DISTRIBUTION AND ACTIVITY OF CAPA-LIKE PEPTIDES IN THE BLACK-LEGGED TICK, *IXODES SCAPULARIS*

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

GRADUATE PROGRAM IN
BIOLOGY

YORK UNIVERSITY
TORONTO, ONTARIO
December 2017

ABSTRACT

Ixodes scapularis is a chief vector for a range of diseases. Due to their obligate blood feeding strategy, it is crucial for *I. scapularis* to maintain ionic and osmotic homeostasis. In insects, CAPA peptides act as either diuretic or anti-diuretic hormones controlling the movement of excess ions, water, and metabolic wastes secreted in the primary urine. Immunohistochemical techniques were used to localize CAPA-like peptides to the synganglion, midgut, reproductive tissues, and hindgut. Given the presence of CAPA-like immunoreactivity in the hindgut, i*n vitro* bioassays were conducted. A significant increase in the frequency of hindgut contractions were observed when exposed to 1 μM IxoscCAPA-PVK. RT-PCR techniques, however, solely localized the CAPA transcript to the tick synganglion. The immunoreactivity observed in the peripheral tissues, including the midgut, are indicative of the RFamide-related peptide, sNPF, as the transcript coincides with the immunoreactivity, and ELISA results revealing affinity of the primary antibody (rabbit anti-*Rhopr*CAPA-2α).

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to Dr. Jean-Paul Paluzzi, for first providing me with the opportunity to conduct research for my undergraduate honours thesis in his laboratory, and for taking me in once more for my graduate research. His expertise and ingenuity has made this the most rewarding experience, as I have gained a plethora of knowledge. His kindness, encouragement, and continued support throughout the years will always be greatly appreciated. I would also like to thank Dr. Andrew Donini for his valuable input and guidance that helped shape my research. Sincere thanks to the Donini lab members for their feedback and support.

These acknowledgments would not be complete without mentioning my lab mates for their inputs and support: Melanie, Carmela, Dave, Azizia, Andreea (Cristina), Aryan, Ali O., Farwa, Daniel, and Fidan.

Finally, I would like to thank my family for their continued support, motivation, and love throughout my academic pursuits. Thank you to Dr. Ian Orchard for kindly providing me with the anti-*RhoprCAPA-2* antibody, Dr. Angela Lange for the *Rhopr*SIFa peptide, and Lesia Szyca for the illustrations.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I:

The tick: Organ systems, physiology and neurochemicals

Ixodes scapularis

Ixodes ticks have four life stages which include the developing embryo and three stages that require a blood meal; larval, nymph and adult stage (Balashov, 1998; Sonenshine and Roe, 2014). Mated female *Ixodid* ticks have two feeding periods, consisting of the slow feeding and rapid engorgement phases. In addition, female *Ixodid* ticks have two post-feeding stages, which include preoviposition and oviposition periods, after which they die (Tarnowski and Coons, 1989). There is a gradual increase in the weight of the tick starting from the beginning of the slow-feeding period until the onset of the rapid engorgement phase. The rapid engorgement period occurs after mating and lasts from 24 to 72 hours. For this duration, there is a dramatic increase in the weight of the tick (Sauer and Hair, 1972; Tarnowski and Coons; 1989). Once fully engorged, the tick detach from the host.The adult female then undergoes oogenesis prior to oviposition. The total blood-feeding period at 30˚C spans approximately 3-4, 4-7 and 8-12 days for the larval stage, nymphs and females, respectively (Balashov, 1998; Sonenshine and Roe, 2014). The adult males feed approximately for 2-3 days when females are present. Males detach once the female-produced sex pheromone is detected and start mating with the attached feeding female (Sonenshine et al., 1982). After mating, males may or may not resume feeding. If no females are present, males remain attached to the host and either feed slowly or do not feed at all (Tarnowski and Coons; 1989).

During feeding, tick salivary secretions are crucial for the manipulation and suppression of the vertebrate host's immune responses. The salivary pharmacological cocktail has been shown to contain anticoagulants, immunosuppressive agents and anti-inflammatory factors that inhibit haemostatic defences (Narasimhan et al., 2002; Wikel et al., 1994; Ribeiro, 1987). These properties allow the tick to blood-feed without blood coagulation or host immune responses. As

a consequence, the decreased host immune defence allows efficient transmission of pathogens to the new host.

Ixodes scapularis are potent vectors for a range of diseases. Lyme disease is a bacterial infection that was originally believed to be a form of arthritis as it causes swelling and pain within the large joints of the body (Steere et al., 1977). The tick spirochetal bacterium, *Borrelia burgdorferi,* was identified to be transmitted by various vectors such as the black-legged tick, *I. scapularis* (Burgdorfer et al., 1982). It is suggested that ticks acquire these bacteria by feeding on infected rodents during their larval stage and are able to transmit this pathogen to a new host in the next developmental stage (Donahue at al., 1987; Mather et al., 1989). However, it is highly unlikely that these spirochetes are passed on trans-ovarially to offspring (Patrican, 1997). The initial infections by *B. burgdorferi*, reside mainly in the midgut diverticula (De Silva and Fikrig, 1995; Piesman et al., 2001). During blood-feeding in their next life stage, the spirochetes undergo a dramatic change and rapidly multiply. This is followed by the migration of the bacterium into the haemolymph and then to the salivary glands, from which they are then transmitted to the vertebrate host during the second phase of blood-feeding (De Silva and Fikrig, 1995; Piesman et al., 2001; Benach et al., 1987). Studies spanning the period from 1993 to 2012, focusing on the north east and north central USA have shown a 320% and 250% increase in Lyme disease cases, respectively (Kugeler et al., 2015).

Osmoregulation

Osmoregulation is the process by which a delicate balance is maintained between uptake and loss of solutes and water, which allows organisms to inhabit a vast array of ecological niches and thrive in the face of various physiological stressors. Terrestrial arthropods, for example,

compensate for unfavourable conditions by behavioural and physiological responses that are an extension of their adaptations to terrestrial life (Cloudsley-Thompson, 1975). These include diurnal and seasonal rhythms of activity, diapause, and diuretic or anti-diuretic strategies (Cloudsley-Thompson, 1975). Terrestrial arthropods can be divided into two main groups; the first group consists of those that are susceptible to water loss through their integument, thus are essentially nocturnal and/or cryptozoic. The second group is comprised of arthropods that have epicuticular layers of wax that function to restrict transpiration allowing them to exploit a wide range of terrestrial habitats, both at night and during the day (Cloudsley-Thompson, 1975). These groups also differ by the means by which they excrete nitrogenous waste products. Organisms that belong in the first group eliminate nitrogen mainly as ammonia; as opposed to those of the second group that excrete insoluble purines uric acid and guanine, respectively (Cloudsley-Thompson, 1975). As obligate blood-feeders, it is essential for black-legged ticks to maintain osmotic and ionic homeostasis after blood engorgement. One of the main pathway by which they remove excess ions and water from a blood meal is via the salivary glands (Tatchell, 1967). During a blood meal, excess water passes across the gut epithelium to the haemolymph, then to the salivary gland where it is returned to the host through secretion (Tatchell, 1967; Kaufman an Philips, 1972). Approximately 80% of excess water and ions are excreted during on-host feeding, of which 75% is excreted by the salivary glands, about 3% through evaporation from the integument and spiracles and the remainder through the rectal sac and Malpighian tubules via guanine crystals (Kaufman an Philips, 1973). The movement of the main ions and water during a blood meal are summarized in Figure 1.1.

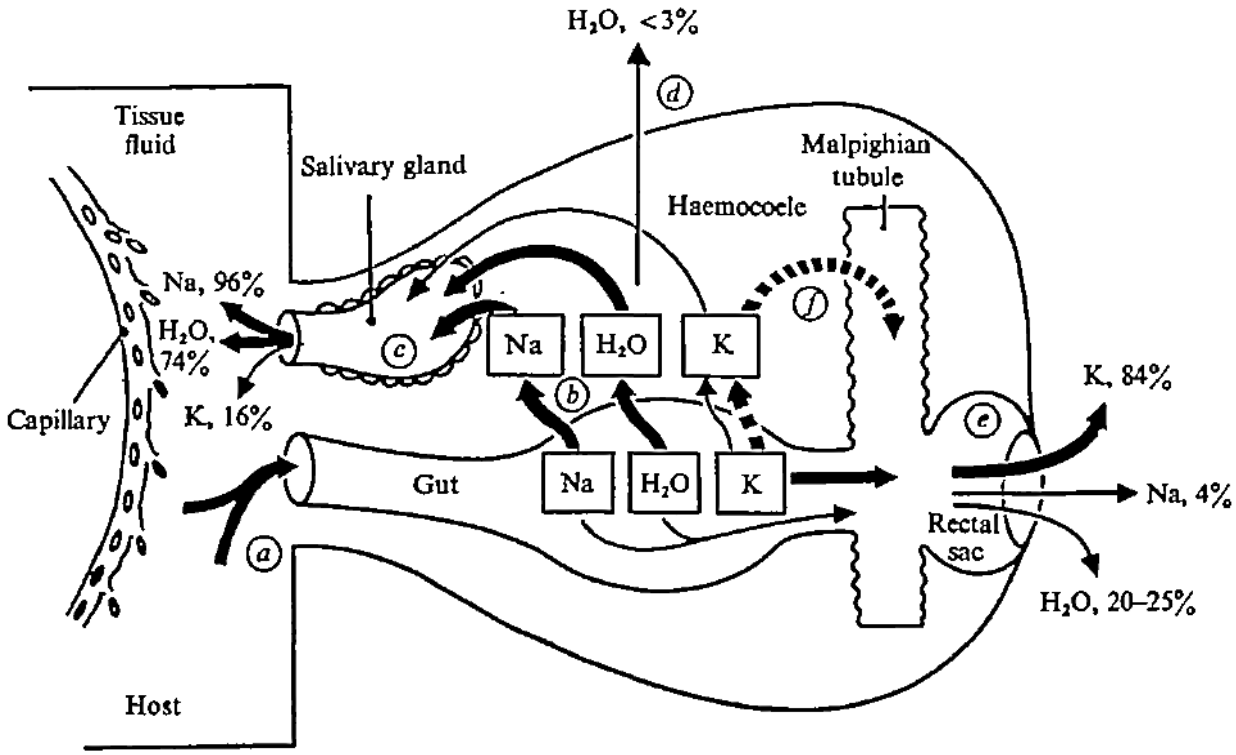

Figure 1.1: Summary of the ingestion and elimination of water and ions in the female *Ixodid* tick during a blood meal. Thick solid arrows indicate major routes. Thin solid arrows denote minor routes. Heavy broken arrows suggest a possible major route. Percentages represent the proportions of the total amount of ions excreted for the duration of the blood meal. (a) The blood meal, consisting of a mixture of whole blood and other tissue fluids are taken up into the tick. (b) Na^+, water, and minor amounts of K^+ enter the haemolymph via the midgut diverticula. (c) Na^+ (as NaCl), water, and some K^+ (those in excess of the tick's requirements) are transferred back into the host through salivary secretions. (d) A small quantity of water is evaporated via the surface of the integument. (e) Large quantity of K^+ is either passed directly from the midgut diverticula to the rectal sac, via the intestine, and excreted bythe anus, or (f) K^+ enters the haemolymph from midgut diverticula and then into the Malpighian tubules to be excreted as faecal matter via the anus. Reproduced with permission from Kaufman and Phillips, 1973.

Organ Systems in the Tick

Salivary Glands

The multifunctional salivary glands (SGs) are crucial in the biological success of ticks and play a vital role in the transmission of pathogens (Sauer et al., 1995). They constitute the second largest organ, originating from the ventral anterior region of the tick, which divides into two laterally localized salivary ducts that project posteriorly and branch into numerous lobular ducts (a branch from the main salivary duct) each leading to an alveolar duct in the alveoli (acini; Figure 1.2; Coons and Roshdy, 1973; Sauer et al., 1995). The main salivary duct is a chitinous tube comprised of a layer of epithelial cells that arejoined by septate desmosomes, located on a basement membrane (Coons and Roshdy, 1973). These epithelial cells have elongated nuclei and various cytoplasmic organelles that include free ribosomes, mitochondria, and copious amounts of microtubules (Coons and Roshdy, 1973). The epithelium is lined by a thick cuticular layer, which forms three distinct regions: a thin, electron dense outer region facing the lumen; a larger, less dense middle region of complex laminations; and an electron-dense inner region (Coons and Roshdy, 1973).

The SGs consist of three different types of acini; type I, II and III (Figure 1.3; Binnington, 1978). The agranular acini (type I) can be found attached to the anterior region of the main salivary duct and do not change in size during feeding (Binnington, 1978; Sauer et al., 1995). Type I acini secrete hygroscopic solutions, containing salts from increasingly concentrated haemolymph onto the mouthparts to take up moisture until rehydration and subsequent re-ingestion is possible (Rudolph and Knulle; 1979; Bowman and Sauer, 2004; Sonenshine and Roe, 2014).

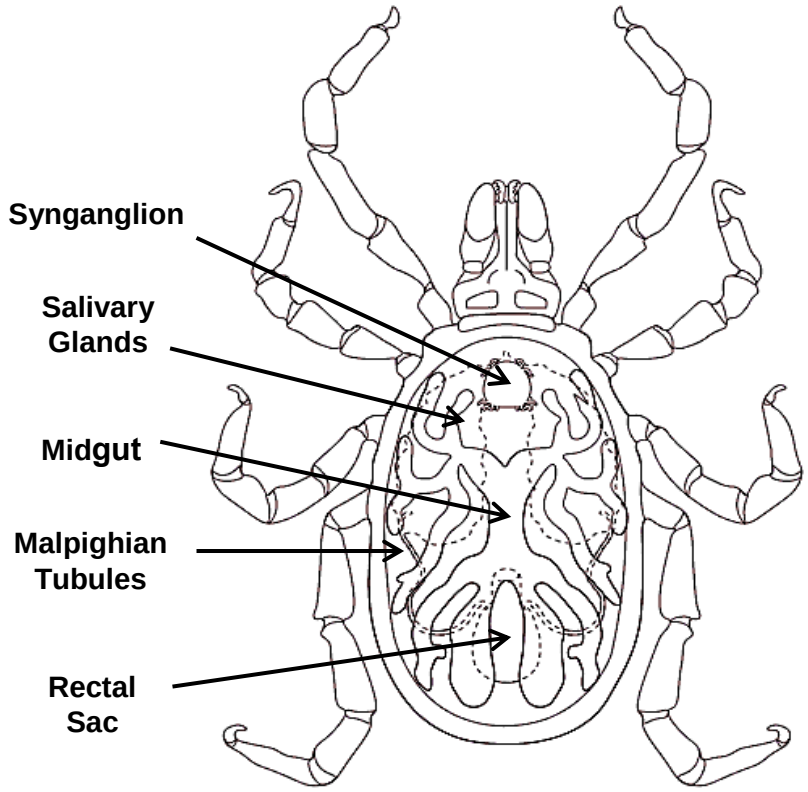

Figure 1.2: Schematic overview of the internal anatomy of adult *Ixodes scapularis*. Broken lines represent organs that are localized most ventrally. The synganglion is localised antero-medially, followed by laterally localized pair of salivary glands (ventral to the midgut). These salivary glands project posteriorly. The midgut is localized most dorsally. It is segmented into two regions, the midgut diverticula (lateral branches) and the midgut stomach (medial body). The posterior region of the midgut stomach has an intestinal projection that goes ventrally and connects to the anterior region of the rectal sac. The rectal sac is also connected anteriorly to a pair of Malpighian tubules that project anteriorly. Reproductive system not shown for simplicity. Drawing by Lesia Szyca.

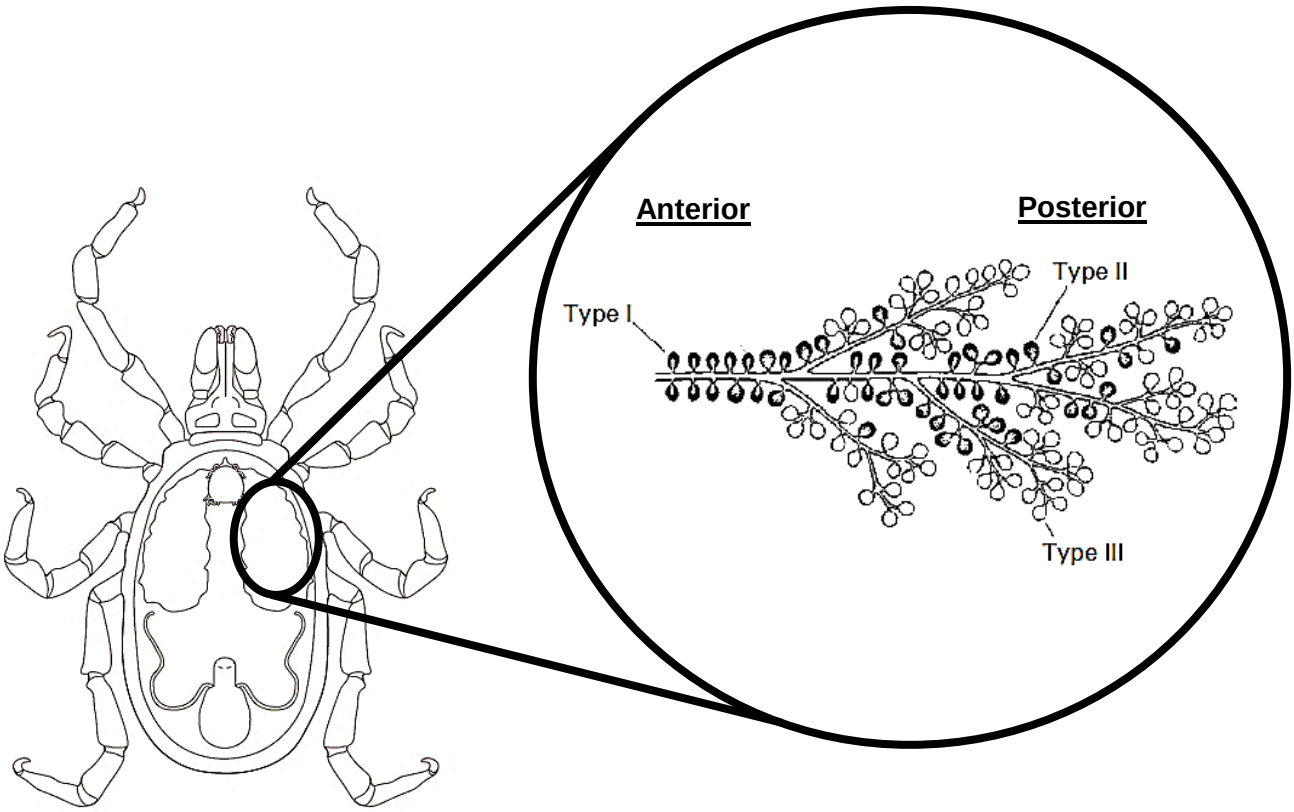

Figure 1.3: Illustration of the acini arrangement in the salivary glands of the female, *Ixodes scapularis.* The different acini are distinguished by the shading. The Type I acini, are found on the anterior region attached directly on the main salivary duct and some on the principal branches of that duct. Type II acini are more abundant and are proximally localized on the large branches. Type III acini are prominent in the distal regions of these branches (Adapted with permission from Binnington, 1978; Bowman and Sauer, 2004). Drawing by Lesia Szyca.

There are three recognized types of granular acini in *Ixodid* ticks; types II and III, which are present in both sexes, and the male-specific type IV acini (Binnington, 1978). Both types II and III are granule-secreting cells and change in size during feeding. Furthermore, both type II and III secrete the cement proteins that bind the tick's mouthparts to the host skin (Binnington, 1978; Sonenshine and Roe, 2014). Type II acini have a cuticular valve into a short lobular duct, which then attaches to the main duct. It is composed of six cell types 'A', 'B', and 'C_1-C_4' based on morphology and reactions to specific staining (Binnington, 1978; Bowman and Sauer, 2004). Type II acini dramatically increase in size during feeding, despite granules in the granular cells being depleted at the end of feeding (Bowman and Sauer, 2004). Type III acini bind to the main duct via a longer lobular duct, but in comparison to the type II acini, have only three cell types: granular cell types 'D', 'E', and 'F' (Binnington, 1978; Bowman and Sauer, 2004). Type III acini also increase greatly in size during feeding, but it is cell type 'F' that undergoes drastic proliferation of the plasma membrane and mitochondria (Bowman and Sauer, 2004; Sonenshine and Roe, 2014). The transformed 'F' cell is believed to be responsible for the majority of the fluid secretions across the salivary glands, which enables the tick to eliminate much of the water and salts taken up during a blood meal so as to concentrate the nutritive portion of the blood (Bowman and Sauer, 2004; Sonenshine and Roe., 2014). Thus, these morphological and physiological specializations allow the salivary glands to be the primary osmoregulatory organ in *Ixodid* ticks (Sonenshine and Roe, 2014).

