. 2-6) in CHO-K1 cells transiently expressing *A. aegypti* PK1-R. Additionally, the *A. aegypti* PK1-R was responsive to the *R. prolixus* PK2 orthologs, PK2a and PK2b (EC_{50} = 1.57 and 0.4465 µM, respectively) encoded by the *R. prolixus ADA83379.1* gene (Jurenka and Nusawardani, 2011), albeit 12- to 42-fold reduced compared to *Aedae*PK1. Structurally-related peptides (*Aedae*CAPA-1 and -2) encoded by *A. aegypti AAEL005444* gene were also effective in activating PK1-R at high concentrations. The efficacy of these other peptides however was orders of magnitude lower than *Aedae*PK1, which elicited a luminescent response significantly greater than that achieved with all other tested peptides. Notably, at 10 nM, *Aedae*PK1 was the only peptide which elicited a significant luminescent response different from controls treated with BSA assay media alone (Fig. 2-6B).

Cells transiently expressing the *A. aegypti* PK2-R (Fig. 2-6C) were significantly more responsive to the PK2 peptides, PK2a and PK2b (EC_{50} = 17.47 and 8.54 nM, respectively), particularly at lower doses of 10 nM (Fig. 2-6D) since no other tested peptide at this concentration had a significant effect on *A. aegypti* PK2-R. Although *Aedae*PK1 was still able to activate PK2-R at high concentrations (EC_{50} = 158 nM), it elicited an over four-fold greater potency on PK1-R expressing cells (Fig. 2-6A, C). Similar to results observed in PK1-R functional expression, the structurally related CAPA peptides (*Aedae*CAPA-1 and -2) were only active on PK2-R at very high concentrations and did not achieve over 25% activation relative to the highly potent pyrokinins (Fig. 2-6C, D). These results confirm the specificity of PKs for their authentic receptors (Table 2-1). No luminescence was detected in cells transfected with

pcDNA3.1^{+} containing mCherry (Fig. 2-7), indicating that responses observed with PK1-R and PK2-R leading to Ca^{2+} mobilization were specifically a result of receptor binding and activation.