As already mentioned, type IV acini have been found only in males, in addition to the aforementioned acini types I- III (Bowman and Sauer, 2004). The males also have 'F' cells, but do not undergo as dramatic a change since adult males take up very little blood (or none at all), in comparison to females (Bowman and Sauer, 2004; Sonenshine and Roe, 2014). It has also

been noted that type IV male-specific acini may be involved in the copious salivation of ticks during copulation (Feldman-Muhsam et al., 1970; Bowman and Sauer, 2004).

Once blood enters the midgut, excess water passes through the gut epithelium to the haemolymph, then to the salivary gland where it is returned to the host through secretions (Tatchell, 1967; Kaufman an Philips, 1972). In addition to the removal of excess water, of the total excess Na^+ excreted, approximately 96% is through the salivary secretions, in addition to some excess Cl^- (Figure 1.1; Kaufman an Philips, 1972). This is a result of the midgut epithelium being able to transport Na^+ and Cl^-, but being relatively impermeable to K^+ (Kaufman an Philips, 1972).

Midgut

Blood ingested during a blood meal is drawn into the preoral canal by the action of a powerful sucking pharynx and passed into the midgut via the oesophagus (Figure 1.2; Sonenshine and Roe, 2014). The midgut in ticks is the largest organ in the body and is the site for the development of blood-borne parasites transmitted by ticks (De Silva and Fikrig, 1995; Piesman et al., 2001). It is comprised of two sections; the midgut stomach and distal branches from the midgut stomach, known as the midgut diverticula (Sonenshine and Roe., 2014). The midgut wall is made of an epithelial inner layer and a thin layer of elongated smooth muscle cells. In blood-sucking insects, digestion occurs rapidly in the lumen of the intestine. In ticks, however, the process of blood-meal digestion is a slow intracellular process, taking place in the endo/lysosomal vesicles of gut cells (Balashov, 1972; Sonenshine and Roe, 2014). The narrow gut lumen of an attached unfed tick is surrounded by the midgut epithelium, which is comprised by undifferentiated reserve cells (stem cells) and digestive cells remaining from previous instar feeding (Agbede and Kemp, 1985; Franta et al., 2010). During the second day of attachment,

there is the differentiation of the reserve cells into initial digestive cells, called prodigest cells (Agyei and Runham, 1995). On the fourth day of feeding, the digestive cells enlarge and begin to take up haemoglobin for digestion. Notably, the by-product of haemoglobin digestion, haeme, is a potentially toxic molecule. Minor amounts are absorbed through the gut, while the majority of haeme undergoes haeme detoxification by aggregating into organelles called haemosomes (Lara et al., 2003). Additionally, large endosomes and lipid vacuoles start to appear. During this phase of slow feeding, signs of detachment of the first digestive cells from the midgut epithelium and an increase in haemoglobinolytic activity are observed (Franta et al., 2010). At approximately six days post attachment, which marks the end of the slow feeding phase, dramatic changes take place in the midgut. These include the full extension of digestive cells, filled with residual bodies, large endosomes and lipid inclusions. The detached digestive cells are released into the lumen without lysis for possible removal by defecation (Franta et al., 2010). The next 24-48 hours comprises the rapid-engorgement phase, which is then followed by detachment from the host. The midgut epithelium is covered with flattened sessile digestive cells that remain associated with the epithelium. However, stem cells or initial digestive cells are no longer detected following the adult blood feeding event (Franta et al., 2010).

Hindgut

The hindgut in ticks consists of the intestine, the rectal sac, the anal canal, and the anus (Figure 1.2; Sonenshine and Roe, 2014). There is a pair of Malpighian tubules (MTs) that originate at the junction of the intestine and rectal sac. The intestine is a short, narrow tube that is a postero-ventral extension from the midgut stomach. It is comprised of a single layer of columnar or cuboidal epithelial cells on a fine basement membrane (Balashov, 1972; Sonenshine

and Roe, 2014). The function of the intestine is to transport undigested wastes from the midgut to the rectal sac. When it is filled with waste fluids, it distends and through peristaltic movements by a thin layer of circular and longitudinal muscles, the undigested materials are transported into the rectal sac. Groupings of circular muscles form a sphincter at the junction of intestine and the rectal sac, which prevents backflow of undigested residues to the midgut and excretory waste from the MTs (Balashov, 1972; Sonenshine and Roe, 2014).

The MTs and rectal sac encompass the remaining excretory organs in black-legged ticks in addition to the anal canal and anus (Kaufman and Phillips 1973; Balashov, 1972; Simo and Park, 2014). These organs are structurally and functionally homologous to those of insects; whereby the MTs of most terrestrial insects are responsible for nitrogenous waste elimination by producing the primary urine that goes to the hindgut (rectal sac). In insects, such as the haematophagous insect, *Rhodnius prolixus*, the hindgut then further modifies its luminal contents by reabsorption and secretion via the ileum and rectum prior to excretion as dry or semi-dry urine (Coast 2009).

Some insects such as Dipterans, have two epithelial cell types in the MTs; the first large predominant cell type with a tall brush border, are known as the principal cells. The principal cells are involved in the transepithelial secretion of Na^+ and K^+ (Beyenbach et al., 2010). The second cell type that comprises only a minor percentage of cells are the small, thin stellate cells (Beyenbach et al., 2010). These cells are involved in regulating the translocation of Cl^- through a paracellular pathway (Beyenbach et al., 2010). The iso-osmotic urine is formed in the MTs, by transporting water and ions (mainly K^+) from the haemolymph to the lumen by crossing the tubular epithelium. This process is achieved by taking advantage of the osmotic pressure gradient between the tubular lumen and the haemolymph. In most insects, like the mosquito *Aedes*

aegypti, the process is initiated by producing a relatively iso-osmotic solution containing KCl, NaCl, and water at the distal regions of the MTs (Beyenbach et al., 2010). Vacuolar-type H^+ ATPase (V-type ATPase) located at the apical, brush border membrane of principal cells powers the transepithelial secretion of electrolytes by pumping H^+ into the lumen (Pannabecker, 1995; Beyenbach et al., 2010). The protons in the lumen are cycled back into the cell through a cation-H^+ exchanger, an antiporter that results in the movement of Na^+ out of the cell and into the lumen, and the reverse process occurs for K^+ (Pannabecker, 1995; Beyenbach et al., 2010). The ouabain-sensitive Na^+-K^+ ATPase, a secondary active transporter, is located on the basolateral and apical membranes of MTs (Hegarty et al., 1991). The basolateral membrane of the MTs also contain Na^+/ K^+/$2Cl^-$ co-transporters, allowing the movement of chloride, sodium, and potassium ions from the haemolymph into the cell cytoplasm (Hegarty et al., 1991). MTs also aid in the removal of nitrogenous wastes from the haemolymph. In the kissing bug, *R. prolixus*, uric acid is formed and bound to K^+, forming an insoluble crystal, potassium urate (O'Donnell et al., 1983; Sonenshine and Roe, 2014), which is further concentrated by the removal of excess K^+ back into the haemolymph, but Na^+ is concentrated. Further concentration occurs in the rectal sac and resorption in the rectum lumen (O'Donnell et al., 1983)

In ticks, nitrogenous waste is eliminated in the form of guanine (2-amino-6-oxypurine) from the MTs (Sonenshine and Roe, 2014). These guanine-rich excreta from the MTs and undigested waste from the midgut are further concentrated in crystalline form in the rectal sac and expelled from the anus (Sonenshine and Roe, 2014). Very little is known about the molecular processes responsible for excretion in ticks, except recent studies pertaining to aquaporins. Aquaporins, are molecules that form pores in the cell membrane that facilitate water transport. Recently, the aquaporin, *Rs*AQP1, was found to be present in the gut, MTs, and most

abundant in salivary gland tissues in the partially fed brown dog tick, *Rhipicephalus sanguineus* (Ball et al., 2009). This is in correlation with previous literature which has shown that the midgut and salivary glands are tissues that are involved in high water flux in ticks. Since then, a homolog of *Rs*AQP1, was also identified in *Ixodes ricinus* (*Ir*AQP1) and was shown to be present in the rectal sac, alongside the gut and salivary glands (Campbell et al., 2010).

Reproductive System

The female reproductive organs are comprised of the ovary, filled with numerous white oocytes of varying size, the paired oviducts a single uterus, the vagina, and the seminal receptacle (Roshdy, 1969; Sonenshine and Roe, 2014). The tick ovary is located at the posterior region of the body and is of the panoistic type (lacking nurse cells in the germarium), and lacks follicular cells (Denardi et al., 2004; Saito et al., 2005). In an unfed adult female, the ovaries appear as a thin band of cells. Upon feeding, the ovaries become larger as the oocytes expand greatly and fill with innumerable brown yolk vitellin globules (Sonenshine and Roe, 2014). At the point when a fully engorged female drops from its host, the brown ovary occupies most of the interior of the body. The oviduct connects the vagina to the uterus, followed by a connecting tube joining the uterus to the cervical vagina. The cuticle-lined vestibular vagina connects the reproductive system with the external genital pore (Roshdy, 1969; Sonenshine and Roe, 2014).

The male reproductive system is comprised of the paired testes, the vasa deferentia, the seminal vesicle, the ejaculatory duct, and a large, complex, multi-lobed male accessory gland (Sampieri et al., 2016; Sonenshine and Roe, 2014). The testes are the site of primary spermatocyte formation and, like the ovaries, are located at the posterior of the body (Kiszweski et al., 2001; Sonenshine and Roe, 2014). The testes are joined to the common seminal vesicle,

located in the anterior region of the male accessory gland, via the vasa deferentia. During feeding, the testes and the vas deferentia enlarge and are soon filled with numerous spermatids (Oliver 1989; Sonenshine and Roe, 2014). The cuticle-lined ejactulatory duct connects the seminal vesicle with the external genital pore (Kiszweski et al., 2001; Sonenshine and Roe, 2014). The accessory glands have approximately 7-8 lobes of varying size and shape. It is responsible for the secretion of protein that comprises the spermatophore and the seminal fluid that nourishes the spermatids and facilitates fertilization.

Central Nervous System

In ticks, the central nervous system is fused into a compact synganglion, located in the antero-ventral region of the body (Figure 1.2). It is surrounded by an acellular neural lamella below a periganglionic sheath, possibly acting as a selective membrane for various nutrients and ions transporting from the haemolymph to the thick, amorphous perineurium and synganglion cortex layer below (Coons et al., 1974; Lees and Bowman, 2007). Peripheral nerves project from the synganglion to the various appendages and regions of the body. It has been noted that there are 18 centers where neurosecretory cells are localized throughout the cortex of the synganglion (Chow and Wang, 1974; Obenchain and Oliver, 1975; Lees and Bowman, 2007). The oesophagus subdivides it into the supra- and suboesophageal regions (Obenchain and Oliver, 1975; Lees and Bowman, 2007; Sonenshine and Roe, 2014).

The supraoesophageal region of the synganglion lies anterior and dorsal to the oesophagus and is comprised mainly of paired protocerebral, cheliceral, and palpal ganglia with associated paired nerve projections for all (Figure 1.4; Obenchain and Oliver, 1975; Lees and Bowman, 2007; Sonenshine and Roe, 2014). The supraoesophageal region is the smaller of the

two regions and houses the protocerebral ganglia, which combines to form a large dorsal mass of loosely organised neuropile and small areas of glomeruli (Figure 1.4; Lees and Bowman, 2007). Nerves from this region also innervate the salivary glands, pharynx oesophagus and eyes. A retrocerebral organ complex (ROC) surrounding the oesophagus at its junction with the midgut on the dorsal side of the supraoesophageal region was noted (Obenchain and Oliver, 1975; Sonenshine and Roe, 2014). This complex is believed to act as a neurohaemal organ, homologous to that of insects.

The suboesophageal region of the synganglion comprises the largest part and it resides posteriorly and is located ventral to the oesophagus (Lees and Bowman, 2007). It contains the four pairs of pedal ganglia with their trunk-like lateral extensions that innervate the four pairs of legs, and the unpaired opisthosomal ganglion with its nerves projecting laterally on each side (Figure 1.4; Obenchain and Oliver, 1975; Lees and Bowman, 2007; Sonenshine and Roe, 2014). There are numerous association-motor neurons in this region, which is suggestive of a primary role in motor coordination (Obenchain and Oliver, 1975; Sonenshine and Roe, 2014).

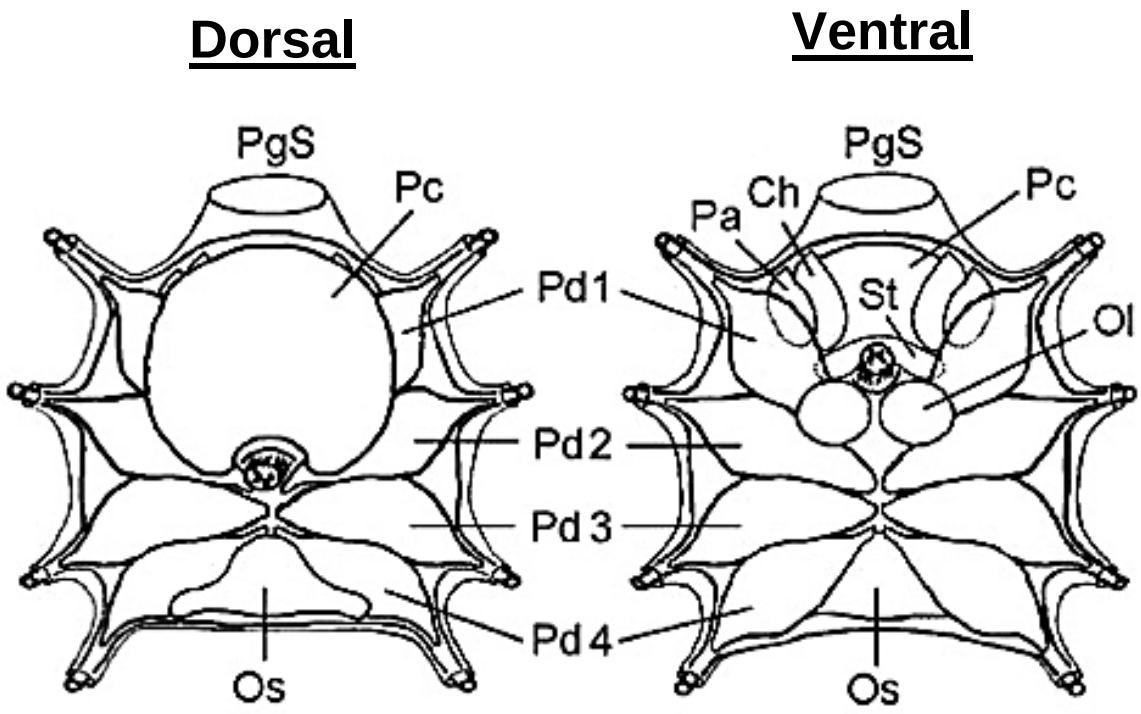

Figure 1.4: Diagrams of (dorsal and ventral views) of the various ganglia and lobes in the synganglion of the tick. Starting at the anterior region (top of image), there is the periganglionic sheeth (PgS); cheliceral ganglion (Ch); palpal ganglion (Pa); stomodeal lobe (St); protocerebral ganglion; pedal ganglia (Pd 1-4); olfactory lobes (Ol); opisthosomal lobe (Os). Reproduced with permission from Simo et al., 2009a.

Neurochemicals controlling tick physiology

The physiology of ticks are mainly governed by peptides and hormones, playing crucial roles in growth and development, reproduction, and water and ion balance (Caers et al., 2012; Gäde and Goldsworthy, 2003). As terrestrial arthropods and obligate blood feeders, it is essential for *I. scapularis* to maintain ionic and osmotic homeostasis (Coast and Garside 2005; Coast, 2009). In comparison to blood-sucking insects, it is expected that similar regulatory mechanisms are at play, whereby volumic, osmotic and ionic challenges are tightly controlled by diuretic (DH) and antidiuretic hormones (ADH).

The salivary glands (SGs) in ticks are their primary osmoregulatory organ, therefore they need to be under tight regulatory control. Axonal projections from the synganglion have been shown to innervate different acini types, thus regulating their function directly (Kaufman and Harris, 1983; Simo et al., 2009a and 2009b; Simo et al., 2017). SG fluid secretion assays (*in vivo* and *in vitro*) have shown dopamine, a catecholamine, to be a potent activator of fluid secretion via SGs, followed by norepinephrine and epinephrine, and isoproterenol in potency, respectively (Kaufman, 1977, 1978; Sauer et al., 2000; Simo et al., 2017). Dopamine has been suggested to elicit its function through autocrine/paracrine signalling (Simo et al., 2011; Koči et al., 2014; Simo et al., 2017). It binds to two related receptors expressed in the acini types II and III of the SGs: the dopamine receptor (D1) and the invertebrate-specific D1-like dopamine receptor (InvD1L; Simo et al., 2011; Simo et al. 2014; Simo et al., 2017). The D1 receptor, a G-protein coupled receptor (GPCR), leads to the activation of adenylate cyclase causing activation of the cyclic adenosine monophosphate (cAMP)-dependent pathway, resulting in fluid secretion (Sauer et al., 2000; Simo et al., 2011, 2014). The D1 receptors have been localized to the cell junctions on the luminal surface of acini types II and III, regulating the inward transport of fluid into the

acini (Simo et al., 2011, 2014, 2017; Kim et al., 2014). The InvD1L receptor exclusively activates the calcium-dependent pathway leading to the secretion of prostaglandin E_2 (PGE_2) into the salivary cocktail (Qian et al., 1998; Sauer et al., 2000; Simo et al., 2011, 2014). PGE_2 may subsequently induce the exocytosis of anticoagulant proteins through paracrine signal in tick SG (Qian et al., 1998; Sauer et al., 2000; Simo et al., 2011, 2014). Furthermore, the InvD1L receptor was found to be expressed within theaxon terminals in proximity to the myoepithelial cells, possibly regulating the expulsion of the acinar content into the connecting ducts of acini types II and III (Simo et al., 2011, 2014, 2017; Kim et al., 2014).

Moreover, axonal projections associated with myoinhibitory peptide (MIP; also called allatostatin B) and SIFamide have also been shown to innervate acini types II and III (Simo et al., 2009a and 2009b). Similar observations with respect to the neuroregulatory control of the hindgut have been determined, involving the antagonistic activity between SIFamide and MIP (Simo and Park., 2014). Specifically, axonal projections from the opisthosomal lobe in the posterior region of synganglion have been shown to have immunoreactive staining for SIFamide and MIP peptides, which innervate the anal sphincter. In this region of the hindgut, these two peptides were shown to have antagonistic effects on the motility/contractions of the rectal sac and anal canal, with SIFa stimulating and MIP inhibiting contractions (Simo and Park, 2014).

Members of the MIP family are characterized by the highly conserved Trp residues at positions 2 and 9, and an amidated C-terminus. They were first shown to have an inhibitory effect in the locust, *Locusta migratoria*, suppressing the spontaneous contraction of the hindgut and oviduct (Schoofs et al., 1991). Since then these peptides have been found in many other organisms, including the adult tobacco hornworm, *Manduca sexta* (Blackburn et al. 1995; Blackburn et al., 2001), and the silkworm, *Bombyx mori* (Hua et al., 1999). The insect SIFamide

family was first identified in the grey fleshly, *Neobellieria bullata,* where it exhibited myotropic activity (Janssen et al., 1996). The SIFamides are highly conserved between crustaceans and flies, with a 91.67% C-terminal sequence homology between the crustacean SIFamide (GYRKPPFNGSIFamide) and the *Drosophila melanogaster* SIFamide (AYRKPPFNGSIFamide; Vanden Broeck, 2001b; Yasuda et al., 2004). SIFamide peptides have also been shown to act as a neuromodulator in the stomatogastric ganglion, activating the pyloric motor pattern, resulting in an increase in both burst amplitude and duration in the pyloric dilator neurons, in the American lobster, *Homarus americanus* (Christie et al., 2006). Furthermore, they have been shown to affect sexual behaviour in both sexes in *Drosophila* (Terhzaz et al., 2007). The structurally related , sex peptide in *D. melanogaster* is the natural ligand affecting sexual behaviour in this organism (Kim et al., 2010; Poels et al., 2010). The activation of the sex peptide receptor, a GPCR, by both of these structurally related peptides arises from their highly conserved tryptophan residues (Kim et al., 2010; Poels et al., 2010). In the tick *I. scapularis*, with respect to the salivary gland type II and III acini, MIP and SIFamide have been suggested to act in an antagonistic manner, where MIP elicits an inhibitory function and SIFamide plays a stimulatory role (Simo et al., 2009a and 2009b).

CAPA

There are several diuretic and antidiuretic factors that have been shown to regulate the secretory activity of Malpighian tubules in insects (Coast, 2007). One of these factors are the CAPA peptides, which were initially proposed to be cardioacceleratory peptides (CAPs) as it was found to increase the rate and the amplitude of contraction of the heart of the tobacco hawkmoth, *Manduca sexta* (Tublitz and Truman, 1985a, b, and c). Two cardioacceleratory

peptides (CAP-1 and CAP-2) were identified in the ventral nerve cord of *Manduca sexta* (Tublitz and Truman, 1985a). CAPs 1 and 2 have been shown to increase the heart rate during wing inflation after adult emergence and during flight via inositol 1, 4, 5 trisphosphate signalling (Tublitz and Truman, 1985b; 1985c; Tublitz, 1988; Davies et al., 2013). CAP2 is also responsible for hindgut contraction in *M. sexta* (Tublitz et al., 1992; Davies et al., 2013). It was later identified that there were two peptides making up the partially purified CAP-1 sample (CAP-1a and CAP-1b, with unknown sequences) and 3 peptides comprised the CAP-2 sample (CAP-2a, CAP-2b and CAP-2c; Cheung et al., 1992; Davies et al., 2013). CAP2a and Cap2b were found to be structurally different; with their respective sequences being PFCEAFTGC-NH_2 and pyroQLYAFPRV-NH_2 (Cheung et al., 1992; Huesmann et al., 1995). CAP2a was also identified to be the same peptide as the crustacean CAP (CCAP; Stangier et al., 1987). It was later shown that CAP2b is a potent regulator of Malpighian tubule activity whereby it stimulates secretion (diuresis) in *D. melanogaster* and inhibits fluid secretion (anti-diuretic) in *Rhodnius prolixus* (Davies et al., 1995; Quinlan et al., 1997).

With the availability of the *Drosophila* genome, the first gene that encoded CAP2b-like peptides was identified, termed the *capability (capa)* gene since it is "capable" of encoding CAPA peptides (Kean et al., 2002). This allowed for the discovery of a third peptide that is encoded by the *capa* gene, CAPA-3 or CAPA-PK-1 (Kean et al., 2002). Both CAPA1 (CAPA-PVK1) and CAPA2 (CAPA-PVK2) are similar to the *M. sexta* CAP2b, whereby the C-terminal motif is conserved containing an FPRV-NH_2 (Table 1; Kean et al., 2002; Davies et al., 2013). In contrast, CAPA3 (CAPA-PK-1) is part of a pyrokinin/pheromone biosynthesis-activating neuropeptide (PBAN) /diapause hormone family with a C-terminal motif consisting of FXPRL-NH_2 (Table 1; Jurenka and Nusawardani, 2011; Davies et al., 2013; Kean et al., 2002).

In contrast to the diuretic effect of CAP2b in *D. melanogaster*, in *R. prolixus* and some other insects it has been shown to elicit a dose-dependent antidiuretic effect at lower concentrations and diuretic at higher concentrations (Davies et al., 1995; Quinlan et al., 1997; Coast and Garside, 2005; Paluzzi and Orchard, 2006; Ionescu and Donini, 2012).

With respect to the Malpighian tubules, CAPA (both CAPA 1 and 2; referred to also as CAPA-PVKs) peptides elicit their function in *D. melanogaster* by acting on the L-type voltage gated Ca^{2+} channels, which leads to an influx of Ca^{2+} ions (Kean et al., 2002). The increased intracellular Ca^{2+} causes the production of endogenous nitric oxide (NO) via stimulation of nitric oxide synthase (NOS). Subsequently, NO stimulation of guanylate cyclase causes increased production of cyclic guanosine monophosphate (cGMP), which activates V-type H^{+} ATPase on the apical membrane, thus causing an increase in the rate of fluid secretion (Kean et al., 2002; Davies et al., 1997). It was shown that the physiological functioning of CAPA peptides with respect to fluid homeostasis in *D. melanogaster* is conserved in other Dipteran insects (Pollock et al., 2004).

The first attempt at identifying the sequence of the tick CAPA/PVK peptides was done using MALDI-TOF/TOF and mass spectrometry on *Ixodes ricinus* and *R. microplus* with the sequence structure identified as PALIPFPRV-NH2 (Neupert et al., 2005). It was later sequenced in *Ixodes scapularis* and found to be pQGLIPFPRVa (Christie, 2008; Neupert et al., 2009). CAPA peptides have been shown to be associated with the neurosecretory system in the abdominal ventral nerve cord in insects (Predel and Wegener, 2006). Furthermore, they are synthesized in the median neurosecretory neurons of the abdominal ganglia and are released as hormones from the abdominal perisympathetic organs (PSOs) near nerves or muscles (Predel and Wegener, 2006)

The first CAPA-related peptide receptor in insects was functionally characterized after the genome sequencing of *D. melanogaster (*Adams et al., 2000; Park et al., 2002; Iverson et al., 2002) and was shown to belong to the G protein-coupled receptor superfamily. G protein-coupled receptors (GPCRs) are seven transmembrane spanning proteins that elicit an intracellular signalling pathway by activating heterotrimeric G proteins consisting of α, β and γ subunits (Vanden Broeck, 2001a). Binding of an agonist on the extracellular side stabilizes the active receptor conformation interacting with a heterotrimeric G protein to form a ternary complex; agonist, receptor and G-protein. This interaction allows for the exchange of GTP for GDP on the α-subunit of the G-protein, leading to the dissociation of the Gα-GTP and Gβγ from the receptor (Vanden Broeck, 2001a). The Gα-GTP and Gβγ now are able to regulate the activity of various cellular proteins, such as enzymes to elicit various functions. Upon hydrolysis of GTP to GDP by intrinsic GTPase activity of the Gα-subunit; the α, β and γ subunits reassociate (Vanden Broeck, 2001a).

Recently, the first CAPA/PVK tick receptor was functionally characterized in the cattle tick, *Rhipicephalus microplus*, showing typical rhodopsin-like GPCR characteristics (Yang et al., 2013). These include the seven transmembrane α-helices containing highly conserved sequence motifs and a canonical D/E-R-Y/F motif sequence between the third transmembrane domain and the second intracellular loop (Yang et al., 2013). Tissue expression profiles revealed a high transcript level in the tick synganglion, followed by relatively lower levels in the Malpighian tubules, salivary glands, and ovaries (Yang et al., 2013). In contrast, the receptor transcript was either not detected or was present in low abundance in the midgut and rectal sac (Yang et al., 2013).

FMRF-related Peptides (FARPs)

Another group of peptides which share some limited structural similarity to the CAPA peptides, particularly at the C-terminus, are the FMRFamide-related peptides (FaRPs). Like CAPA peptides, FaRPs elicit their various functions by binding to GPCRs (Meeusen et al., 2002). FMRFamide (Phe-Met-Arg-Phe-NH_2)-related peptides normally terminate with the sequence Arg-Phe-NH_2 but with variable amino terminal sequences (Duttlinger et al., 2003). FMRFamide was first identified in the sunray venous clam, *Macrocallista nimbus* (Price and Greenberg, 1977). The FaRP gene often encodes multiple structurally-related peptides and usually there is more than one FaRP gene present in the genome of any given animal species (Duttlinger et al., 2003). They act as modulators and messengers for a variety of biological functions. FaRPs are also present and have been shown to elicit their function via GPCRs in the mosquito *A. aegypti* (Meeusen et al., 2002); Duttlinger et al., 2003). In *D. melanogaster,* FaRPS have been shown to induce myogenic contractions in a Ca^{2+}-dependent manner. FMRFamides have also been shown to increase the amplitude and frequency of oviduct contractions in the locust, *Locusta migratoria* (Peeff et al., 1993). The mechanism including the secondary messengers involved is still not known (Milakovic et al., 2014). However, it has been shown that FaRP-induced contractions via GPCRs in muscle cells do not appear to act via cAMP, cGMP, IP3, PLC, CaMKII or arachidonic acid (Milakovic et al., 2014).

Short neuropeptide-F (sNPF) is a distinct subfamily of FaRPs characterized by a conserved C-terminus consisting of RXRF-NH_2, where X indicates a variable residue, but is usually found to be leucine (Christie, 2008). SNPF was originally described in *D. melanogaster* as the homolog to the vertebrate neuropeptide Y (NPY), which is a neuromodulator that is expressed predominantly in the mammalian brain to control feeding among other physiological

processes (Wahlestedt and Reis, 1993; Zimanyi et al., 1998). In *D. melanogaster*, sNPF expression in the nervous system was observed to control food intake and regulation of body size (Lee et al., 2004). Recently, information from predicted neuropeptide precursor sequences found in EST databases was used to analyze the neuropeptides in the synganglion of *I. scapularis* using MALDI-TOF mass spectrometry (Christie, 2008; Neupert et al., 2009). One of the neuropeptides that was detected was sNPF (Christie, 2008; Neupert et al., 2009).

Another subfamily of FaRPs that was detected in the synganglion of *I. scapularis* was the sulfakinins (Christie, 2008; Neupert et al., 2009). Sulfakinin isoforms, sulfakinin-1 and sulfakinin-2, have a conserved C-terminal motifs of QDDDY$_{(SO3H)}$GHMRF-NH$_2$ and SDDY$_{(SO3H)}$GHMRF-NH$_2$, respectively (Christie, 2008; Neupert et al., 2009). These isoforms have been shown to have neuromodulatory effects in other organisms, where exogenous application of either of the synthetic isoforms of the peptide to the isolated heart of the American lobster, *Homarus americanus*, induced both increased frequency and amplitude of spontaneous heart contractions (Dickinson et al., 2007).

Research Objectives and Hypothesis

The black-legged tick, *I. scapularis*, has been shown to be a chief vector for a range of disease. In mammals/vertebrates Lyme disease, caused by the transmission of the spirochetal bacterium *Borrelia burgdorferi*, results in arthritis-like symptoms (Steere et al., 1977; Burgdorferi et al., 1982). Despite increasing prevalence of Lyme disease, very little is known about the neuroendocrine system that regulates physiological activities in this important human disease vector (Kugeler et al., 2015). Furthermore, as terrestrial arthropods living in arid environments and as obligate blood-feeders taking up substantial amount of water and ions, it is crucial that these organisms uphold ionic and osmotic homeostasis to ensure their survival. In ticks, the salivary glands have a primary role during blood feeding in regulating the removal of excess ions (mainly sodium) and water.A secondary role during the off-host periods is played by the hindgut. In blood-sucking insects, it has been identified that CAPA peptides elicit a diuretic or antidiuretic action on the Malpighian tubules (Davies et al., 1997; Kean et al., 2002; Paluzzi and Orchard, 2006; Pollock et al., 2004; Quinlan et al., 1997).

The aim of my research was to identify the distribution of CAPA peptides in the nervous system and peripheral tissues of the black-legged tick, *I. scapularis*. Determining the localization of this important neuropeptide will aid in determining the potential functions of CAPA peptides in *Ixodid* ticks. The first objective was to determine the distribution of CAPA-like peptides in the synganglion and peripheral tissues using immunohistochemical methods, to determine the sites of synthesis and putative sites of action. As a result of a previous neuropeptidomics study that was conducted on the synganglion of *I. scapularis*, which revealed the presence of CAPA peptides (Neupert et al., 2009), it was hypothesized that CAPA-like immunoreactivity would be detected in the tick nervous system. Furthermore, as a result of a CAPA/PVK receptor transcript

being detected in the synganglion of another *Ixodid* species, *Rhipicephalus (Boophilus) microplus* (Yang et al., 2013), it was suggested that the tick CAPA peptide may act as a neurotransmitter in the synganglion. Thus, examining its detailed distribution within the synganglion, particularly within the neuropile, would support its suggested role as a neurotransmitter or neuromodulator. Lastly, it was hypothesized that CAPA-like immunoreactivity may also be present in the salivary glands, MTs, and ovaries, once again due to the fact that the CAPA/PVK receptor transcript was detected in these tissues in the cattle tick (Yang et al., 2013).

The second objective was to confirm the site of CAPA peptide synthesis, quantify the amount of peptide present by using fluorescent *in situ* hybridization methods and enzyme-linked immunosorbent assay (ELISA), respectively. Since CAPA-precursors have been detected solely in the central nervous system (CNS) of insects, including, for example, in the mosquito, *A. aegypti* (Predel at al., 2009), *D. melanogaster* (Park et al., 2008; Nässel and Winther, 2010) and *R. prolixus* (Paluzzi et al., 2008), it was hypothesized that CAPA peptide transcript would also be found solely in the nervous system (i.e. synganglion) of the *Ixodid* tick. Furthermore, given the expected strict localization of the transcript to the nervous system, I hypothesized that CAPA-like material would be quantifiable most abundantly within extracts of the nervous system.

The third objective of my research was to examine potential physiological roles of the endogenous CAPA peptide in *I. scapularis* by investigating its bioactivity on peripheral tissues where CAPA-like immunoreactive staining or expression was detected in my earlier objectives. Thus, for this component of my thesis, I hypothesized that the native *I. scapularis* CAPA peptide would have bioactivity on peripheral tissues that contained CAPA-like immunoreactivity.

To my knowledge, limited information is available on the function or localization of CAPA peptides in *I. scapularis* (black-legged tick), therefore this study will aim to be the first to deduce the location and suggest possible functions of this conserved neuropeptide. The results obtained will broaden our knowledge on the neurochemicals and their functions in ticks, better our understanding of the physiology of these organisms and potentially uncover mechanisms to prevent these organisms from acting as vectors of disease.

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Chapter II:
DISTRIBUTION AND ACTIVITY OF CAPA-LIKE PEPTIDES IN THE BLACK-LEGGED TICK, *IXODES SCAPULARIS*

Abstract

Ixodes scapularis transmit a variety of pathogens causing serious ailments. In the present study, the distribution of CAPA-like peptides was localized in various tissues of adult *I. scapularis* by using immunohistochemical procedures. CAPA-like immunoreactivity was localized in cells and processes in the synganglion, midgut endocrine-like cells, reproductive tissues and the posterior region of the rectal sac. RT-PCR was used to verify the distribution of the CAPA peptide-encoding transcript in various tissues. However, it was detected exclusively in the synganglion. The transcript encoding a peptide sharing structural similarity at its C-terminus, short neuropeptide F (sNPF), demonstrated a complete overlap in the transcript tissue distribution to that demonstrated by the CAPA-like immunoreactive staining. Finally, contraction assays were conducted on the hindgut, which revealed CAPA had myostimulatory effects.

These findings have determined novel physiological functions of the CAPA-related peptides in this important human disease vector.

Introduction

As an obligate blood-feeder, the black-legged tick, *Ixodes scapularis,* transmits a variety of pathogens, including the Lyme disease-causing spirochete, *Borrelia burgdorferi* (Burgdorfer et al., 1982). Lyme disease is a bacterial infection that was originally believed to be a form of arthritis as it causes swelling and pain within the large joints of the body (Steere et al., 1977). Studies focusing in northeastern and northcentral states in the U.S. from 1993-2012 have shown a significant increase in Lyme disease cases (Kugeler et al., 2015). Therefore, it is of great interest to enhance the knowledge and expand fundamental studies on *I. scapularis* in order to possibly develop improved means of preventing the transmission of pathogens by this important disease vector.

These ectoparasites have three post-embryonic life stages: larval, nymphal stage and adult, with each requiring a blood meal (Balashov, 1998; Sonenshine and Roe, 2014). At 30˚C, the blood-feeding duration is approximately 3-4, 4-7 and 8-12 days for the larval stage, nymphs and adult females, respectively (Balashov, 1998; Sonenshine and Roe, 2014). The Lyme disease causing agent, *Borrelia burgdorferi,* is usually taken up by the *Ixodid* tick during the larval stage and is transmitted to a new host in the next instar (Donahue at al., 1987; Mather et al., 1989). There has been uncertainty concerning the transovarial transmission of *B. burgdorferi* spirochetes to offspring, but recent evidence has shown that the antigenically and phylogenetically-related, *Borrelia miyamotoi,* is the transovarially transmitted agent that some studies have reported and not *B. burgdorferi* (Patrican, 1997; Rollend at el., 2013). *Borrelia miyamotoi* is more closely related to the relapsing fever-group of *Borrelia* typically transmitted by soft ticks (*Argasidae*; Rollend at el., 2013).

As terrestrial blood-sucking arthropods, *Ixodid* ticks are constantly faced with various stressors that challenge their ionic and osmotic homeostatic mechanisms (Cloudsley-Thompson, 1975; Sonenshine and Roe, 2014). These include environmental factors, such as arid environments that promote susceptibility to water loss through the integument. Additionally, the bulk influx of water and ions during a blood meal causes stress on the excretory system to maintain haemolymph hydromineral balance (Tatchell, 1967; Kaufman and Philips, 1973; Sonenshine and Roe, 2014).

I. scapularis maintain hydromineral balance during a blood meal via the salivary glands, hindgut and Malpighian tubules (MTs), and evaporation through integuments (Tatchell, 1967; Kaufman and Philips, 1973). Approximately 80% of excess water and ions are excreted during a blood meal (remaining 20% excreted off-host, after the blood meal), of which 75% is excreted by the salivary glands (back into the host), about 3% through evaporation from the integument and spiracles, and the remainder through the Malpighian tubules, eventually leading to defecation via the rectal sac (Kaufman and Philips, 1973). This process is first initiated by the movement of excess water passing across the gut epithelium to the haemolymph, which then enters the salivary glands where it is returned to the host through secretions (Tatchell, 1967; Kaufman and Philips, 1973). The removal of the excess water through the salivary glands is accompanied by the excretion of about 96% of the total excess Na^+, and minor amounts of excess Cl^- (Figure 1.1; Kaufman and Philips, 1973). This is a result of the midgut epithelium being able to transport Na^+ and Cl^-. However, it is relatively impermeable to K^+ and thus its movement into the haemolymph is prevented (Kaufman and Philips, 1973).

Recently, aquaporins have been shown to be involved in the secretion of water by forming pores in the cell membranes in the various excretory organs. This includes the aquaporin

*Rs*AQP1, which was detected in the gut, MTs, and most abundantly in salivary gland tissues in the partially fed brown dog tick; *Rhipicephalus sanguineus* (Ball et al., 2009). A homolog of *Rs*AQP1, *Ir*AQP1, was recently found to be present in the rectal sac, in addition to the gut and salivary glands of *Ixodes ricinus* (Campbell et al., 2010). Therefore, the presence of aquaporins suggests that the midgut, salivary glands, and rectal sac are tissues that are involved in high water flux in ticks.

The hindgut in ticks consists of the intestine, the rectal sac, the anal canal, and the anus (Balashov, 1972; Sonenshine and Roe, 2014). Additionally, there is a pair of MTs that project anteriorly from the junction between the intestine and rectal sac (Sonenshine and Roe, 2014).

The MTs are structurally and functionally homologous to those of insects; whereby the MTs of most terrestrial insects are responsible for nitrogenous waste elimination by producing the primary urine that goes to the hindgut (Balashov, 1972; Coast 2009; Sonenshine and Roe, 2014). The intestine in ticks seems to have a passive transport role, involved in the passage of undigested waste from the midgut to the rectal sac (Sonenshine and Roe, 2014). In blood-sucking insects the undigested waste (in the hindgut) is further modified by reabsorption and secretion via the ileum and rectum prior to excretion as dry or semi-dry urine (Balashov, 1972; Coast 2009; Sonenshine and Roe, 2014). Moreover, the rectal sac in ticks also plays an important role in body water regulation by concentrating urine from the MTs (Sonenshine and Roe, 2014).

Despite being an important human disease vector, there is limited knowledge on the neuroendocrine or neuromodulatory systems in the *Ixodid* tick. This is particularly noteworthy since neuropeptides in insects have been shown to coordinate various physiological processes, including maintaining ionic and osmotic homeostasis (Gäde and Goldsworthy 2003; Coast, 2009; Caers et al., 2012). Diuretic (DH) and antidiuretic hormones (ADH) in blood-feeding and non-

blood-feeding insects are involved in the regulation of secretion by Malpighian tubules and reabsorption across the hindgut, which together function to alleviate the ionic and osmotic challenges especially after a blood meal (Wheelock et al., 1988; Eigenheer et al., 2002; Coast and Garside 2005; Coast, 2009).

The cardioacceleratory peptide 2b (CAP2b)/ periviscerokinin (PVK) has been excessively studied in insects, as it has been shown to regulate fluid secretion (Davies et al., 1995; Quinlan et al., 1997; Pollock et al., 2004; Coast and Garside, 2005; Paluzzi and Orchard, 2006; Ionescu and Donini, 2012). The *capability* (*capa*) gene in the *Drosophila* genome was the first gene found to encode CAP2b-like peptides, named as a result of its "capability" to produce CAPA peptides (Kean et al., 2002). The sequencing of the *capa* gene allowed the discovery of a third peptide, CAPA3 or CAPA-PK-1 (Kean et al., 2002). CAPA3 (CAPA-PK-1) is a peptide associated with the pyrokinin/pheromone biosynthesis-activating neuropeptide (PBAN)/diapause hormone family, with a conserved C-terminal motif consisting of FXPRL- NH_2 (Jurenka and Nusawardani, 2011; Davies et al., 2013; Kean et al., 2002). Both CAPA1 (CAPA-PVK1) and CAPA2 (CAPA-PVK2) are similar to the *M. sexta* CAP2b, with respect to the highly conserved C-terminal sequence of FPRV- NH_2 (amide) (Table 1; Kean et al., 2002; Davies et al., 2013). CAP2b was found in many other organisms with species-specific effects; anti-diuretic effects (stimulating fluid secretion) in *R. prolixus*, and in some insects, having an anti-diuretic effect (inhibiting fluid secretion) at lower concentrations and diuretic at higher concentrations (Davies et al., 1995; Quinlan et al., 1997; Coast and Garside, 2005; Paluzzi and Orchard, 2006; Ionescu and Donini, 2012).