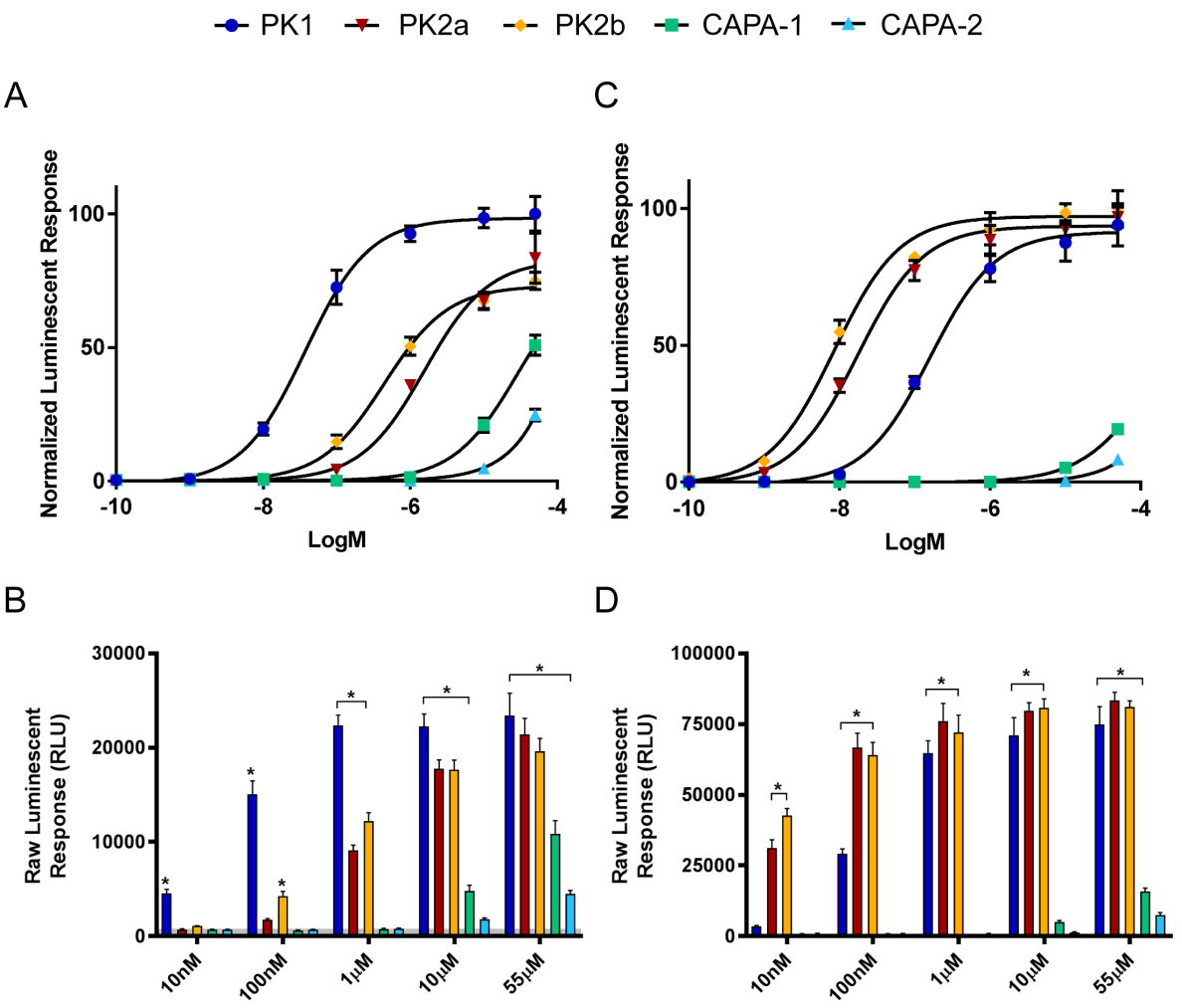

Figure 2-6: Luminescent response of CHO-K1 cells expressing the *A. aegypti* PK1 **(A, B)** and PK2 **(C, D)** receptors. Transient expression of *A. aegypti* PK1-R in CHO-K1 cells was used to examine receptor functional activation by *Aedae*PK1 demonstrating a dose-dependent luminescent response following peptide application **(A, C)**. Structurally-related peptides, including two PK2 analogs derived from the *R, prolixus ADA83379.1* gene along with endogenous *AAEL005444* gene-derived CAPA anti-diuretic peptides demonstrated significantly lower activity on the heterologously-expressed *A. aegypti* PK1 receptor **(B)**. Although *Aedae*PK1 was able to activate both receptors, PK2-R displayed more selective activation by the PK2 analogs derived from the *R, prolixus ADA83379.1* gene **(C, D)**. Luminescent responses were monitored after peptide treatment, with data representing average luminescence (mean ± standard error) over the first 10 seconds immediately following peptide application. Raw luminescent responses significantly different from background responses (BSA media alone shown in the gray shaded region) are denoted by an asterisk, as determined by a one-way ANOVA and Dunnett's multiple comparison post-hoc test ($p<0.05$).

Table 2-1. Summary of peptide activity in eliciting a luminescent response in PK1-R and PK2-R functional assay.

Peptide	**Sequence**	**EC_{50}**	
		PK1-R	**PK2-R**
*Aedae*CAPA-1	GPTVGLFAFPRV-NH_2	> 10 μM	> 55 μM
*Aedae*CAPA-2	pQGLVPFPRV-NH_2	> 55 μM	> 55 μM
*Aedae*PK1	AGNSGANSGMWFGPRL-NH_2	37.6 nM	158 nM
*Rhopr*PK2a	NTVNFSPRL-NH_2	1.57 μM	17.47 nM
*Rhopr*PK2b	SPPFAPRL-NH_2	446.5 nM	8.54 nM

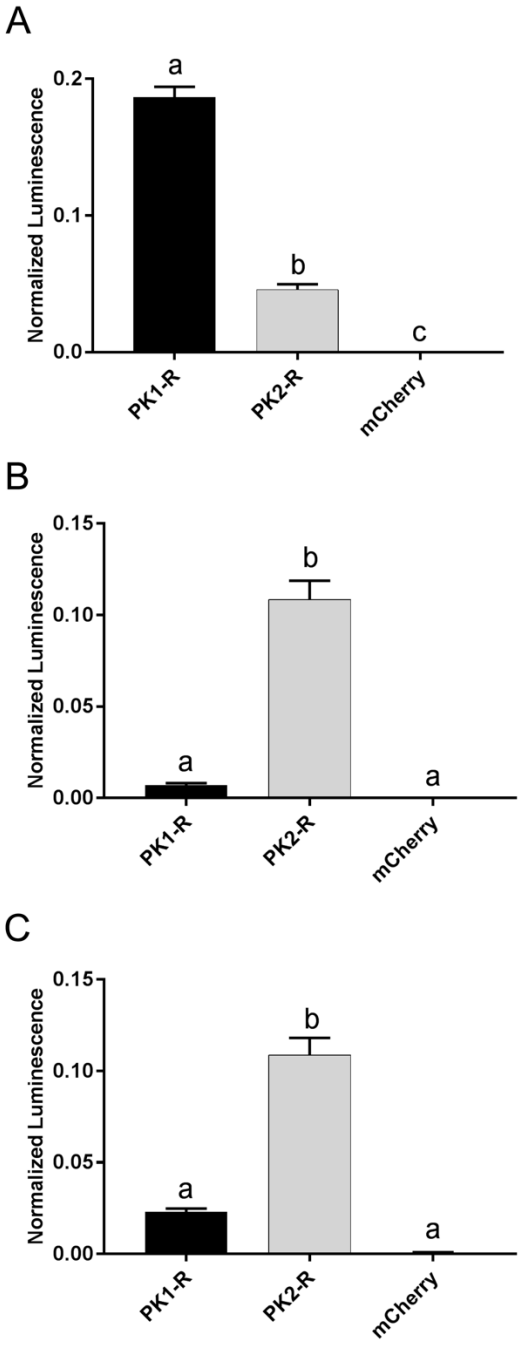

Figure 2-7: Validation of PK1-R and PK2-R activation by their proposed ligands. The bioluminescence response (mean 0-10s) of CHO-K1 cells co-transfected with aequorin and either *A. aegypti* PK1-R, PK2-R or mCherry was recorded in response to 10^{-7} M *Aedae*PK1 **(A)**, 10^{-7} M *Rhopr*PK2a **(B)** and 10^{-7} M *Rhopr*PK2b **(C)**. Luminescence was subtracted from BSA control and normalized to the ATP response. Significant differences in normalized luminescent responses are denoted with different letters, as determined by a one-way ANOVA and Tukey post test ($p<0.01$).

2.4.3 Pyrokinins on mosquito hindgut motility

Having verified specific sites of *A. aegypti* PK1-R and PK2-R transcript enrichment and functional activation of these receptors with greatest sensitivity to PK1 and PK2 peptides, we sought to determine the potential myotropic activity of these neuropeptides on the rectum and ileum, respectively. Serotonin (5-HT), a known myostimulator of *A. aegypti* hindgut (Messer and Brown, 1995), was used as a positive control to validate functionality of the bioassay setup. Application of 5-HT typically stimulated greater contractile activity compared to unstimulated baseline activity (Fig. 2-8A) reflecting a 1.97-fold increase in contraction rate for female recta (Fig. 2-9A) and a 2.48-fold increase for male recta (Fig. 2-9B). *Aedae*PK1 had no apparent effect on contractile activity (Fig. 2-8B) with no significant change in contraction rate (Fig. 2-9).