Table 1: Summary of select *I. scapularis* neuropeptides of interest and their respective C-terminal sequences (adopted from Christie, 2008; Neupert et al., 2009)

Peptide Name	Peptide Sequence
CAPA-PVK	pQGLIPFPRVa
Pyrokinin-1	RSNNFTPRIa
Pyrokinin-2	GSFVPRLa
Short Neuropeptide F (sNPF)	GGRSPSLRLRFa
Sulfakinin-1	QDDDY$_{(SO3H)}$GHMRFa
Sulfakinin-2	SDDY$_{(SO3H)}$GHMRFa

The mechanism by which CAPA (both CAPA1 and CAPA2; also referred to as CAPA-PVKs) peptides maintain hydromineral balance is conserved in Dipteran insects (Pollock et al., 2004). CAPA peptides in insects are synthesized in the median neurosecretory neurons of the abdominal ganglia and are released as hormones from the abdominal perisympathetic organs (PSOs) near nerves or muscles (Predel and Wegener, 2006). With the availability of the *D. melanogaster* genome, it was deduced that CAPA peptides bind to members of the G protein-coupled receptor (GPCR) superfamily (Adams et al., 2000; Vanden Broeck, 2001; Park et al., 2002; Iverson et al., 2002). GPCRs are seven transmembrane spanning proteins that elicit intracellular signalling pathway by activating heterotrimeric G proteins consisting of α, β and γ subunits (Vanden Broeck, 2001). When an agonist with the conserved C-terminal PRX- NH_2 motif binds to the extracellular side the receptor, this results in stabilization of the active receptor conformation interacting with a heterotrimeric G protein to form a ternary complex; agonist, receptor and G-protein. That promotes the exchange of GTP for GDP on the α-subunit of the G-protein, leading to the dissociation of the Gα-GTP and Gβγ from the receptor (Vanden Broeck, 2001). The Gα-GTP and Gβγ are then able to regulate the activity of various cellular proteins and enzymes, leading to the manipulation of various functions. When intrinsic GTPases hydrolyze GTP to GDP, the Gα-subunit; the α, β and γ subunits reassociate (Vanden Broeck, 2001). In the *D. melanogaster* MTs for example, the receptor downstream signalling then activates L-type voltage gated Ca^{2+} channels that cause an influx of Ca^{2+} ions (Kean et al., 2002). The increased intracellular Ca^{2+} then stimulates nitric oxide synthase (NOS), leading to the production of endogenous nitric oxide (NO). This then signals the production of cyclic guanosine monophosphate (cGMP) via guanylate cyclase, to activate V-type H^+ ATPase on the apical membrane promoting fluid secretion (Kean et al., 2002; Davies et al., 1997).

In ticks, CAP2b/PVK peptides have recently been the focus of a variety of studies. Initial sequencing studies in *Ixodes ricinus* and *R. microplus* revealed a C-terminal sequence of PALIPFPRV-NH_2 (Neupert et al., 2005). Peptidomic studies then revealed the sequence in *Ixodes scapularis*, pQGLIPFPRV-NH_2 (Christie, 2008; Neupert et al., 2009).

As a result of some shared C-terminal sequence similarity to CAPA peptides, this study will also look at the FMRFamide (Phe-Met-Arg-Phe-NH_2)-related peptides (FaRPs), as they also elicit their various functions by binding to GPCRs (Table 1, Meeusen et al., 2002). FaRPs have a characteristic C-terminal sequence of Arg-Phe-NH2 but with variable amino terminal sequences (Duttlinger et al., 2003).

FMRFamide was first identified in the sunray venous clam, *Macrocallista nimbus* (Price and Greenberg, 1977). The FaRP gene often encodes multiple structurally related peptides and usually there are multiple FaRP genes present in the genome of an animal species (Duttlinger et al., 2003). They act as modulators and messengers for a variety of biological functions. FaRPS binding to GPCRs are conserved between species, including in the mosquito *A. aegypti* (Meeusen et al., 2002; Duttlinger et al., 2003). FMRFamides have been shown to increase the amplitude and frequency of oviduct contractions in the locust, *Locusta migratoria* (Peeff et al., 1993). FaRPs have been shown to induce contractions via a G-protein coupled FMRFamide receptor in muscle cells of *D. melanogaster*, but does not appear to act via cAMP, cGMP, IP3, PLC, CaMKII or arachidonic acid (Milakovic et al., 2014). The mechanism including the secondary messengers involved is still not known (Milakovic et al., 2014). Short neuropeptide F (sNPF) belongs to a subfamily of FaRPs characterized by a conserved C-terminus consisting of –RXRF-NH_2, with 'X' denoting a variable residue, but leucine is most commonly found at that position (Christie, 2008). It was originally described in *D. melanogaster* as being homologous to

the vertebrate neuropeptide Y (NPY), which is expressed predominantly in the mammalian brain and acts as a neuromodulator to control feeding among other physiological processes (Wahlestedt and Reis, 1993; Zimanyi et al., 1998).

In *D. melanogaster*, sNPF expression in the nervous system was observed to control food intake and regulation of body size (Lee et al., 2004). Recently, information from predicted neuropeptide precursor sequences found in EST databases was used to analyze the neuropeptides in the synganglion of *I. scapularis* using MALDI-TOF mass spectrometry (Christie, 2008; Neupert et al., 2009). One of the neuropeptides that were detected in the synganglion of *I. scapularis* was sNPF (Christie, 2008; Neupert et al., 2009).

Another member of the FaRP family that was also detected in the synganglion of *I. scapularis* was sulfakinin (Christie, 2008; Neupert et al., 2009). Sulfakinin isoforms, sulfakinin-1 and sulfakinin-2, have the conserved C-terminal motifs of $QDDDY_{(SO3H)}GHMRF\text{-}NH_2$ and $SDDY_{(SO3H)}GHMRF\text{-}NH_2$, respectively (Christie, 2008; Neupert et al., 2009). In the American lobster, *Homarus americanus*, sulfakinin-1 ($GGGEY_{(SO3H)}DDY_{(SO3H)}GHLRF\text{-}NH_2$) and sulfakinin-2 ($pEFDEY_{(SO3H)}GHMRF\text{-}NH_2$; Christie et al., 2015), have been shown to have neuromodulatory effects, where exogenous application of either synthetic isoform of the peptide to the isolated heart induced both increased frequency and amplitude of spontaneous contractions (Dickinson et al., 2007).

Overall, there have been limited studies pertaining to the function, localization and activity of CAP2b/PVK in the black-legged tick, *I. scapularis*. Studies conducted in related species showing the presence of CAP2b/PVK in various tissues, provide evidence for the expectation of their presence in *I. scapularis* tissue (Neupert et al., 2005; Christie, 2008; Neupert et al., 2009; Yang et al., 2013). Additionally, as a result of conserved *I. scapularis* CAP2b

receptor characteristics and the high sequence homology to the homologous receptors in insects, it is possible that CAP2b in *I. scapularis* may also be involved in the regulation of gut tissues, including the Malpighian tubules and hindgut, which are known targets of these peptides in insects (Neupert et al., 2005; Yang et al., 2013; Predel and Wegener, 2006). Furthermore, it is of great importance to obtain a clear understanding of the roles of CAP2b/PVK within the black-legged tick to further our understanding of their physiology, therefore providing vital information in devising potential mechanisms to eradicate these disease vectors and minimize pathogen transmission.

For the purpose of gaining knowledge pertaining to the function of CAP2b/PVK in *I. scapularis,* this study will focus on determining the localization and activity of these neuropeptides within the nervous system (i.e. synganglion) and peripheral tissues and organs. This will be accomplished by isolating the various organs of adult *I. scapularis* and processing them using immunohistochemistry (IHC). This will aid in providing plausible sites of expression for CAP2b/PVK-like peptides. Furthermore, as a means of confirming the distribution of cells producing CAPA peptides as well as peptides sharing some structural similarity, the tissues will also be analyzed using fluorescence *in situ* hybridization (FISH) and reverse transcriptase PCR (RT-PCR). An enzyme-linked immunosorbent assay (ELISA) will be used to quantify the amount of CAP2b/PVK-like peptides in these various tissues and organs. Finally, to delineate CAPA-related functionality in *I. scapularis,* bioassays will be conducted on the hindgut to determine if CAPA plays a myomodulatory role in this tick species. The results from these various experiments will provide a definitive map of where CAP2b/PVK peptides are expressed and potentially elucidating possible physiological functions.

Materials and methods

Animals

Black-legged tick, *Ixodes scapularis,* colonies were obtained from Oklahoma State University (OSU) at the Tick Rearing Facility (Stillwater, OK). Adult ticks were obtained from OSU and maintained (~23°C; room temperature) at York University. They were kept in 50mL falcon tubes with a cotton ball on the open end to allow gas exchange. To ensure proper moisture control; falcon tubes were places in a body of sterile water, and a filter paper was placed inside the falcon tubes to absorb extra moisture. Only unfed adult ticks were used for all experiments, approximately 3-5 months after the nymphal-adult molt.

Immunohistochemistry

Ticks were dissected in ice-cold nuclease-free Dulbecco's phosphate-buffered saline (DPBS). The dorsal cuticle was removed with a sharp micro scalpel exposing the internal anatomy of the tick. The tissues of interest were dissected and transferred to a microcentrifuge tube containing fixation solution (40% filter-sterilized paraformaldehyde diluted in DPBS for a final concentration of 4%) and incubated overnight at 4°C. Following washes with DPBS, all tissues samples were then permeabolized in DPBS containing 4% TritonX, 2% BSA, and 10% normal sheep serum for one hour at room temperature (~23°C)**.** Tissue samples were then washed with 1X DPBS changing the solution three times and subsequently incubated in the primary antibody (1:1000 rabbit anti-*Rhopr*CAPA- α2 EGGFISFPRV; a generous gift by Prof. Ian Orchard, University of Toronto Mississauga; Paluzzi et al., 2008) for 48 h, at 4°C. The samples were once again washed with DPBS changing the solution three times (~5 minutes each time) and mixing for approximately 5 minutes each time. After these wash steps, tissue samples were then

incubated in the secondary antibody (1:200 Cy3 labelled sheep anti-rabbit immunoglobulin G, IgG (Sigma-Aldrich, Oakville, ON) in 10% normal sheep serum in DPBS) for 24 h (blocking from light), at 4°C. The samples were again washed in DPBS three times mixing the contents for approximately 5 minutes each time the solution was changed, and then mounted on slides by incubating the tissue in 1:500 dilution of 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; in 1:1 solution of glycerol and DPBS; Sigma-Aldrich, Oakville, ON) for 10 minutes (blocking light). The tissue samples were washed again with DPBS with three buffer changes and mixed for approximately 5 minutes each wash, and then the samples were mounted in glycerol on regular microscope slides or depression slides. The prepared slides were then observed under a Lumen Dynamics X-Cite™ 120Q Nikon fluorescence microscope (Nikon, Mississauga, ON, CA) using TRITC and DAPI filters.

A control experiment was prepared by staining tissues with antibody (*Rhopr*CAPA- α2) previously incubated with IxoscCAPA-PVK (50 μmol l^{-1}; Genscript, Piscataway, New Jersey) at 4˚C and using the same volume of antisera as described earlier (see Paluzzi and Orchard, 2006). This also allowed us to determine the antibody's specificity for *I. scapularis* CAPA-PVK. No positive staining was observed. 1

Primer Design

Primers used for FISH and RT-PCR were designed using CAPA sequences that were predicted and confirmed in previous studies on *I. scapularis* (Gondalia et al., 2016; Gulia-Nuss et al., 2016; Neupert et al., 2009). Two forward primers CAPAF1 and CAPAF2 with sequences of TGCTGATGAGGGCTTTCTGG and GAACTGCTCACCCTGGACAC, and two reverse primers CAPAR1 and CAPAR2 with sequences of AGGAGCGGGAATTTGAGTCC and

TAGGTGTAAAGGAGCCTCGC, respectively, were commercially synthesized (Table 2; Sigma-Aldrich, Oakville, Ontario, Canada).

Primers for sNPF and sulfakinin were designed in a similar fashion. Briefly, primers for sNPF were designed using an *I. scapularis* EST from previous studies (Accession No. EW855501; Christie, 2008; Neupert et al., 2009). Four primers were designed and synthesized: Forward 1(46F) and Forward 2 (42F) with sequences AGCCTCATAATGGTTTCGCTC and CTCGAGCCTCATAATGGTTTC, respectively (see Table 2); Reverse 1 (548R) and Reverse 2 (624R) with the sequences of TCAGGCGTGTTAAAGACTTGC and ACACTCCTCAAAAAGCATAACGG, respectively (see Table 2). Primers for sulfakinin were designed using *I. scapularis* EST (Accession No. EW941557) and Primer3 software, (Christie, 2008; Untergasser et al., 2012; Koressaar and Remm, 2007; Neupert et al., 2009). The two forward primers for *I. scapularis* sulfakinin amplification were Forward 1 (23F) with a sequence of GTGTTTCGGTAAAATAGAAGAGTGC and Forward 2 (218F) with the sequence of ATAAAGACCTGGATTCGAGACC (see Table 2). The two reverse sulfakinin primers were Reverse 1 (610R) and Reverse 2 (743R) with the sequence of AACCTCATGTGTCCGTAGTCG and TTTTTATACCAGAACCATGCCG, respectively (see Table 2).

Probe Preparation for Fluorescent in situ Hybridization (FISH)

In order to support results from the immunohistochemistry experiments, FISH was conducted to localize the CAPA peptide expressing cells at the transcript level. The probes used for FISH were designed using the different combinations of the forward and reverse primers mentioned earlier (see Table 2) and *I. scapularis* synganglion cDNA prepared following manufacturer guidelines (iScript™ Reverse Transcription Supermix, Bio-Rad, Mississauga, ON).

Table 2: Primer information for oligonucleotides used for RT-PCR and FISH

Oligo Name	Oligo Sequence (5'-3')
CAPA	
CAPAF1	TGCTGATGAGGGCTTTCTGG
CAPAF2	GAACTGCTCACCCTGGACAC
CAPAR1	AGGAGCGGGAATTTGAGTCC
CAPAR2	TAGGTGTAAAGGAGCCTCGC
sNPF	
46F	AGCCTCATAATGGTTTCGCTC
42F	CTCGAGCCTCATAATGGTTTC
548R	TCAGGCGTGTTAAAGACTTGC
624R	ACACTCCTCAAAAAGCATAACGG
Sulfakinin	
23F	GTGTTTCGGTAAAATAGAAGAGTGC
218F	ATAAAGACCTGGATTCGAGACC
610R	AACCTCATGTGTCCGTAGTCG
743R	TTTTTATACCAGAACCATGCCG

PCR amplification of the RNA probe template involved use of Invitrogen *Taq* DNA Polymerase reagents and protocol. Some minor adjustments to the protocol included performing 40 cycles instead of the recommended 25-35 cycles of PCR amplification, 60˚C annealing temperature, and extension time of only 1 min. The amplified PCR products were column purified and were then ligated to a pGEM T vector and used for transformation of competent *Escherichia coli* (JM109 strain) bacteria following the manufacturer's recommendations (Promega, Madison, WI). Plasmid DNA was isolated using a miniprep kit (BioBasic, Markham, ON) from overnight bacterial cultures containing plasmid with the CAPAF1 and CAPAR2 combination PCR products. Miniprep plasmid DNA was sent for sequencing at the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, Ontario, Canada) to confirm base accuracy and directionality of inserts in the vector. Sense and antisense DNA templates for RNA probes were then synthesized using pGEMT7-long (AATTGTAATACGACTCACTATAGGGCG) and CAPAR2, and pGEMT7-long and CAPAF1, respectively, using a standard *Taq* DNA polymerase protocol (Invitrogen, Burlington, ON). DIG-labeled RNA sense and anti-sense probes were synthesized as previously described (Paluzzi et al., 2008). The aforementioned steps were taken for designing RNA probes specific for sNPF as well, where the gene-specific primers used for preparing the DNA template were 42F and 548R.

Fluorescent in situ Hybridization (FISH)

Tissues were dissected in nuclease-free DPBS and transferred to 1.5mL microcentrifuge tubes containing fixation solution (40% filter-sterilized paraformaldehyde diluted in DPBS for a final concentration of 4%) where they were fixed at room temperature for 30 minutes on a rocker (Paluzzi et al., 2008). Tissues were subsequently washed 5 times with 0.1% Tween-20 in DPBS

(PBT) and treated with 1% H_2O_2 (diluted in DPBS) for 10 minutes at room temperature to quench endogenous peroxidase activity. Tissues were then incubated in 4% Triton X-100 (Sigma-Aldrich, Oakville, Ontario, Canada) in PBT for one hour at room temperature to and then washed for 5 minutes with PBT to stop the digestion. Tissues were placed in fixative once more for 20 minutes at room temperature on a rocker and then washed with PBT to remove all traces of fixative. Tissues were transferred into PCR tubes and rinsed in a 1:1 mixture of PBT-RNA hybridization solution (50% formamide, 5x saline sodium citrate, 100 µg/ml heparin, 100 µg/ml sonicated salmon sperm DNA, and 0.1% Tween 20; filter sterilized through a 0.2-µm filter, and stored in aliquots at –20°C), followed by placing them in 100% RNA hybridization solution. Aliquots (400 µl/sample) of hybridization solution were boiled at 100°C for 5 min and then cooled on ice for a minimum of 5 min and used as the pre-hybridization solution. Tissues were incubated in the pre-hybridization solution in an incubator set at 56°C for 1 hour. 400 ng of antisense RNA probe (or sense RNA probe for controls) in an aliquot of hybridization solution were heated at 80°C for 3 minutes to denature the probe and cooled on ice for a minimum of 5 minutes. At the end of the pre-hybridization incubation, the solution was removed and replaced with hybridization solution containing labeled probe and incubated approximately 16 hours (overnight) at 56°C. Tissues were then washed with solutions preheated to 56°C; twice with hybridization solution and then with pre-warmed 3:1, 1:1, and 1:3 (vol/vol) mixtures of hybridization solution: PBT for approximately 5 minutes each. The tissues were then washed once with pre-warmed PBT (56°C) and acclimatized to room temperature. To reduce non-specific staining, tissues were blocked with PBTB (PBT with 1% blocking reagent; Invitrogen, Carlsbad, CA) for one hour on a rocker (at room temperature). The tissues were then incubated in a solution of PBTB and a 1:400 dilution of a biotin-SP-conjugated IgG fraction monoclonal

mouse anti-digoxin antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 2 hours at room temperature (~22˚C) on a rocker protected from light. In attempts to optimize detection and signal, the following RNA probe concentrations were tested for CAPA and sNPF: 500ng 700ng, and 1000ng.

The primary antibody incubation was followed by several washes of tissues with PBTB over the course of 1 hour. The tissues were subsequently transferred to a 1:100 dilution of horseradish peroxidase-streptavidin stock solution (Molecular Probes, Eugene, OR) in PBTB and incubated for 1 hour at room temperature with constant mixing and protected from light. Tissue samples were then washed for 1 hour with PBTB followed by two brief washes in PBT and once with DPBS. Toward the end of the final wash, Alexa Fluor 568 (or 647) tyramide working solution was prepared by diluting stock solution 1:1000 in amplification buffer containing 0.0015% H_2O_2. After the last DPBS wash, the solution was removed from the samples and replaced with 100 µl of the prepared tyramide working solution and incubated in the dark for 1 hour at room temperature with constant mixing. The tyramide solution was then removed and the tissues were washed with DPBS several times over the course of an hour. Following the completion of these washes, the tissues were placed in DPBS overnight at room temperature on a rocker to further reduce the non-specific background signal. Tissues were then mounted on slides in ProLong Diamond Antifade Mountant with 4', 6'- Diamidino-2-phenylindole dihydrochloride (DAPI) and slides were viewed using Cy5 filter and DAPI filter (for visualizing cell nuclei) under the EVOS FL Auto live-cell imaging system (Life Technologies, Burlington, ON, CA).

Tissue-Specific Spatial Expression Analysis Using RT-PCR

To verify the immunoreactive staining obtained, reverse transcription-polymerase chain reaction (RT-PCR) was conducted to determine the presence or absence of CAPA gene at the transcript level. The midgut, salivary glands, reproductive tissues, and hindgut (intestine, rectal sac, and MTs) were isolated from unfed adults *I. scapularis* and stored in RNA lysis solution overnight at -20˚C. The tissues were then ground using a mortar and pestle (Ambion, Austin, TX). The ground tissues were then used in the total RNA isolation procedure as discussed above. These samples were then used to make cDNA using iScript™ Reverse Transcription Supermix for RT-PCR (Bio-Rad Laboratories Inc., Mississauga, Ontario, Canada). Slight modifications were made to the recommended protocol; 10 µL reaction volumes were used and the reaction protocol consisted of priming at 25°C for 5 minutes, reverse transcription at 46°C for 20 minutes, RT inactivation at 95 °C for 1 minute, then cDNA samples were held at 4°C until use. The cDNA was used as template for PCR with standard *Taq* DNA Polymerase (Life Technologies, Burlington, ON) using CAPAF1 and CAPAR2 primers for CAPA, and 42F and 548R primers for sNPF (see Table 2). For sulfakinin, four different primer combinations were utilized: 23F and 610R, 23F and 743R, 218F and 610R, and 218F and 743R (see Table 2). For positive control, the housekeeping gene ribosomal protein 49 (RP49) previously reported for use in *I. scapularis* (Gondalia et al., 2016), was used.

Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of CAPA-like material was done using ELISA methodology. 100 µL/well of carbonate buffer solution (15 mM Na2CO3-H2O and 35mM NaHCO3 in water; pH 9.4) was put in column 1. The remaining wells of a 96-well plate were coated with 100 µL/well of a 1:1000

dilution of rabbit anti-RhoprCAPA- α2 primary antibody made up in carbonate buffer solution and incubated overnight at 4 °C. The following day, the plate contents were discarded, the plates were inverted and gently tapped on a paper towel (blotted) to remove any remaining contents in the wells. The plate was then washed three times with 250 µL/well of wash solution (0.05% Tween-20 (v/v), 350 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 5.15 mM Na2HPO4-H2O in nuclease-free water). Subsequently, wells were blocked for 1.5 hours at room temperature (RT) with 250 µL/well of block solution (0.5% skim milk powder (w/v) and 0.5% BSA (w/v) in PBS). Later, the block solution was discarded and the 96-well plate was blotted. Thereafter, the wells were incubated with 100 µL/well of either standard IxoscCAPA-PVK antigen at various doses, protein extracts from the various tissues (see next section below) of *I. scapularis,* or other peptides sharing some structural similarity to IxoscCAPA-PVK (*eg. Ixodes PK*-1 and -2, and *Rhopr*-sNPF; see table 3), which were used to determine their binding affinity to the RhoprCAPA- α2 antibody (all made up in block solution) and incubated on a rocker for 45 minutes at RT. Afterwards, 100 µL/well of $5x10^{-9}$M biotinylated- IxoscCAPA-PVK antigen (prepared in block) was added and incubated overnight at 4°C. Plate contents were later discarded, the plates were blotted, and washed four times with 250 µL/well wash solution. This was followed by incubation with 100 µL/well Avidin-HRP in block solution (1:2000; Bio-Rad, Mississauga, ON, CA) for 2 hours at 4ºC. Once again the plate contents were discarded, the plates were blotted and washed four times with 250µL/well of wash solution. Incubation with 100 µL/well of 3,3',5,5'-tetramethylbenzidine (TMB) solution (Sigma-Aldrich, Oakville, ON) followed for 10-15 minutes at RT and observe colour development. Finally, the reactions were stopped with 100 µL/well of 2N HCl and absorbance was measured at 450 nm using a Synergy 2 Modular Multi-Mode Plate Reader (BioTek, Winooski, VT, USA).

Table 3: C-terminal sequence comparison between select *R. prolixus* and *I. scapularis* peptides (Ons et al., 2009; Neupert et al., 2009).

Peptide Name	Peptide Sequence
sNPF	
Rhopr-sNPF	NNRSPQLRLRFa
Ixosc-sNPF	GGRSPSLRLRFa
SIFa	
RhoprSIFa	TYKKPPFNGSIFa
IxoscSIFa	AYRKPPFNGSIFa

Protein Extraction for ELISA

The midgut, synganglion, reproductive system, and hindgut (consisting of the intestine, MTs, rectal sac, and anal canal) from 25 ticks (for both males and females) were dissected and pooled in 100 µL of ice-cold nuclease-free water and stored at -20˚C for later use. The tissues were then sonicated (QSonica XL, 2000 series, Newtown, CT, USA) on ice and centrifuged at 10,000 x*g* for 10 minutes. The supernatant was collected and dried in a Speed vacuum concentrator (Jouan Inc., RC1010, Winchester, UK). Subsequently, 300 µL blocking solution was added to each of the dried-down samples, and used according to the ELISA protocol described above.

The statistical analysis for the ELISA results consisted of compiling the data in Microsoft Excel and performing a one-way ANOVA.

Impedance contraction assays

Ticks were secured firmly on dental wax (placed on top of an inverted petri dish) and had their dorsal cuticle removed. The midgut and reproductive tissues were dissected out in saline (140 mM NaCl, KCl 5 mM, MgCl2 1 mM, CaCl2 5 mM, NaHCO3 4 mM, and 5 mM HEPES, pH 7.2.; Simo and Park, 2014). Removal of the hindgut (intestine, Malpighian tubules, rectal sac, and anal canal) for the contraction assays entailed cutting the intestine from the midgut and excising the portion of the ventral cuticle containing the rectal opening and placing it ventral side down on the dental wax. The samples were submerged in 30 µL of saline upon initiating the assay. The setup consisted of two probes (held in position using Kanetec stands) that were placed on opposite sides of the hindgut preparation, allowing for the detection of mechanical disruptions caused by the tissue contractions propagating in the saline. This mechanical signal was

transduced into an analog signal using an impedance converter (UFI, model 2991, Morrro bay, California), which was subsequently converted into a digital signal using PowerLab 15T (LTS, ADInstruments) and was visualized using Lab Chart 8 reader program. Upon obtaining the baseline reading of the tissues in saline, 3 μL of saline was removed and added back again (acting as the "true baseline"); for the purpose of taking into consideration the deviations in the signal as a result of external interference. The true baseline was obtained for 2-3 minutes, followed by the removal of 3 μL of saline once more and replaced with 3 μL of 10^{-5} M IxoscCAPA-PVK peptide. Finally, 3 μL of this solution was removed and replaced with 3 μL of 10^{-5} M *R. prolixus* SIFa (*Rhopr*SIFa; a kind gift from Angela Lange, University of Toronto Mississauga), since previous motility assay studies had shown that SIFa elicits an excitatory effect on the *I. scapularis* hindgut (Simo and Park, 2014), thus acting as a positive control. *Rhopr*SIFa (TYKKPPFNGSIF-NH_2) has a high sequence homology (83% identical; see table 3) to the *I. scapularis* SIFa (AYRKPPFNGSIF-NH_2; Neupert et al., 2009).

The statistical analysis for the impedance contraction assays consisted of compiling the data in GraphPad Prism 7 and conducted a repeated measures ANOVA for both males and females.

Results

Immunohistochemistry

The distribution of IxoscCAPA-PVK was determined using whole mount immunohistochemistry. This allowed for the localization of CAPA-like peptides in the synganglion and peripheral tissue of unfed adult *I. scapularis*. Overall, there was CAPA-like immunoreactivity localized in the cells and processes of the synganglion, in endocrine-like cells in the midgut, in the reproductive organs, and immunoreactivity in the posterior regions of the rectal sac. There were some differences observed in CAPA-like immunoreactivity between adult male and female *I. scapularis* in the midgut and reproductive tissues. Overnight preincubation (peptide block) of the antibody with IxoscCAPA-PVK (50 μmol l^{-1}; at 4˚C) eliminated all immunoreactivity, except for non-specific staining associated with guanine crystals in the rectal sac.

Midgut

Immunoreactive CAPA-like cells were observed in the midgut of both male and female adult *I. scapularis* (Figures 2.1-2.4). There were differences in the number and distribution of these cells between males and the females. In females, immunoreactivity was more abundant in comparison to that observed in males, with a greater distribution in the anterior region of the midgut diverticulum than the midgut stomach (Figures 2.1 and 2.2). Additionally, there was very minimal staining observed in the posterior regions of the midgut, in both the stomach and diverticula. In males, there were only sparsely localized immunoreactive cells in the midgut (Figure 2.4). It is of interest to note that the number and distribution of these CAPA-like neuropeptides in the midgut varied between individuals in both males and females,

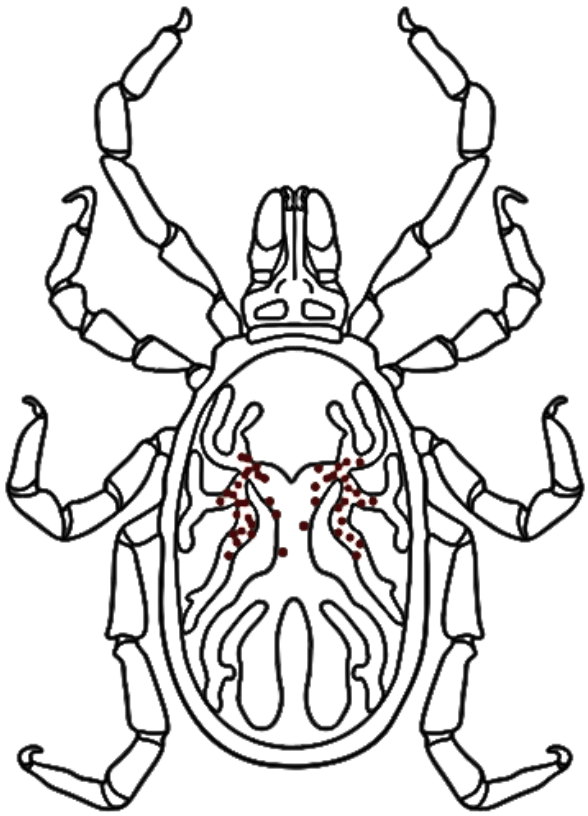

Figure 2.1: Schematic representation of the distribution of CAPA-like immunoreactivity in the female midgut of *I. scapularis* (not to scale). The dots indicate an overall representation of the distribution of CAPA-like immunoreactive cells. Distributions were mapped based on observations from immunohistochemistry. There were variations of the number and distribution of immunoreactive cells between individuals. Drawing by Lesia Szyca.

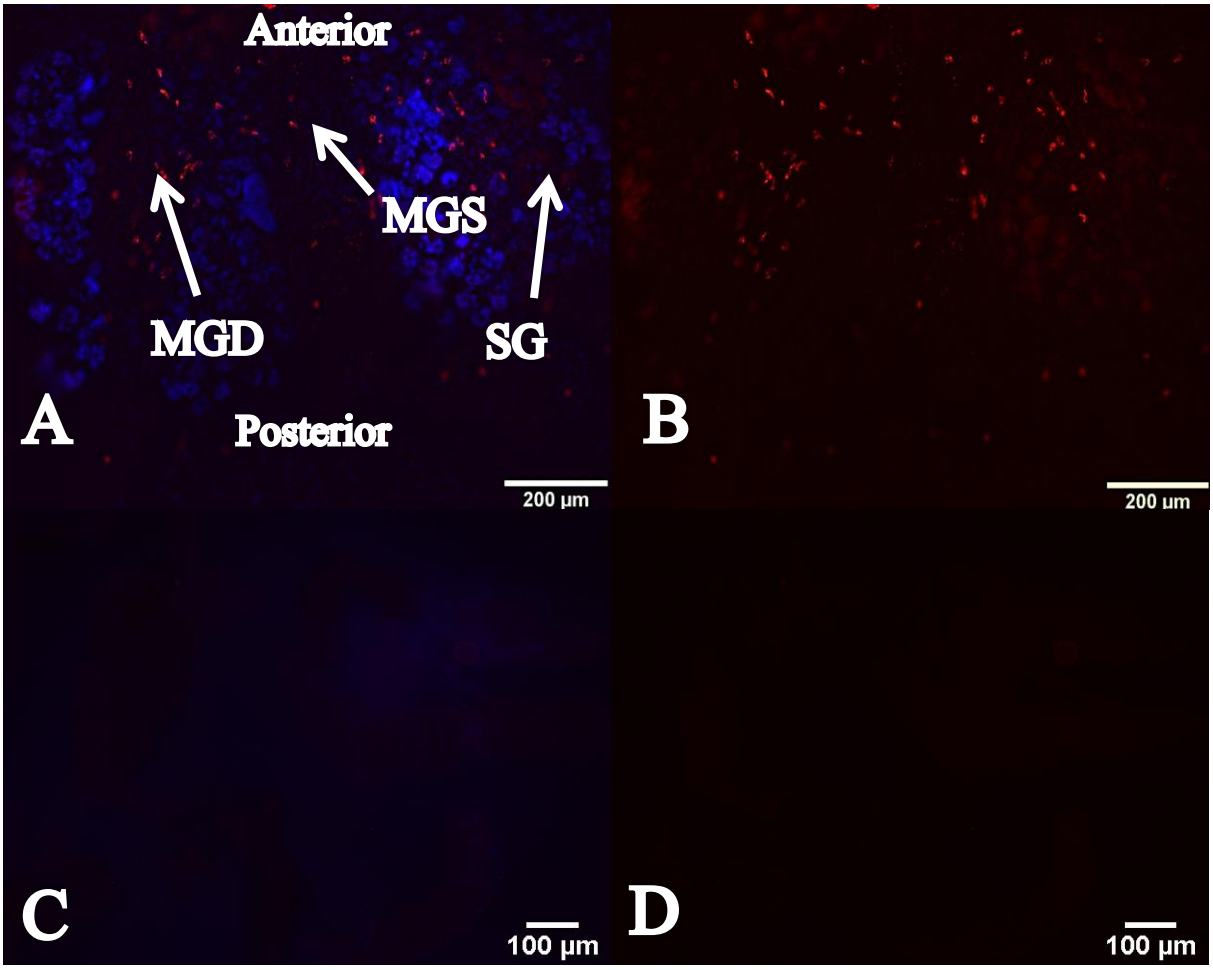

Figure 2.2. CAPA-like immunoreactivity in the midgut of adult female *I. scapularis* (dorsal view; 10X magnification). A) DAPI (blue) and TRITC (red) filter merged together; B) Only TRITC filter. It is evident that the CAPA-like immunoreactive staining is localized in the anterior regions of the midgut. With a higher abundance of staining in the midgut diverticula (MGD) as opposed to the midgut stomach (MGS). With DAPI staining showing the underlying grape-like salivary glands (SG). C) and D) are the controls obtained from peptide blocking; C) DAPI and TRITC filters merged, D) only TRITC filter.

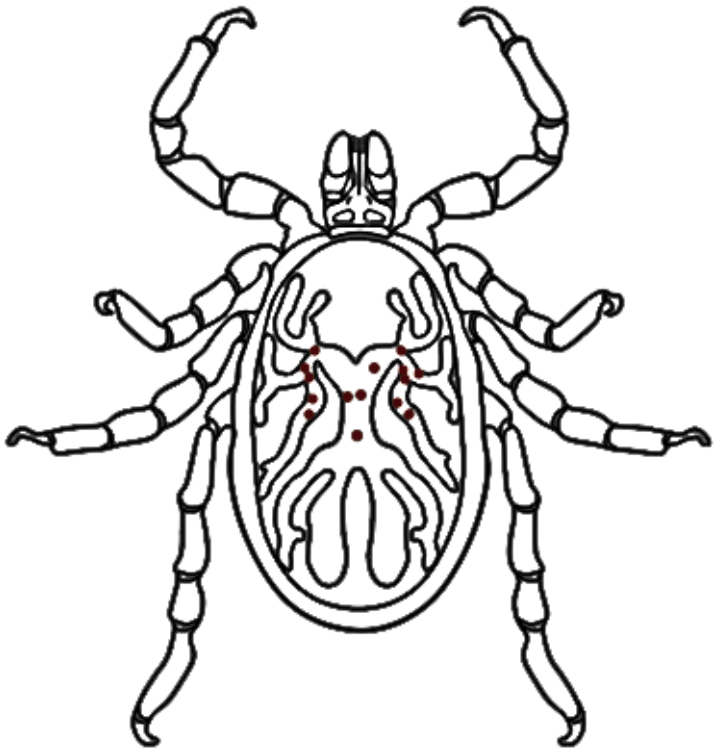

Figure 2.3. A schematic representation of CAPA-like immunoreactive cells within the midgut of adult male *I. scapularis.* The dots denote the average distribution of immunoreactivity in adult males. The quantity and distribution of cells were observed to vary among individuals (not to scale). Drawing by Lesia Szyca.

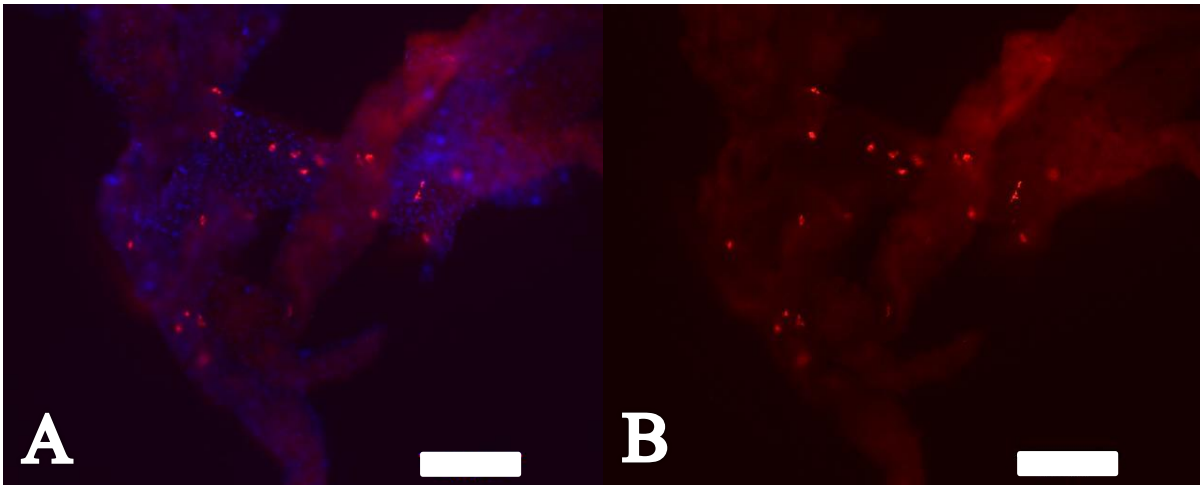

Figure 2.4. CAPA-like immunoreactivity in the midgut of adult male *I. scapularis* (10X magnification). A) DAPI (blue) and TRITC (red) filters merged together and B) only TRITC filter. A small number of CAPA-like immunoreactive cells distributed sporadically within the midgut but localized only to the anterior region. Peptide block abolished all immunoreactivity (data not shown; scale bar= 200 μm).

thus the schematic representation is a general representation of observations in both females (Figure 2.1) and males (Figures 2.3). Furthermore, while observing some of the female midguts it was observed that the heart also showed CAPA-like immunoreactivity (Figure 2.5).

Synganglion

There were no differences observed in CAPA-like immunoreactivity between sexes; the schematic representation illustrates the distribution of cells in the synganglion for both males and females (Figure 2.6). There were approximately 9 pairs of immunoreactive cells observed in both males and female synganglia; two pairs of bilateral cells in the anterior region of the protocerebral lobe, three bilateral cells in the posterior region of the protocerebral lobe, immunoreactive cells in pedal lobes 3 and 4, and a cluster of 2 pairs of cells localized in the opisthosomal lobe (Figures 2.6-2.8). Furthermore, CAPA-like staining is observed in the neuropile of the synganglion.

Salivary glands

In both males and females, there was no CAPA-like immunoreactivity observed in the salivary glands, including absence of staining in the salivary duct and all three types of acini (Figure 2.9).