To assess the role of PK2 along the anterior hindgut, we used both a stimulatory as well as an inhibitory control following *Rhopr*PK2 treatment. Relative to baseline levels, contraction frequency significantly decreased from a 0.72-fold change in saline to 0.55-fold in response to PK2 (Fig. 2-10A). This inhibitory effect was reversed upon 5-HT treatment. Although the duration of each contraction did not significantly differ upon PK2 application, the length of time between each contraction event increased. 5-HT effectively reduced both of these metrics, resulting in a 2.88-fold increase in ileal contraction rate (Fig. 2-10B, C). Due to its inhibitory nature, we further assessed the effects of PK2 relative to a known myoinhibitor (Lange et al., 2012). Although *Rhopr*PK2 significantly reduced ileal contraction frequency, *Rhopr*MIP resulted in further inhibition by 0.38-fold (Fig. 2-11A). Each contraction slowed in response to *Rhopr*PK2 and *Rhopr*MIP by about 1.9-fold (Fig. 2-11B), and the relaxation period between spontaneous contractions was also prolonged by 2.2- and 3.9-fold, respectively (Fig. 2-11C).

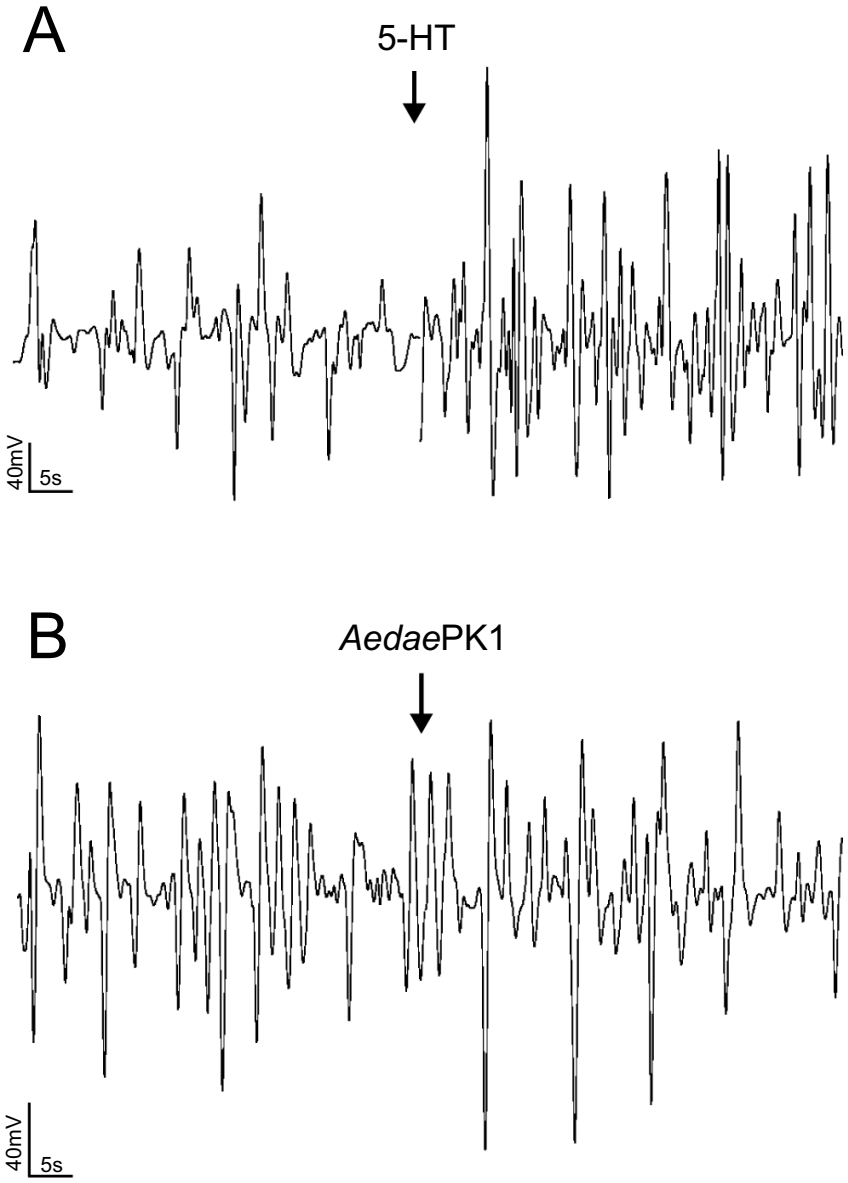

Figure 2-8: Sample traces of 1 μM 5-HT **(A)** and 1 μM *Aedae*PK1 **(B)** on spontaneous rectal contractions. Arrows indicate time of hormone application. Sample data was collected over a two-minute recording interval. Contraction rate increased in response to 5-HT **(A)**, whereas no change in activity was observed following *Aedae*PK1 application **(B)**.

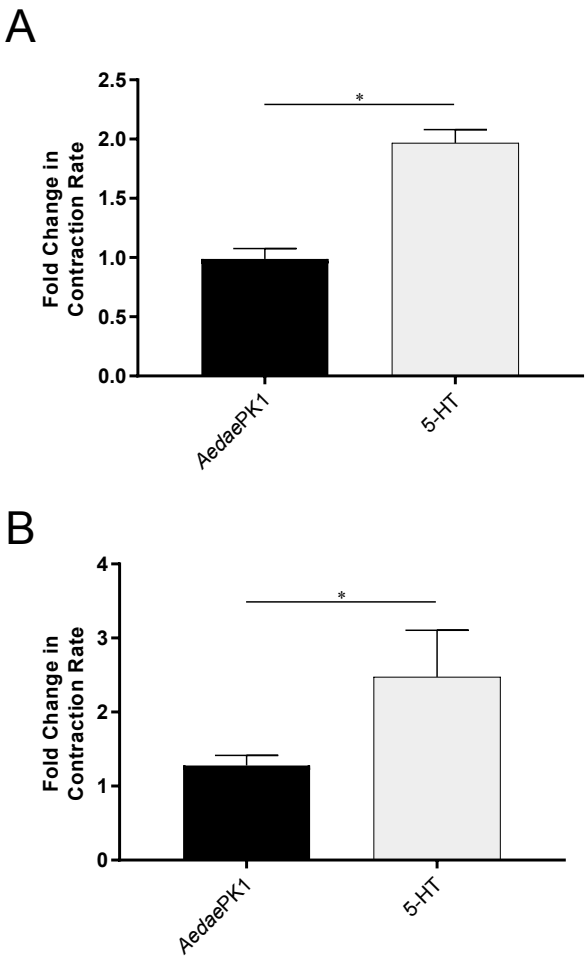

Figure 2-9: Fold change in contraction frequency of recta isolated from female **(A)** and male **(B)** mosquitoes in response to 1 μM 5-HT and 1 μM *Aedae*PK1 relative to baseline activity. Mean ± SEM are obtained from 6–18 preparations, with asterisks representing significant differences, as determined using an unpaired t-test ($p<0.001$).