Reproductive system

In female adult *I. scapularis*, there was CAPA-like imunoreactivity in the anterior region of the reproductive system, more specifically around the lobular accessory gland and

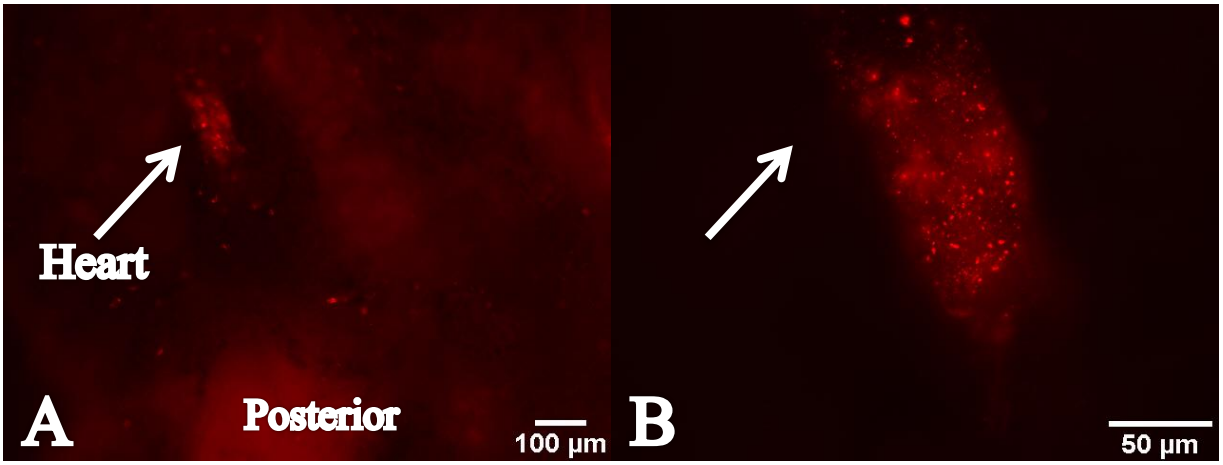

Figure 2.5: CAPA-like immunoreactivity in the heart. The heart is located dorso-medially in the middle region of the midgut stomach in adult *I. scapularis*. A) TRITC (red) filter only (at 10X magnification); B) TRITC filter only (at 40X) magnification).

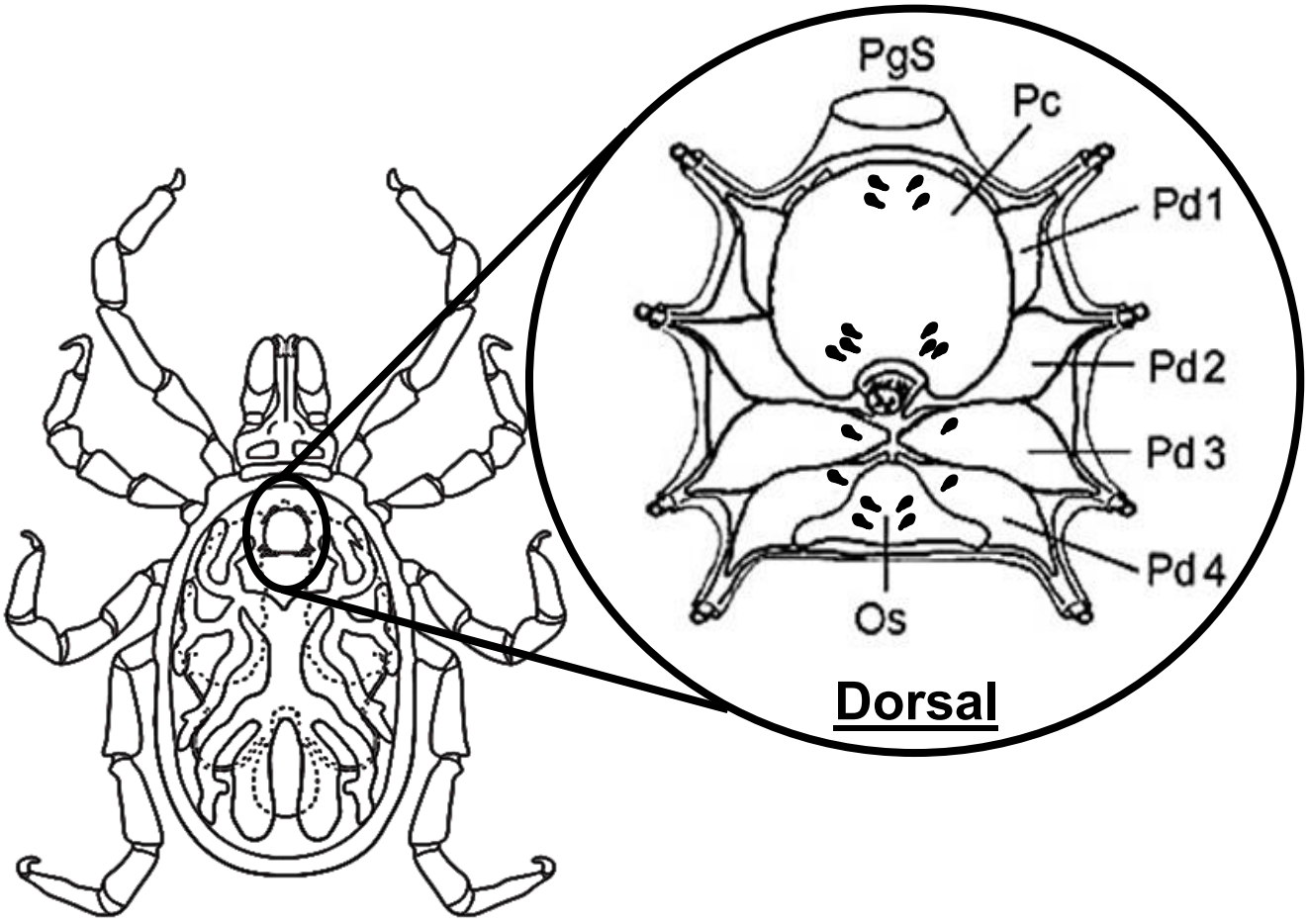

Figure 2.6. An illustration showing CAPA-like immunoreactive cells localized in the synganglion (not to scale). There were 2 pairs of immunoreactive cells in the anterior region of the protocerebral lobe. Furthermore, there was localization of 3 bilateral cells (6 total cells) in the posterior region of the protocerebral lobe (Pc). In the posterior region of the synganglion, there are approximately 4-5 pairs of cells in the opisthosomal lobe (Os) and immunoreactive cells found in pedal lobes 3 and 4 (Pd3 and 4). No immunoreactivity was observed for the periganglionic sheeth (PgS), and Pedal lobes 1 and 2 (Pd1-2). Adapted from Simo et al., 2009a. Drawing by Lesia Szyca.

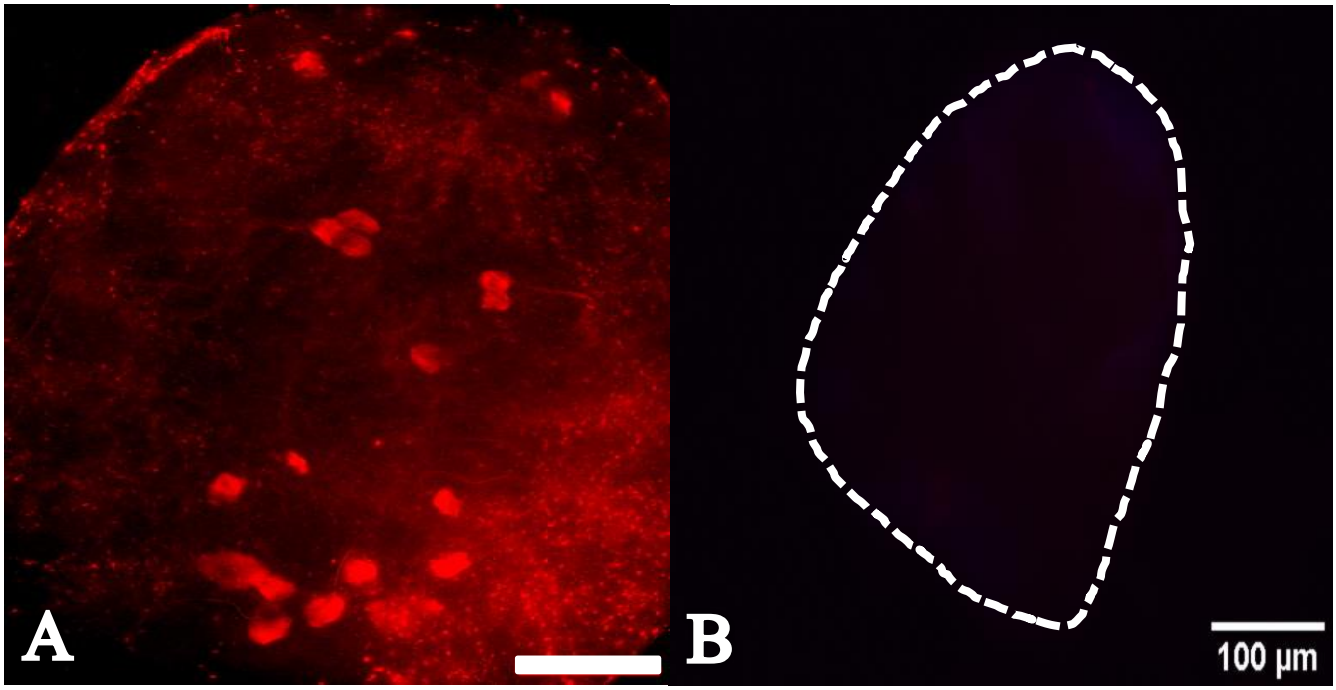

Figure 2.7. CAPA-like immunoreactivity in the female synganglion. A) compressed Z-stack image using TRITC filter, 2 bilaterally paired CAPA-like immunoreactive cells in the anterior region of the protocerebral lobe, 3 bilateral pairs in the posterior region of the protocerebral lobe, groupings of 4-5 immunoreactive cells in the opithosomal lobe, and single immunoreactive cells observed in pedal lobes 3 and 4. Neuropile staining was also observed; bar = 100 µm. B) Peptide block control, showing no CAPA-like immunoreactivity. DAPI (blue) and TRITC (red) filters.

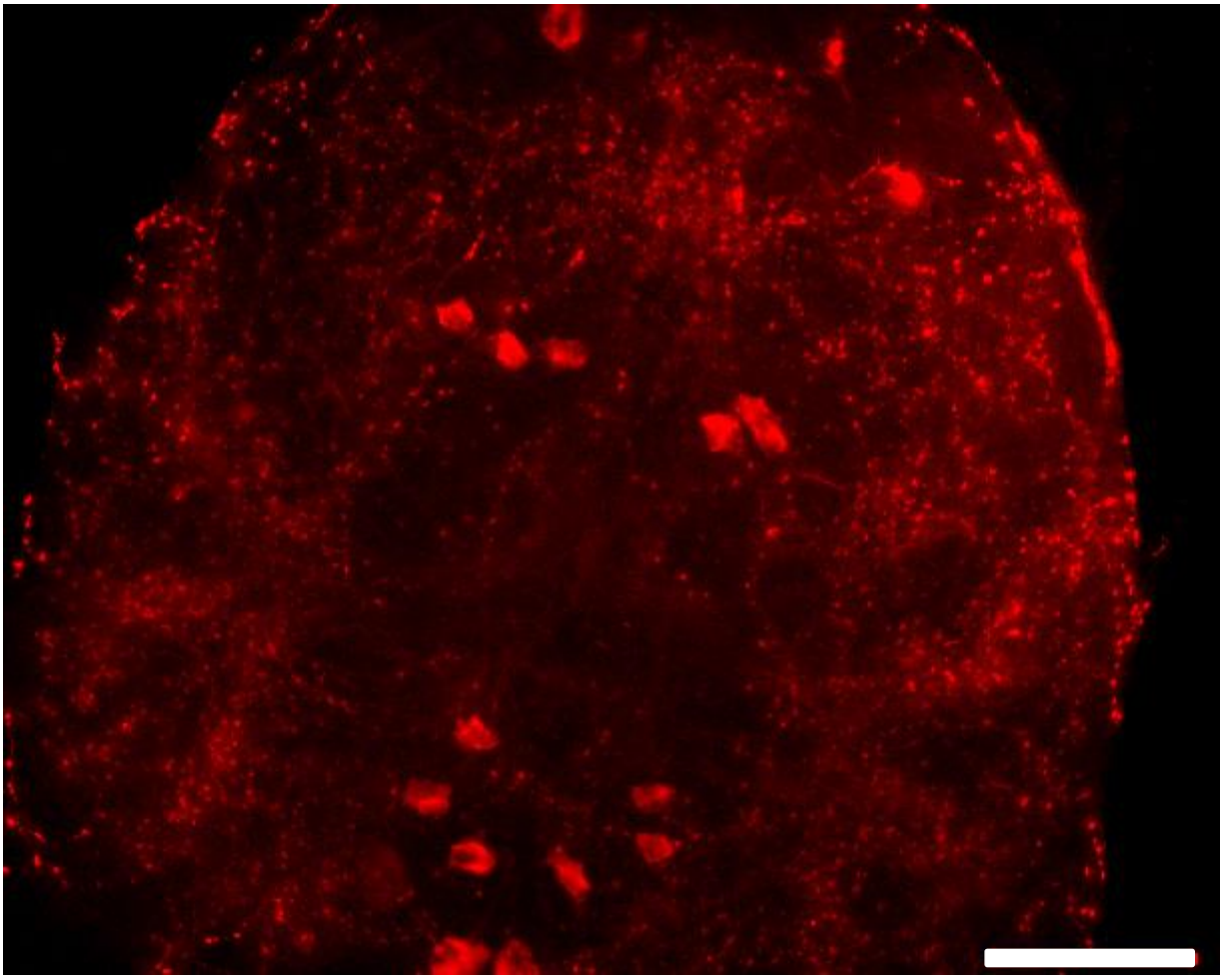

Figure 2.8. CAPA-like immunoreactive cells and processes in the male synganglion of *I. scapularis* (20X magnification). Compressed Z-stack image using TRITC filter, shows 2 pairs of CAPA-like immunoreactive cells in the anterior region of the protocerebral lobe, followed by 3 pairs in the posterior region of the protocerebral lobe. Furthermore, approximately 4 cells localized in the opithosomal lobe, and single immunoreactive cells present in pedal lobes 3 and 4. Neuropile staining can also be seen. Scale bar =100 μm.

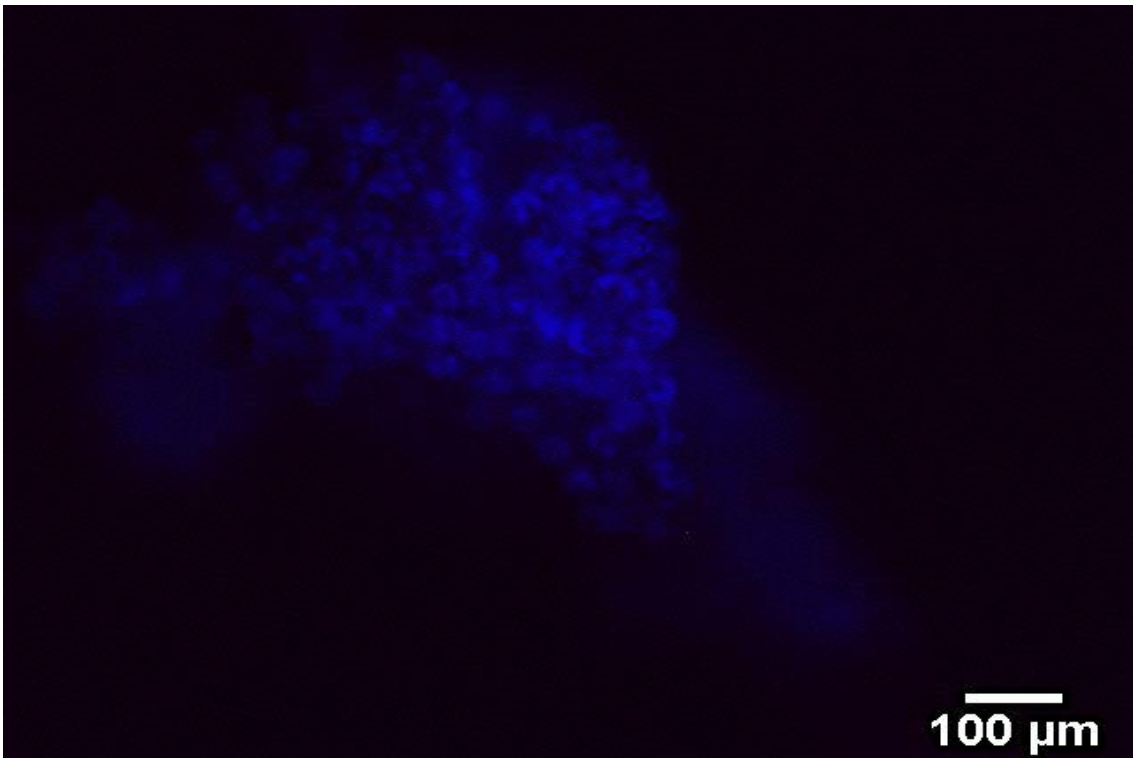

Figure 2.9. Salivary glands of an adult female *I. scapularis* (10X magnification). No CAPA-like immunoreactivity was observed in the salivary glands of both males and females. Image obtained using both DAPI (blue) and TRITC (red) filters.

immunoreactivity in the tubular accessory gland surrounding the vestibular vagina (Figures 2.10 and 2.11). There was no strong CAPA-like immunoreactivity in the posterior region of the female reproductive system; however, after increasing the exposure, CAPA-like immunoreactive cells were observed in the lateral oviduct, common oviduct and also in the cervical region of the vagina (Figure 2.12). Notably, there was no CAPA-like immunoreactivity detected in the ovaries (Figure 2.13).

In the reproductive system of the male adult *I. scapularis*, like the female accessory gland, there was broad staining observed in the male accessory glands (Figure 2.14 and 2.15). Furthermore, there were sparsely localized CAPA-like immunoreactivity in the posterior region of the male reproductive system was.

Hindgut

The hindgut tissues that were processed for immunohistochemistry consisted of the intestine, MTs, rectal sac, and the anal canal (Figure 2.16-2.18). There was no CAPA-like immunoreactivity detected in the intestine and MTs. Guanine crystals located in the rectal sac and MTs were observed to show immunoreactivity (Figure 2.17A), but this staining persisted when the antiserum was pre-incubated overnight with IxoscCAPA-PVK (Figure 2.17B). Notably, CAPA-like immunoreactivity was detected at the junction between the rectal sac and the anal canal, indicative of neural processes, as there were no nuclei present (Figure 2.18B). There were no observed differences for CAPA-like immunoreactivity between the male and female rectal sac of adult *I. scapularis* (Figure 2.16-2.18).

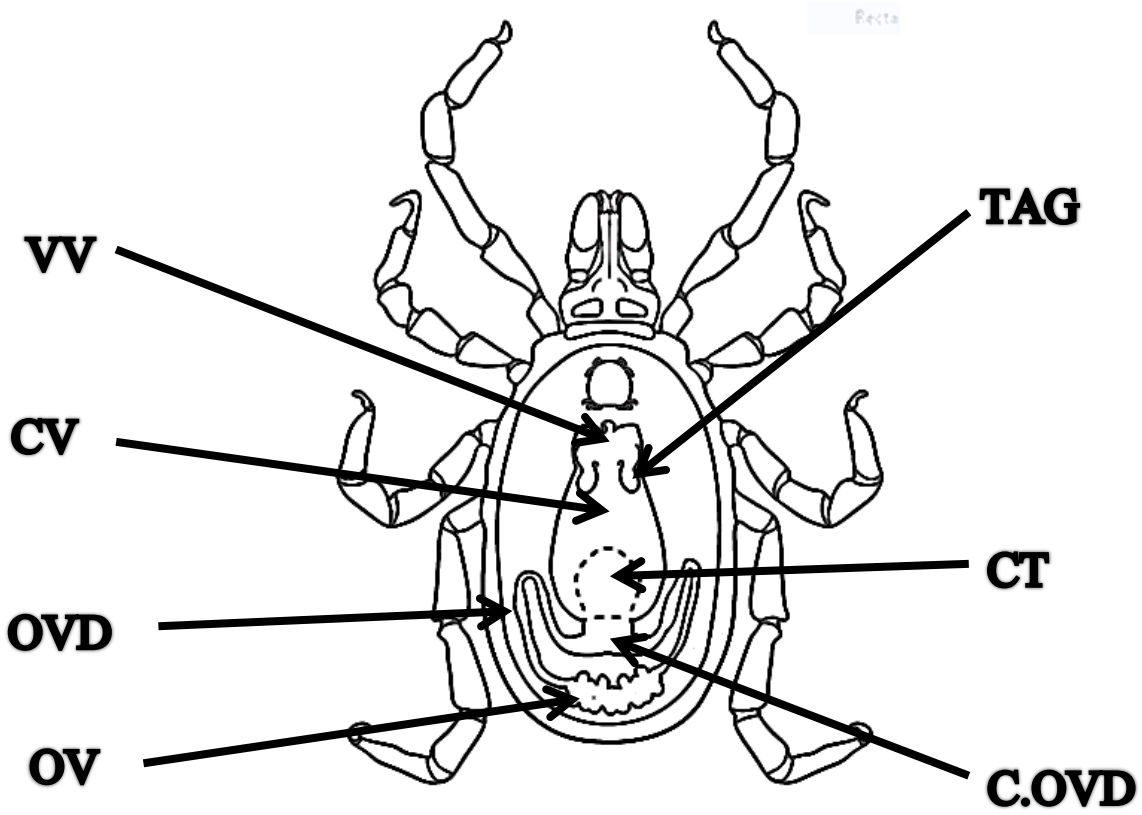

Figure 2.10. Schematic representation of the female reproductive system. The female reproductive system is localized ventrally to the midgut. It consists of the vestibular part of vagina (VV); tubular accessory gland (TAG); cervical part of vagina (CV); connecting tube (CT); common oviduct (C.OVD); oviduct (OVD); ovary (OV). Drawing by Lesia Szyca.

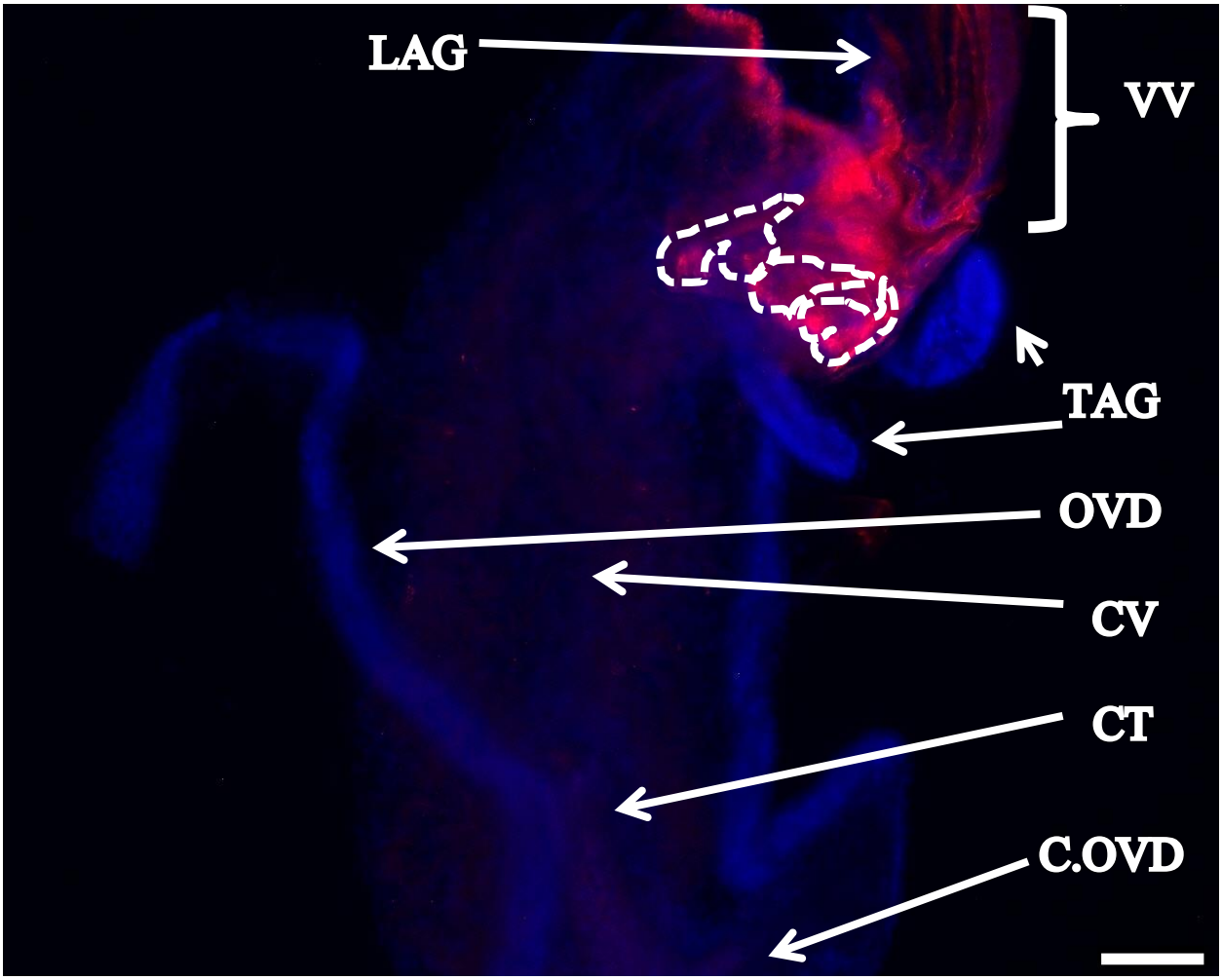

Figure 2.11. CAPA-like immunoreactivity in the female reproductive system (10X magnification). This image contains DAPI (blue) and TRITC (red) filters combined. There is strong CAPA-like immunoreactivity localized in the anterior region of the reproductive system. More specifically, CAPA-like immunoreactivity localized dorsally to each of the tubular accessory glands (TAG). Furthermore, there were broad immunoreactive staining localized around the vestibular part of the vagina (VV), perhaps associated with the lobular accessory gland (LAG). The other regions that can be observed with the DAPI filter are the cervical part of vagina (CV); connecting tube (CT); common oviduct (C.OVD); oviduct (OVD). No immunoreactivity detected upon conducting peptides block (data not shown). Scale bar = 100 μm

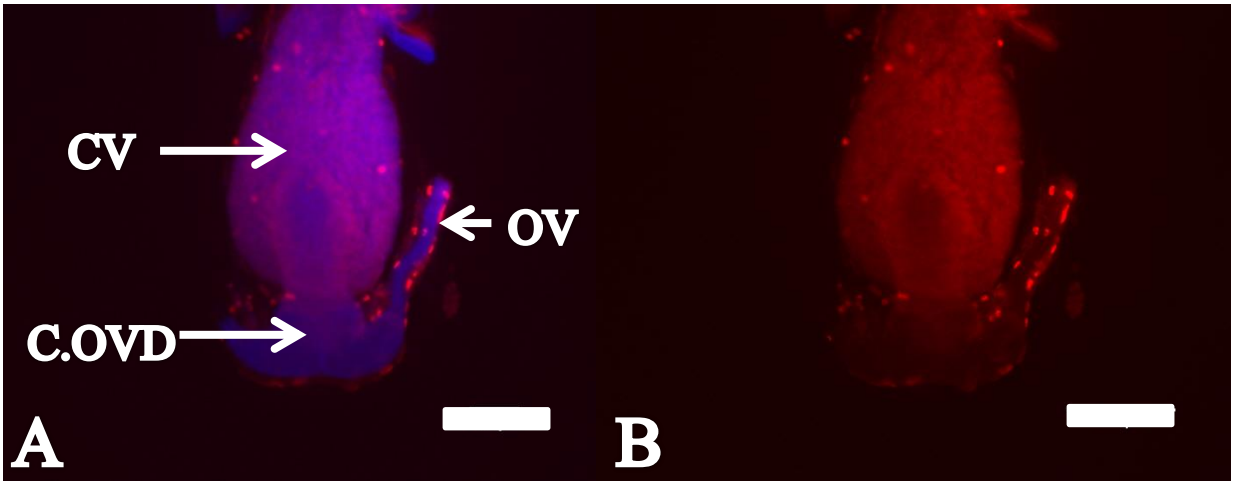

Figure 2.12. Increased exposure of CAPA-like immunoreactive cells in the female reproductive system (10X magnification) A) DAPI (blue) and TRITC (red) filters merged; B) only TRITC filter used. After increasing the exposure time, CAPA-like immunoreactivity is observed in the oviduct (OV), common oviduct (C.OVD), and regions of the cervical part of the vagina (CV). Scale bar = 200μm.

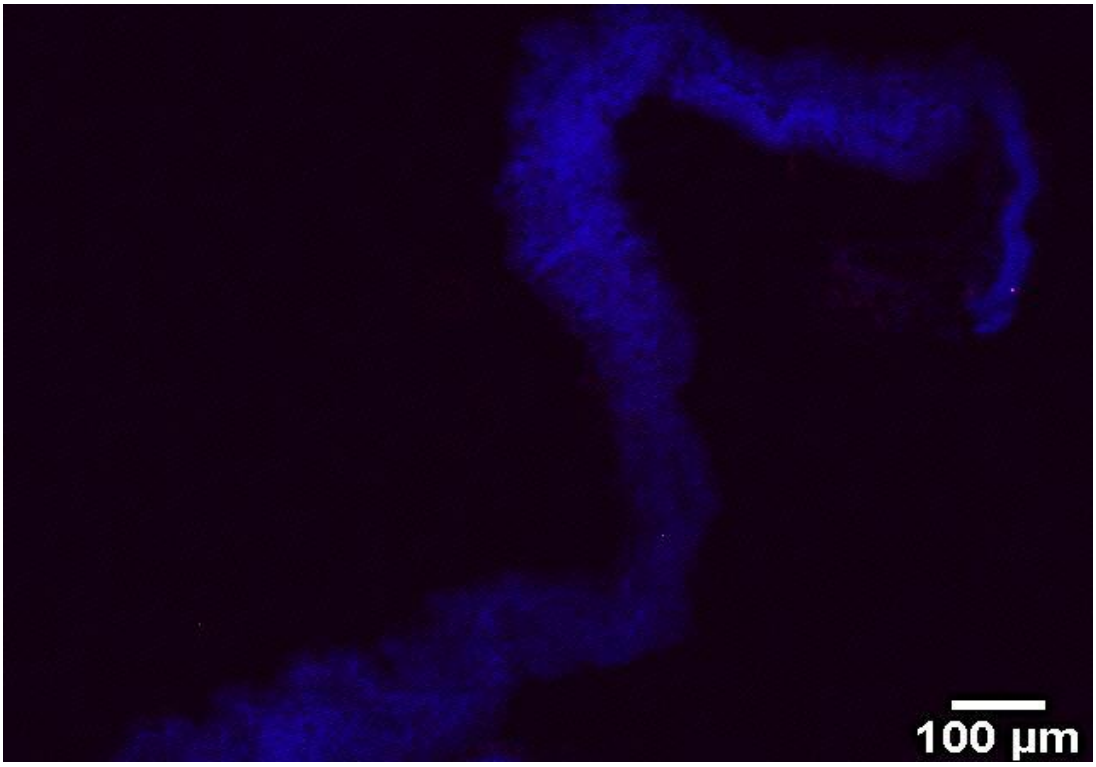

Figure 2.13. Ovaries of an adult female *I. scapularis* at 10X magnification. DAPI (blue) and TRITC (red) filters combined; there is no CAPA-like immunoreactivity detected .

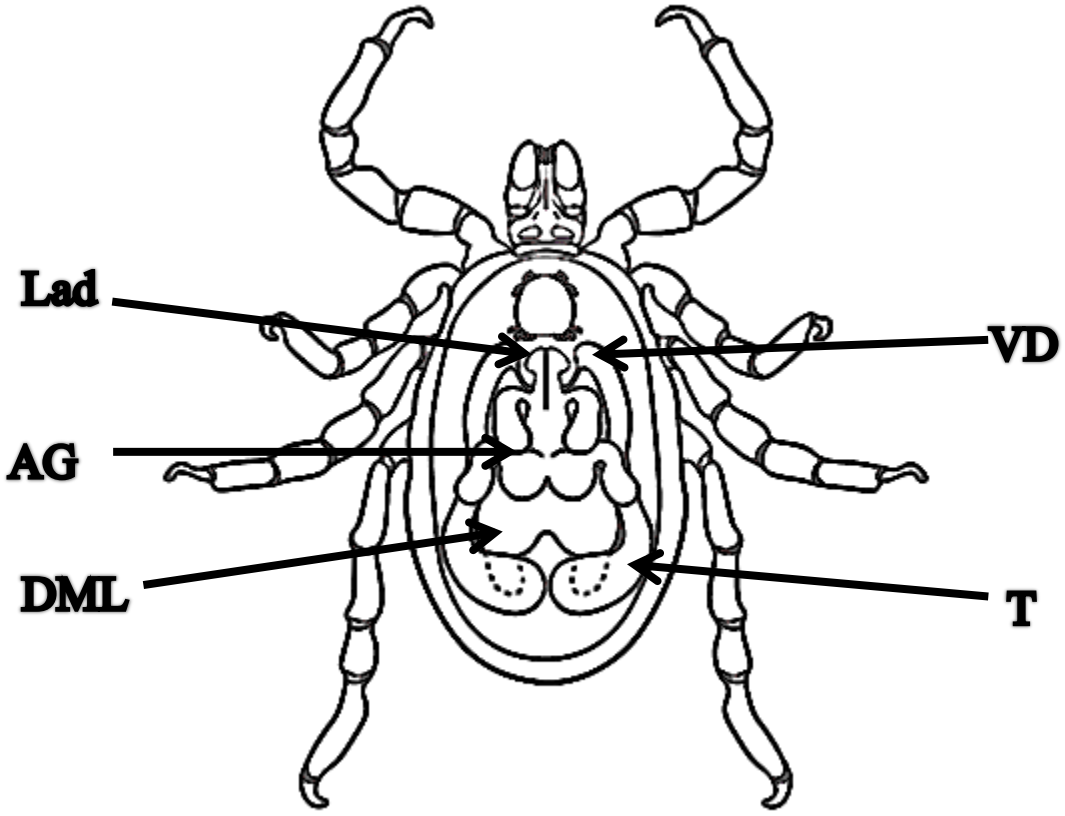

Figure 2.14. Schematic of the male reproductive system. The male reproductive system is located ventrally to the midgut. It consists of the vas deferens (VD), the testis (T), and the multi-lobed accessory gland (AG) which includes the antero-dorsal lobe (Lad) and the dorsal median lobe (DML; not to scale). Drawing by Lesia Szyca.

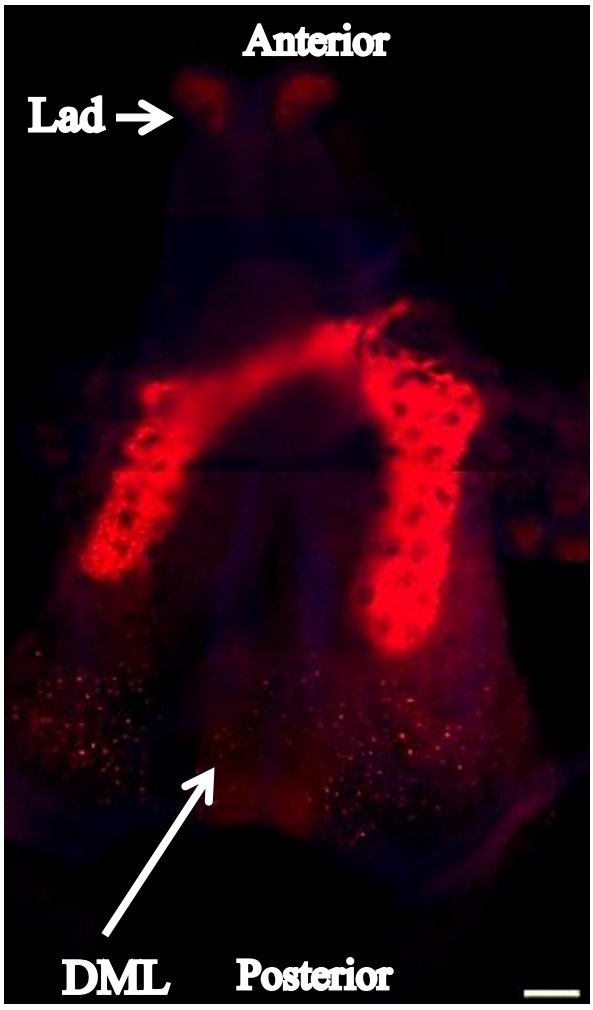

Figure 2.15. CAPA-like immunoreactivity in the male reproductive system. This image was constructed by stitching three separate images of the anterior, mid, and posterior region of the male reproductive system, due to its large size (each at 10X magnification). CAPA-like immunoreactivity is observed in the antero-dorsal lobe (Lad) of the accessory gland, followed by a network of staining in the mid-region (possibly in one of the lobes of the accessory gland), and some immunoreactive cell staining scattered at the posterior region of the dorsal median lobe (DML). Scale bar= 100 μm.

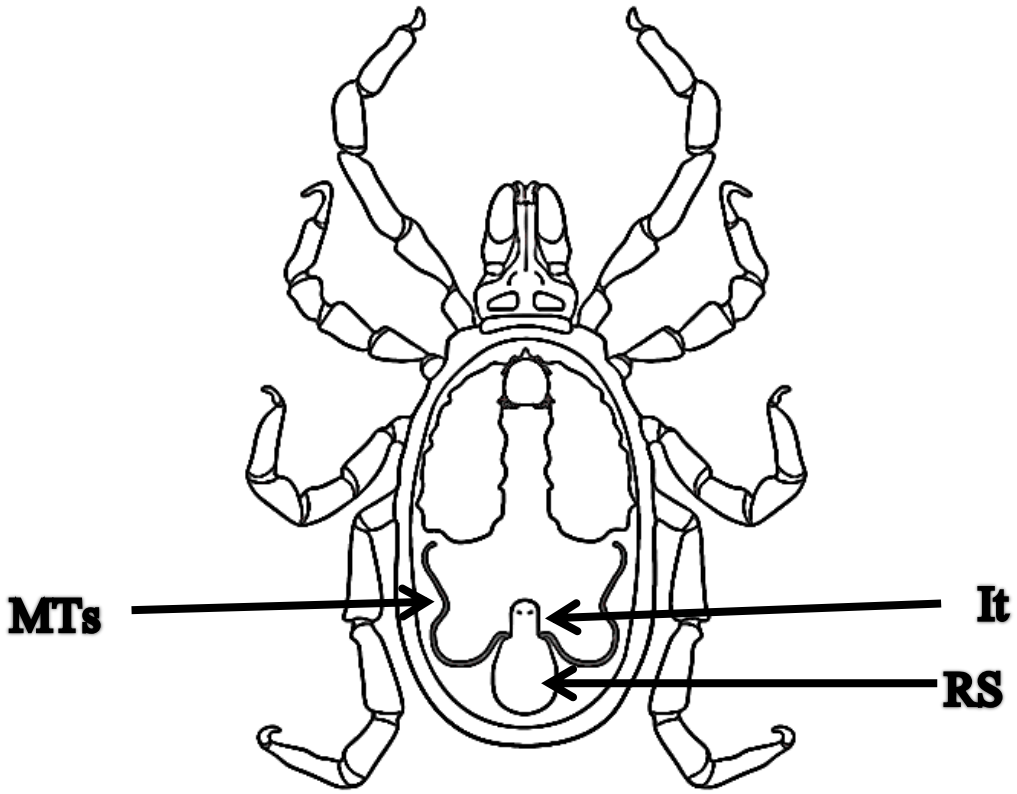

Figure 2.16. Schematic representation of *I. scapularis* hindgut. The hindgut is located posteriorly. Consisting of the intestine (It) that connects the midgut to the rectal sac (RS), followed by the Malpighian tubules (MTs; not to scale). Drawing by Lesia Szyca.

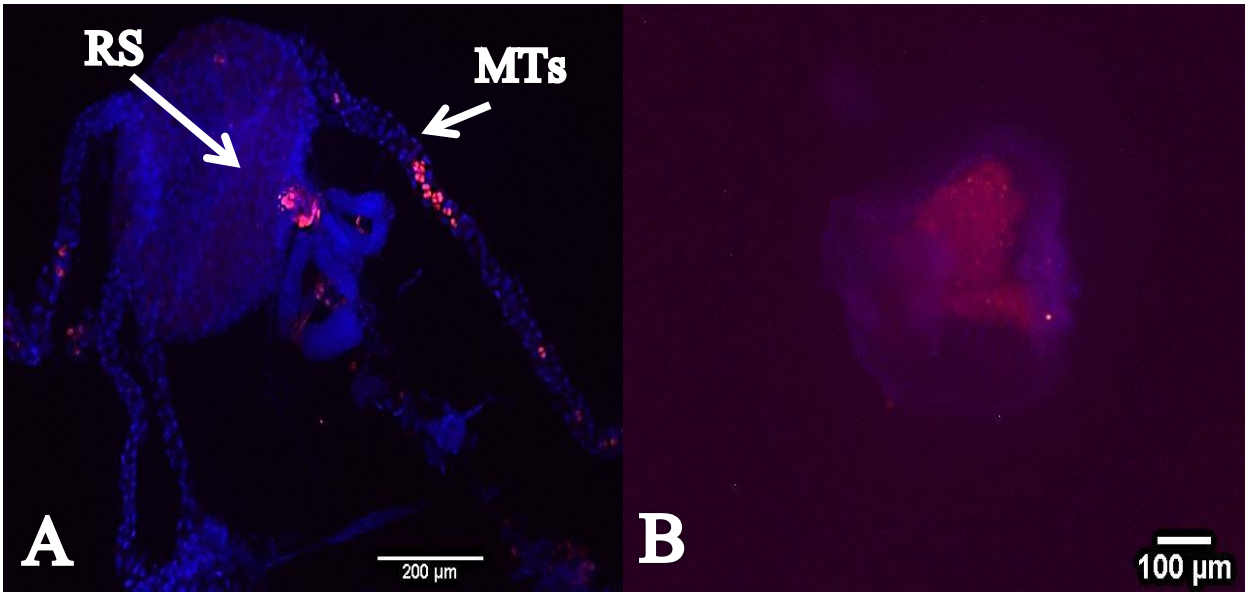

Figure 2.17. CAPA-like immunoreactivity in the female hindgut. Both images are at 10X magnification, using DAPI (blue) and TRITC (red) filters. A) CAPA-like immunoreactivity was detected at the junction of the posterior region of the rectal sac (RS) and the anal canal. Guanine crystal staining in some of the samples, and Malpighian tubules (MTs) showed non-specific immunoreactivity, as they persisted after a peptide block (B) was conducted.