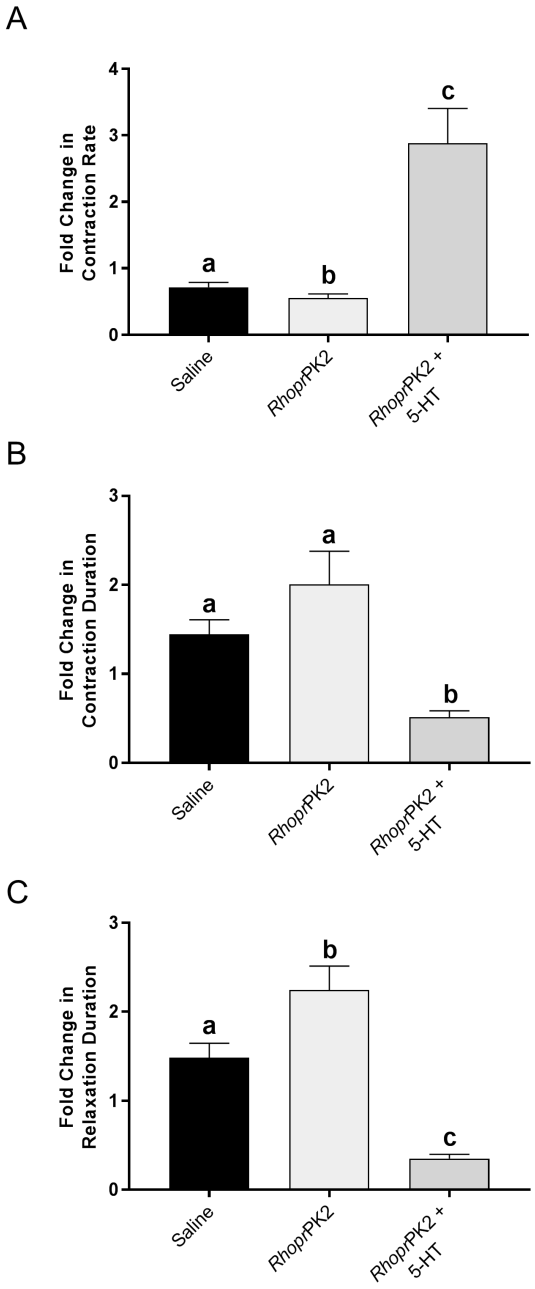

Figure 2-10: Motility of ilea isolated from female mosquitoes in response to added saline (vehicle control), 1 µM *Rhopr*PK2 and 1 µM 5-HT (stimulatory control). The change in contraction frequency **(A)**, duration of contraction **(B)** and length of time between contractions **(C)** was measured relative to baseline recordings. Values are presented as mean ± SEM from 15 preparations. Significant differences between the treatments are denoted by different letters, as determined by a one-way repeated measures ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

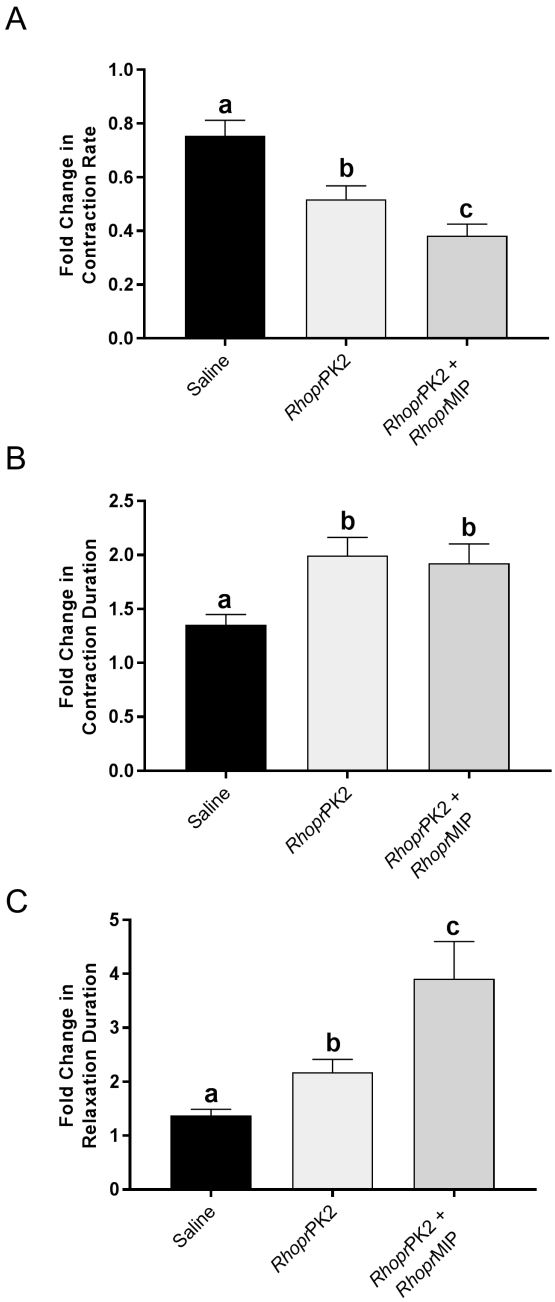

Figure 2-11: Motility of ilea isolated from female mosquitoes in response to added saline (vehicle control), 1 µM *Rhopr*PK2 and 1 µM *Rhopr*MIP (inhibitory control). The change in contraction frequency **(A)**, duration **(B)** and length of time between contractions **(C)** was measured relative to baseline recordings. Values are presented as mean ± SEM from 17 preparations. Significant differences between the treatments are denoted by different letters, as determined by a one-way repeated measures ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

2.4.4 AedaePK1 on hindgut ion transport

As determined by the SIET, most recta (~70%) exhibited haemolymph-directed Na^+ transport (i.e. absorption) in saline prior to treatment, while the remaining recta exhibited lumen-directed transport (i.e. secretion); however, only absorptive rectal preparations were used to examine the potential for *Aedae*PK1 in eliciting an ionoregulatory role. Furthermore, measurements of the preparations displayed variability in baseline transport activity in saline, therefore the difference in ion flux, following application of saline alone or containing peptide, relative to initial transport activity in saline was calculated. The resultant data showed that Na^+ absorption decreased by 52.8±31.4 pmol cm^{-2} s^{-1} after saline application (Fig. 2-12A); however, the net Na^+ transport remained absorptive. An earlier study showed that the receptor for *A. aegypti* kinins (*Ae*KR) was localized to the haemolymph-facing outer rectal pad membrane and *Ae*KR knockdown by RNA interference decreased excretion (Kersch and Pietrantonio, 2011). This result suggested that an available kinin analog (i.e. drosokinin) may decrease reabsorption over the rectal pads, given that mosquito kinins stimulate diuresis by the Malpighian tubules (Veenstra et al., 1997). Indeed, we found that the ionomodulatory effect of drosokinin along the rectum was significant leading to a four-fold decrease in Na^+ absorption (201.6±39.0 pmol cm^{-2} s^{-1}) compared to saline control (Fig. 2-12A, B, C). In response to *Aedae*PK1, Na^+ transport decreased two-fold (104.4±43.2 pmol cm^{-2} s^{-1}), although this was not significantly different from changes in ion transport following treatment with saline alone (Fig. 2-12A, D, E).

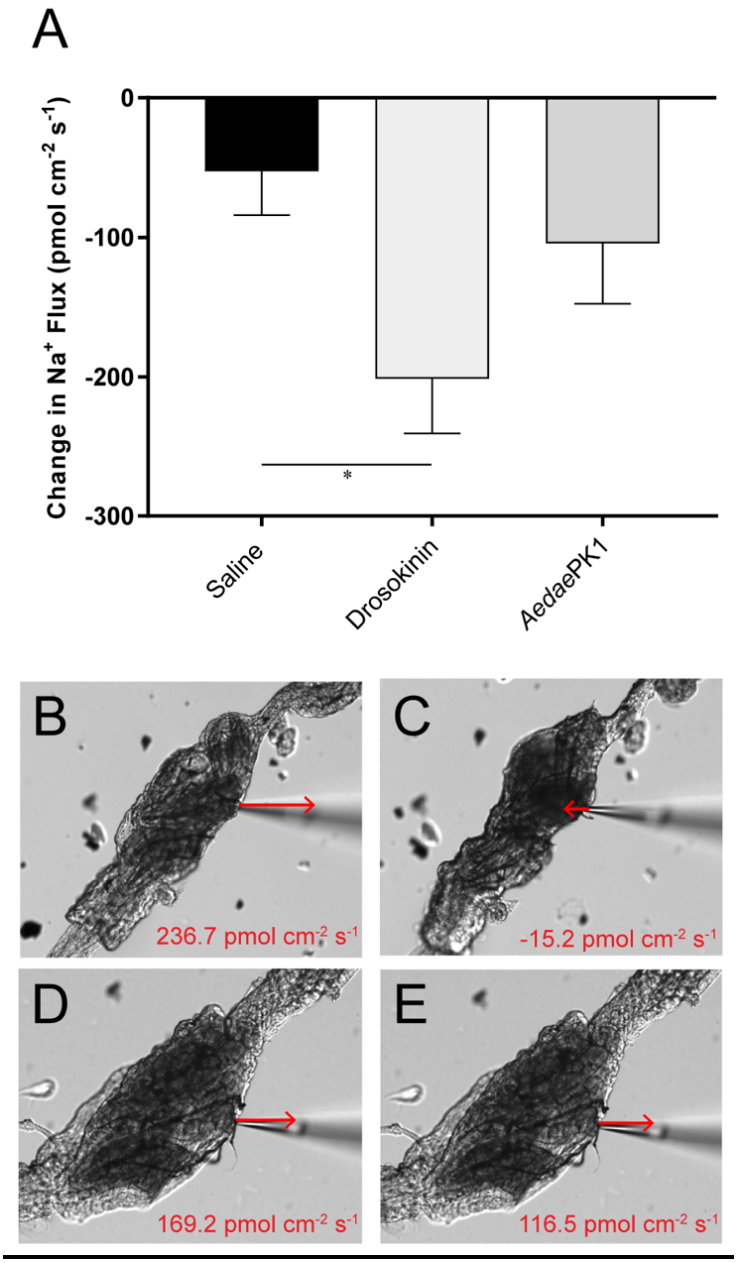

Figure 2-12: Changes in Na^+ transport across female rectal pad epithelia in response to saline (vehicle control), 1 µM drosokinin and 1 µM *Aedae*PK1. Experiments were run only using hindgut preparations that were initially exhibiting haemolymph-directed ion flux (absorption) when unstimulated. Mean ± SEM obtained from 10-14 tissue preparations. There was a significant reduction in Na^+ absorption in response to drosokinin ($p<0.05$); however, ion transport did not significantly change following *Aedae*PK1 treatment in comparison to basal transport activity **(A)**, determined by a one-way ANOVA and Tukey's post-hoc test. Still images of the dissected rectum are demonstrated with ion flux recordings obtained from a sample measurement during baseline activity in saline **(B, D)** followed by either drosokinin **(C)** or *Aedae*PK1 **(E)** application. Arrows indicate the direction and approximate the magnitude of Na^+ transport, but are not drawn to scale. Scale bars, 100 µm.

2.5 Discussion

In this study, we have characterized the functional activation of *A. aegypti* PK1 and PK2 receptors in response to various PKs and related neuropeptides. Our findings indicate that PK1-R is most sensitive to PK1 peptides possessing a WFGPRL-NH_2 carboxyl terminus, also referred to as tryptopyrokinins (Veenstra, 2014), whereas PK2-R is most sensitive to PK2 peptides characterized by their FXPRL-NH_2 motif. This selective activation was similarly observed in *A. aegypti*, *A. gambiae*, *R. prolixus* and the European corn borer, *Ostrinia nubilalis* (Choi et al., 2013; Olsen et al., 2007; Paluzzi and O'Donnell, 2012; Nusawardani et al., 2013), further confirming the binding specificity and selectivity of PK receptors to their subfamily-specific ligands.

Pyrokinins were first discovered in insects based on their effects on hindgut physiology (Holman et al., 1986). The hindgut collects undigested foodstuff passed from the midgut through the pyloric valve, as well as fluid secreted from the Malpighian tubules, and guides these contents along the alimentary canal for waste excretion (Hine et al., 2014). Here we found that relative to other regions examined within the adult mosquito alimentary canal, PK2-R transcript was strongly enriched in the ileum, and PK1-R in the rectum. Our results partially agree with a previous RT-PCR analysis in *A. aegypti* that examined a subset of the tissues/organs we studied herein. Specifically, while enrichment of PK1-R in the midgut and ovaries was not evident as reported earlier (Hellmich et al., 2014), we found this receptor transcript to be significantly enriched in the rectum. For PK2-R, this receptor was previously reported to be expressed within the ovaries in adult females (Hellmich et al., 2014), which is in agreement with our observations showing significant enrichment in female reproductive organs along with the enrichment we observed in the ileum. Peptide immunolocalization further complimented these findings. PK1-

like immunostaining was observed in the axon net encircling the pyloric valve that was contiguous with axonal projections over the ileum and extending towards the rectum, revealing these sites as prospective targets for PKs. Due to the extensive network of musculature in the mosquito hindgut (Rocco et al., 2017), as well as the ion transporters dispersed along the epithelia (Patrick et al., 2006), this structure requires the coordination of ionomodulatory and myotropic activity for controlled waste elimination (Kwon and Pietrantonio, 2013). These processes are regulated by neuropeptides along with other neurochemicals and, considering the distinct expression profile of PK receptors in the hindgut that was herein identified along with PK1-like immunohistochemical staining in association with the hindgut, we examined the potential involvement of pyrokinins in these processes. Although hindgut muscle contractions are myogenic, they can also be modified by neurochemical input from innervation extending from the ventral nerve cord (Audsley and Weaver, 2009). Previously, it was observed that immunostaining for the ovary ecdysteroidogenic hormone (OEH) isolated from *A. aegypti* was associated with the pyloric valve nerve net as well as axonal projections that continued towards the rectum; however, a direct source of this immunoreactivity was not observed although assumed to originate in the ventral nerve cord (Brown and Cao, 2001). Comparatively, cells of the terminal abdominal ganglion innervate the locust hindgut to influence its motility (Donini et al., 2002). Some of these neurochemical factors have been identified as myotropins acting on *A. aegypti* hindgut, including serotonin and diuretic hormone 31 (Messer and Brown, 1995; Kwon and Pietrantonio, 2013), which also promote fluid secretion across the Malpighian tubules (Sajadi et al., 2018). The concerted action of these and other factors suggest coordination between the diuretic response and hindgut motility to regulate urine and blood bolus expulsion in female mosquitoes.

In the current study, we examined the role of PK1 and PK2 peptides in hindgut motility. Although its effects were not as potent as a member of the myoinhibiting peptide family (i.e. *Rhopr*MIP), which is a known myoinhibitor of the insect hindgut (Lange et al., 2012), the ability of *Rhopr*PK2 to significantly reduce ileal motility suggests a role in regulating digestive and excretory processes. Visceral muscle contractions along the hindgut aid in the movement of undigested foodstuff following post-prandial diuresis to eliminate waste (Te Brugge et al., 2008). Inhibition of hindgut motility by PKs therefore warrants further study to determine their functions in these processes and during different feeding states. This is the first study to establish a myoinhibitory role for a PK2 peptide on the insect hindgut, since members of this neuropeptide family sharing the conserved FXPRL-NH_2 carboxyl terminus have previously been characterized as having myostimulatory actions on the hindgut of *L. maderae*, *Periplaneta americana*, *Zophobas atratus*, and *Tenebrio molitor* (Holman et al., 1986; Predel and Nachman, 2001; Marciniak et al., 2012), demonstrating their diverse effects across various insect species.

*Aedae*PK1 did not significantly influence myotropic activity in the mosquito rectum, despite receptor transcript enrichment in this organ, which prompted us to examine other potential functions. The rectum serves as the final site for reabsorbing ions, water and essential metabolites back into the haemolymph, ultimately determining the composition of excreted matter (Coast, 2009; Beyenbach and Piermarini, 2011). PK1-like immunoreactivity revealed axonal projections terminating in close association with cells located within the lumen of the rectal pads (Fig. 2-4), structures that protrude from the rectal epithelium. This close association is indicative of a potential neurotransmitter/neuromodulatory role, in which local release at these synaptic terminals may activate receptors on these uncharacterized cells. To date, morphological studies of the mosquito rectum have been limited. Hopkins (1967) was one of the very few to

study the ultrastructure of the mosquito rectal pads, revealing a single layer of epithelia surrounding a central canal. The central canal of the rectal pads carries a tracheal trunk, which extends into several tracheolar branches throughout the epithelia. Hopkins (1967) proposed the presence of tracheal and glial cells associated with this tracheal trunk. In some insects, cells along this region have been termed medullary cells, situated in close proximity to neurosecretory terminals (Gupta and Berridge, 1966). In *Blattella* and *Blaberus* rectal pads, axonal projections terminate at sites adjacent to the basal surface of secondary cells, which may help regulate fluid reabsorption (Wall and Oschman, 1973). By examining structural changes in the rectal pads following a blood meal in mosquitoes, these sites have been proposed to play crucial roles in maintaining iono- and osmoregulation to help restore haemolymph homeostasis (Hopkins, 1967). The presence of ion transporters within these structures was later revealed, whereby basolateral P-type Na^+/K^+-ATPase and apical V-type H^+-ATPase staining along the rectal pad epithelia supported that they serve as sites for ion transport, enhancing the overall absorption of ions and water back into the haemolymph prior to waste excretion (Patrick et al., 2006).

Although the exact mechanisms of ion and water transport within the rectal pads have not yet been characterized, the *Ae*KR was previously localized along the haemolymph-facing membrane surface of these structures (Kersch and Pietrantonio, 2011). Our results confirm that a structurally related kinin from *Drosophila*, drosokinin, inhibits Na^+ absorption at these sites, showing that ion transport mechanisms along the rectal pads may be regulated by neuropeptides. PK1-R transcript detection along with distinct PK1-like immunoreactivity within the rectum was initially suggestive of an ionoregulatory role at these sites. However, since *Aedae*PK1 did not significantly influence Na^+ transport along the rectal pad epithelia, the function of *Aedae*PK1 at the rectum remains unclear. To better understand its role in mosquito hindgut physiology, it is

critical in future studies to identify and characterize the cells closely associated with PK1-like immunoreactivity within the rectal pad central canal (Fig. 2-4), which are distinct from the epithelial cells where *Ae*KR was immunolocalized previously over the outer rectal pad membrane (Kersch and Pietrantonio, 2011).

The *AAEL005444* gene (Strand et al., 2016) encodes *Aedae*PK1 along with two anti-diuretic hormones (*Aedae*CAPA-1 and -2), with the latter regulating the inhibition of fluid secretion across the Malpighian tubules of larval and adult *A. aegypti* (Ionescu and Donini, 2012; Sajadi et al., 2018). In the adult *A. aegypti*, PK-like immunoreactivity has been previously localized in the central nervous system, including numerous cells in the brain, within three groups of neurons of the SEG, and the abdominal ganglia of the ventral nerve cord (Hellmich et al., 2014). In larval stage *A. aegypti*, genes encoding pyrokinins were molecularly characterized revealing *hugin* gene expression, which encodes both PK1 and PK2 neuropeptides, primarily within the SEG, whereas *capa* gene expression was detected mainly within the abdominal ganglia (Hellmich et al., 2014). In support of this observation, neuropeptidomic analyses have shown the presence of pyrokinins originating from both the *hugin* and *capa* gene in the SEG, whereas only *capa* gene-derived *Aedae*PK1 was found within the abdominal ganglia, which supply the neurohemal perivisceral organs via the unpaired median nerve (Predel et al., 2010). Given that PK1 and anti-diuretic hormones are derived from a common precursor peptide, along with the proposed roles that rectal pads may play in osmoregulatory processes, there could be some functional relatedness between these peptides, such as binding to distinct receptors expressed along different target organs of the alimentary canal to exert similar overall actions. Localization of PK1-like immunoreactivity in close association with cells within the lumen of the rectal pads supports a role for *Aedae*PK1 in the regulation of ion and water absorption owing

to their specific receptor being enriched within this organ. Following nectar or blood feeding, the excess water and ions taken up from the meal pose a challenge to the hydromineral balance of the organism (Coast, 2009). Although *Aedae*PK1 did not elicit changes to Na^+ transport across the rectal pad epithelia of unfed adults, examining other critical processes at these sites, such as anion or water transport, may uncover the role of this neuropeptide in the mosquito rectum. Since the rectal pads have been suggested to play a role in helping to alleviate this insult to haemolymph homeostasis (Hopkins, 1967), *Aedae*PK1 may require the initiation of other signaling pathways involved during postprandial diuresis to exert its physiological actions and aid in the regulation of ion and water balance. However, the control of these processes along the hindgut is not yet well understood and, as a result, updating the current model of the *A. aegypti* rectal pad ultrastructure through modern electron microscopy approaches is necessary. This may in turn help unravel the function of *Aedae*PK1 that activates its receptor *Aedae*PK1-R expressed in this organ, along with other neuropeptides that target these structures.

The presence of *A. aegypti* PK2-R transcript associated with both the ileum and reproductive organs indicate that its PK2 ligands may exhibit pleiotropic actions. Similar to our findings, it was earlier shown by RT-PCR that PK2-R is present in reproductive tissues in *A. aegypti*, where specifically the ovaries were examined (Hellmich et al., 2014), which is consistent with observations on PK receptors in other blood-feeding arthropods, including *R. prolixus, I. scapularis* and the cattle tick, *Rhipicephalus microplus* (Paluzzi and O'Donnell, 2012; Gondalia et al., 2016; Yang et al., 2015). Although their physiological role has not yet been characterized in these hematophagous arthropods, receptor transcript in reproductive organs indicates that PK2 peptides may target these sites to regulate processes critical to mosquito reproduction or development. In other insects, for instance, related PKs were shown to stimulate

L. migratoria, P. americana, *Z atratus* and *T. molitor* oviduct contractions (Schoofs et al., 1993; Predel and Nachman, 2001; Marciniak et al., 2012), which promote the passage of eggs towards the common oviduct for fertilization (Wigglesworth, 1942). They also trigger embryonic diapause by binding to receptors in developing *B. mori* ovaries, upregulating trehalase expression, which promotes glycogen accumulation in oocytes required for the initiation of diapause (Homma et al., 2006; Kamei et al., 2011; Su et al., 1994). Similar roles have been reported in other lepidopteran species, such as the tussock moth, *Orgyia thyellina*, where PK induces embryonic diapause and also promotes ovarian development (Uehara et al., 2011).

In *A. aegypti* mosquitoes, females must feed on blood to initiate egg production within the ovaries (Clements, 2000). Upon feeding, neuropeptides such as insulin-like peptides and OEH are secreted from brain neurosecretory cells to produce and stimulate nutrient uptake into developing oocytes by promoting ecdysone synthesis (Brown et al., 1998, 2008; Helbling and Graf, 1998; Riehle and Brown, 1999). PKs have been previously shown to regulate ecdysteroidogenesis upon receptor activation in *B. mori* prothoracic gland (Watanabe et al., 2007). To assess whether PK2 may be involved in similar processes in mosquitoes, future studies should investigate PK2-R expression upon blood feeding to further delineate the involvement of the PK signaling system in regulating these previtellogenic processes. Although no studies to our knowledge have examined the action of PKs on reproductive success in insects, PK2-R transcript expression associated with this organ warrants further investigation to reveal its putative role in mosquito reproductive biology. Given the importance of these PK receptor-enriched organs in a range of physiological activities, these insights may be useful in developing novel strategies to target processes critical to mosquito survival and reproduction, and could ultimately reduce the burden of these disease vectors.

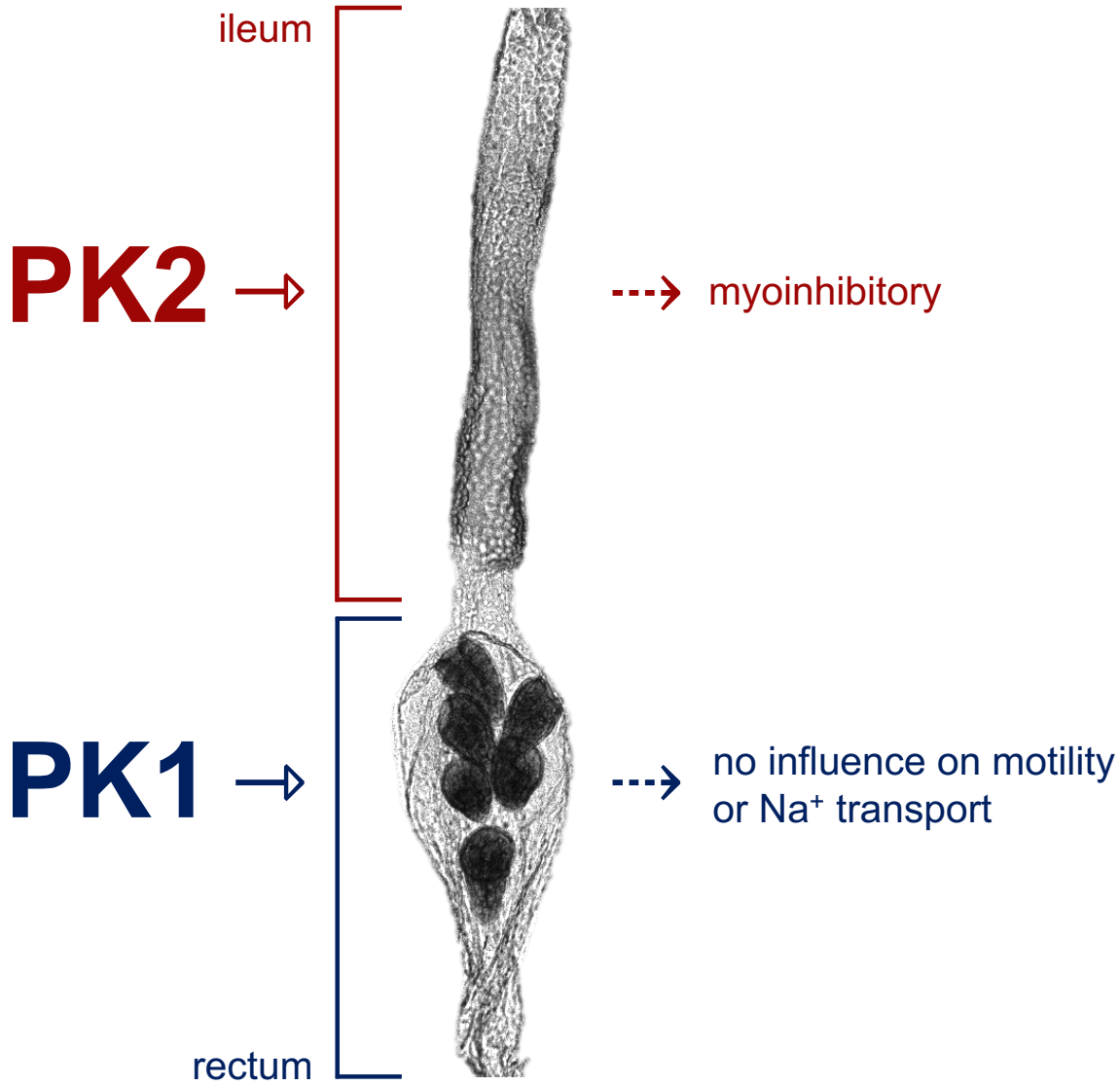

Figure 2-13: Still image used to depict pyrokinin target sites along the hindgut of *Aedes aegypti*. The *A. aegypti* PK2-R is expressed in the anterior hindgut region (ileum) and a pyrokinin-2 (PK2) peptide was shown to inhibit ileum motility, whereas the *A. aegypti* PK1-R is expressed in the posterior hindgut (rectum) indicating a pyrokinin-1 (PK1) peptide, such as *Aedae*PK1, acts on the rectum, although its exact role remains unknown.

2.6 Concluding remarks

This is the first study to elucidate the role of PKs in a haematophagous arthropod and identify sites of neuropeptide action (Fig. 2-13). Although we have discovered a novel function for PK2 in mosquitoes, there is still much to be learned with regards to its specific involvement in processes such as feeding, digestion and excretion. The next steps for future research should therefore examine receptor knockdown using reverse genetics approaches in order to deduce abnormal phenotypes and also uncover the function of PK1, which has yet to be characterized in the mosquito. Gaining a more comprehensive understanding of these pathways can allow us to use the PK signaling system as a potential target for vector control. This knowledge can be harnessed to develop effective mimetic neuropeptide analogs that disrupt these pathways as a means for pest management.

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