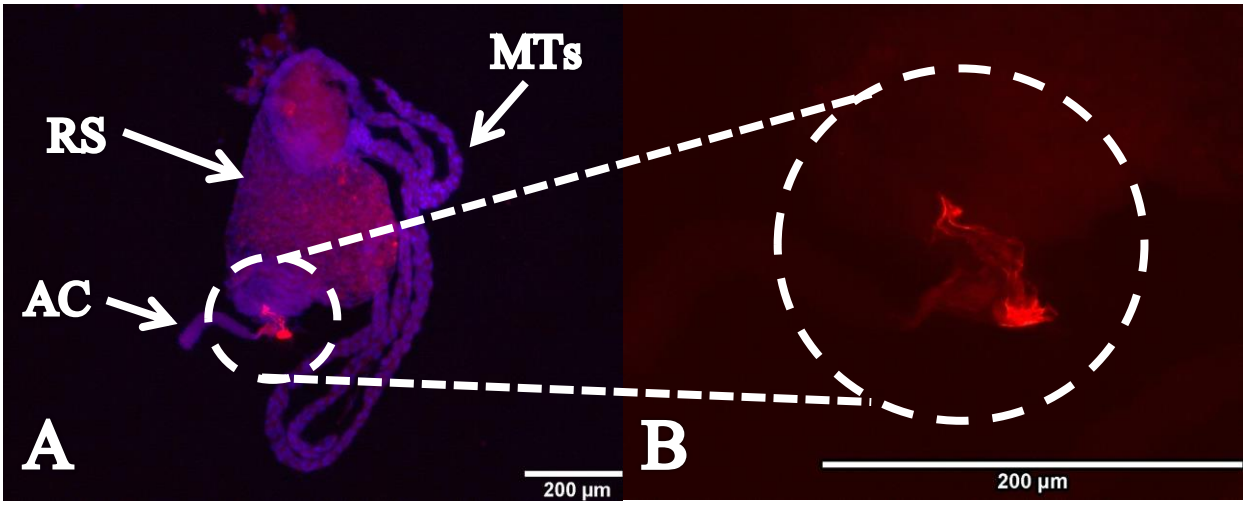

Figure 2.18. CAPA-like immunoreactivity in the male hindgut. (A) Merged DAPI (blue) and TRITC (red) filters (10X magnification), showing CAPA-like immunoreactivity at the junction between the posterior region of the rectal sac (RS) and the anal canal (AC). Malpighian tubules (MTs). (B) 40X magnification of the CAPA-like immunoreactivity.

Fluorescence in situ hybridization (FISH)

CAPA

In order to determine the expression profile of CAPA peptides at the transcript level, I attempted to examine transcript localization using FISH. Despite trying various concentrations and different primer combinations producing CAPA RNA probes, there was no cell-specific expression of the *I. scapularis* CAPA gene observed (Figure 2.19). Similar results were observed for both males and females.

sNPF

As a result of the lack of detection of the cell-specific expression of the *I. scapularis* CAPA transcript and, in conjunction with RT-PCR results (discussed later), *I. scapularis* sNPF RNA probes were designed using 42F and 548R primers (Figure 2.20). Similar to the FISH results obtained for *I. scapularis* using the CAPA RNA probe, there was no detection of the sNPF transcript.

FISH on CNS of Aedes aegypti

In order to deduce whether the absence of cells expressing CAPA and sNPF was a result of problems in the FISH protocol, the same protocol was used to conduct FISH using *Aedes aegypti* CAPA RNA probe since it had been previously conducted successfully (Uyuklu & Paluzzi, unpublished). As observed previously, the FISH protocol in adult *Aedes aegypti* reveals a pair of medially-localized cells expressing CAPA transcript in each of the abdominal ganglia of the central nervous system (Figure 2.21).

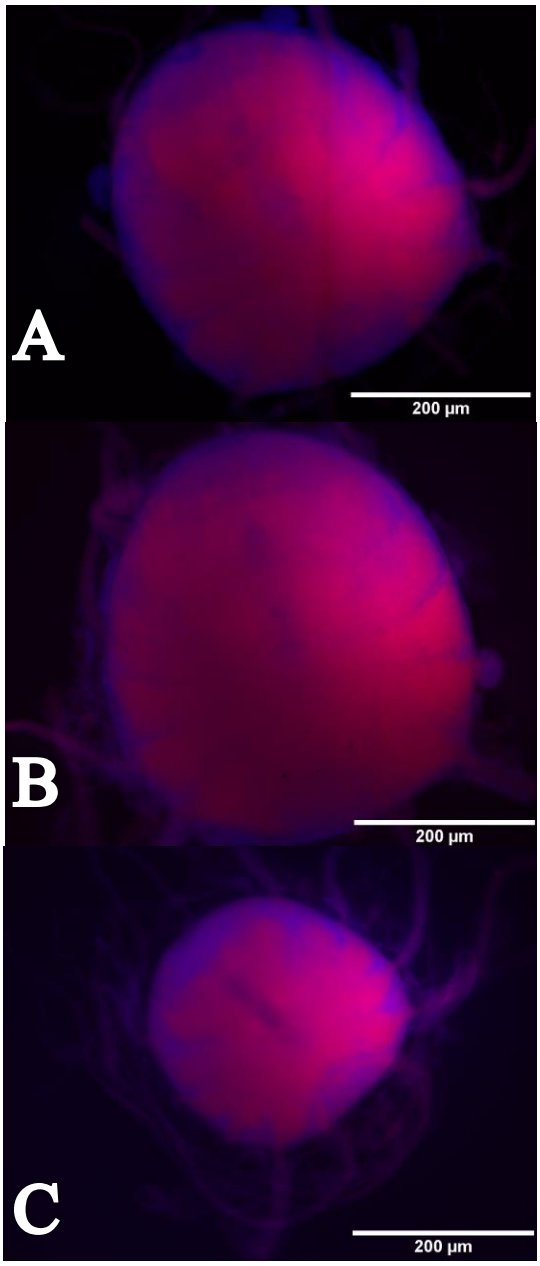

Figure 2.19. FISH using CAPA RNA antisense probe on the female synganglion. Images merged with Cy5 and DAPI filters at 20X magnification. No cell-specific CAPA transcript staining detected for the various concentrations of the RNA probe used: A) 500ng/100µL CAPA RNA probe concentration, B) 700ng/100µL CAPA RNA probe concentration, and C) 1000ng/100µL CAPA RNA probe concentration.

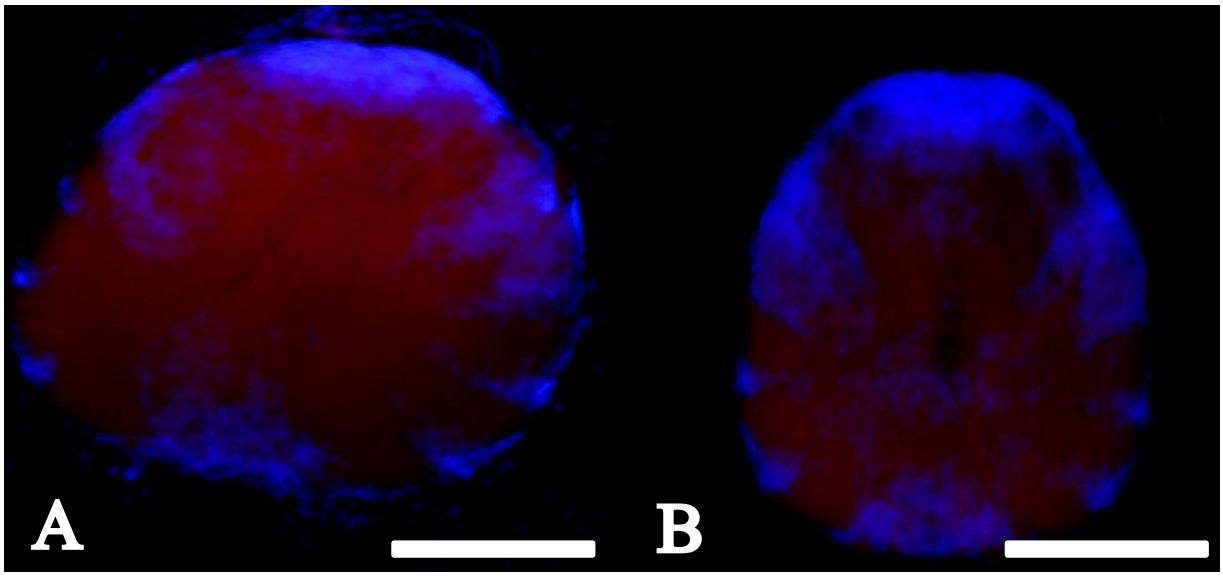

Figure 2.20. FISH using sNPF RNA antisense probe on the female synganglion. Images are merged with Cy5 and DAPI filters at 20X magnification. No cell-specific sNPF transcript staining was observed for the different concentrations of the RNA probe used: a) 400ng sNPF RNA probe concentration, b) 600ng sNPF RNA probe concentration. Scale bar= 200 μm.

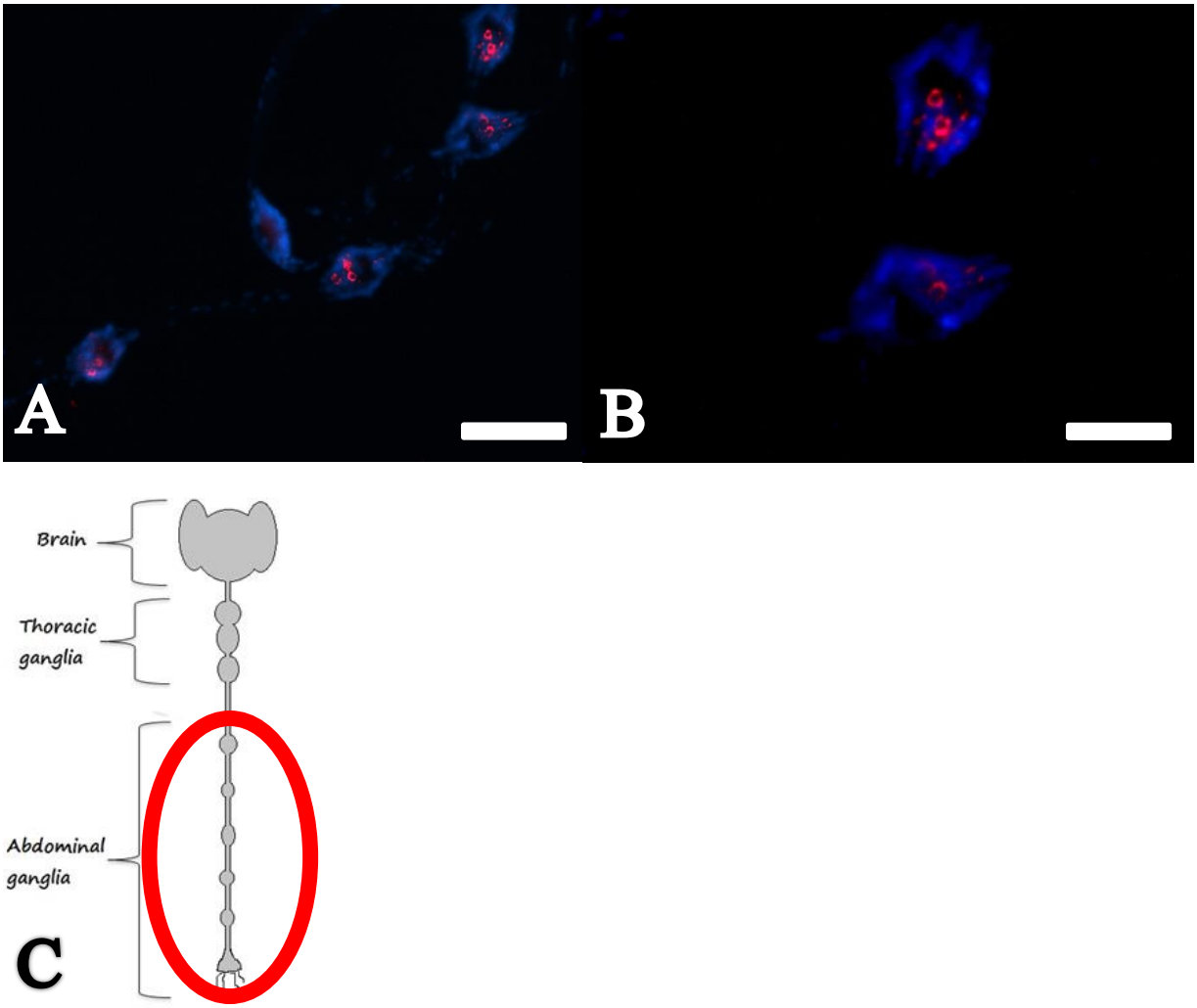

Figure 2.21. CAPA FISH conducted on the abdominal ganglia of adult male *Aedes aegypti*. A) 10X magnification (scale bar= 200μm) and (B) 40X showing each of the abdominal ganglia containing two medially localized cells expressing the CAPA transcript (scale bar= 50 μm) C) Schematic of the CNS of adult *Aedes aegypti*.

RT-PCR

Tissue-specific spatial expression of the CAPA transcript was investigated, revealing the presence of CAPA transcript solely in the synganglion of both male and female *I. scapularis* (Figure 2.22). The band was observed to correspond to approximately 540 bp, matching the predicted size of the CAPA amplicon. To verify the presence and integrity of template cDNA in all tissues samples, a positive control housekeeping gene (RP49) was used.

In order delineate the peptides giving rise to the immunoreactive staining observed in cells outside the nervous system, RT-PCR was also carried out for members of the FaRPs family that were previously detected in the synganglion using peptidomic investigations (Neupert et al., 2009). None of the different primer combinations for sulfakinin revealed its transcript presence in the synganglion of *I. scapularis* female synganglion (Figure 2.23). However, sNPF primer combinations 42F/548R, showed the presence of sNPF transcript in the female synganglion. It was now of interest to determine the presence of sNPF in the other tissues. A band of approximately 507 bp was detected in the midgut, salivary glands, synganglion, reproductive tissues, and hindgut (Figure 2.24).

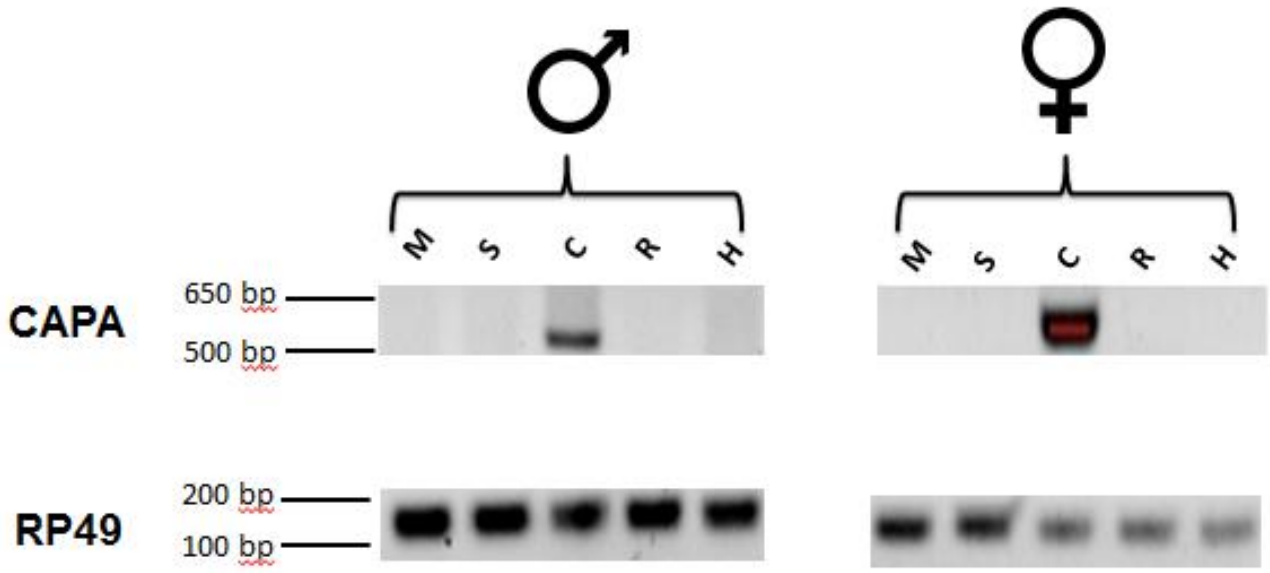

Figure 2.22. Tissue-specific CAPA RT-PCR analysis. RT-PCR of CAPA and RP49 (positive control) in both males and females, showed CAPA transcript to be solely present in the synganglion (C). The approximate size of the band was 540bp. The midgut (M), salivary glands (S), reproductive system (R), and the hindgut (H) did not show any presence of the CAPA transcript.

Figure 2.23. Sulfakinin and sNPF RT-PCR on the female synganglion. Sulfakinin transcript was not detected despite trying different primer combinations (data not shown; predicted size 588 bp). The sNPF transcript was detected with the RT-PCR analysis in the female synganglion, with an approximate size of 507bp.

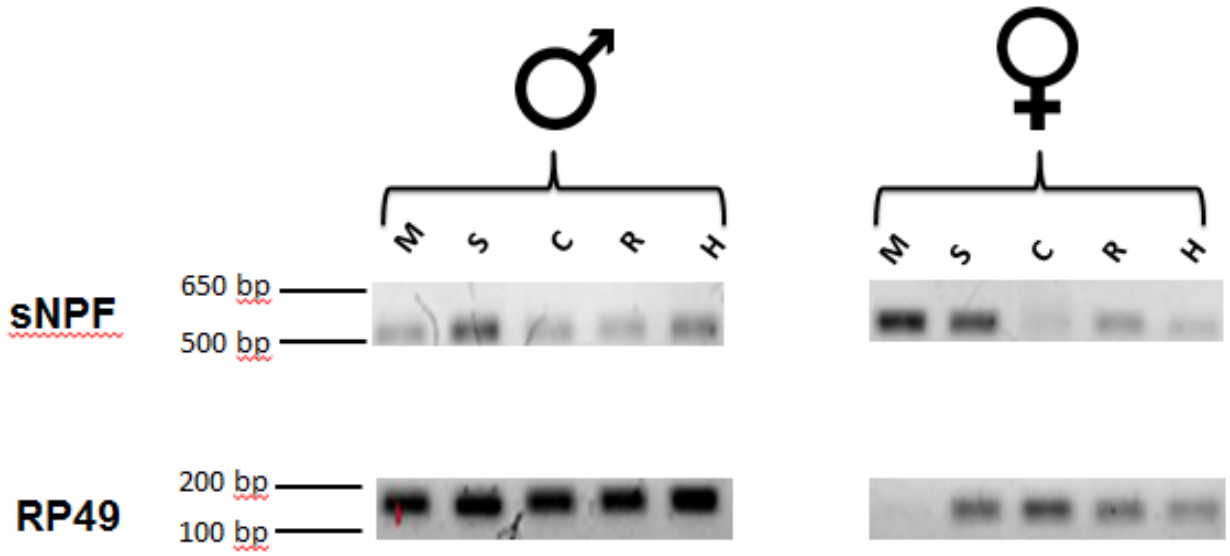

Figure 2.24. Tissue-specific sNPF RT-PCR analysis. Results showed the sNPF transcript was present in all the tissues that were looked at; the midgut (M), salivary glands (S), synganglion (C), reproductive system (R), and the hindgut (H). Once again with an approximate band size of 507 bp.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was conducted in order to quantify the CAPA-like material that was being detected up by whole mount immunohistochemistry. In both the male and female tissues, the reproductive tissues had the most abundant amount of CAPA-like material (0.23 and 0.22 pmol/tick, respectively; Figures 2.25 and 2.26). In the females, the midgut followed with the second highest amount, followed by the synganglion, and hindgut with the lowest (Figure 2.25). In males, the synganglion had the second highest amount of CAPA-like material, and then followed by the midgut, and no CAPA-like material was detected in the hindgut (Figure 2.26). However, this data did not end up being significant between different tissue samples ($P>0.05$; one-way ANOVA). Furthermore, the binding affinity of the anti-RhoprCAPA- α2 peptide to the IxoscCAPA-PVK is approximately 11.2 fold greater than the binding affinity for *Rhopr*-sNPF peptide (Figure 2.27). The binding affinity of the anti-RhoprCAPA- α2 peptide to the IxoscCAPA-PVK was also 9.5 and 10.6 fold greater than the binding affinity for Pyrokinin-1 and Pyrokinin-2 peptide, respectively.

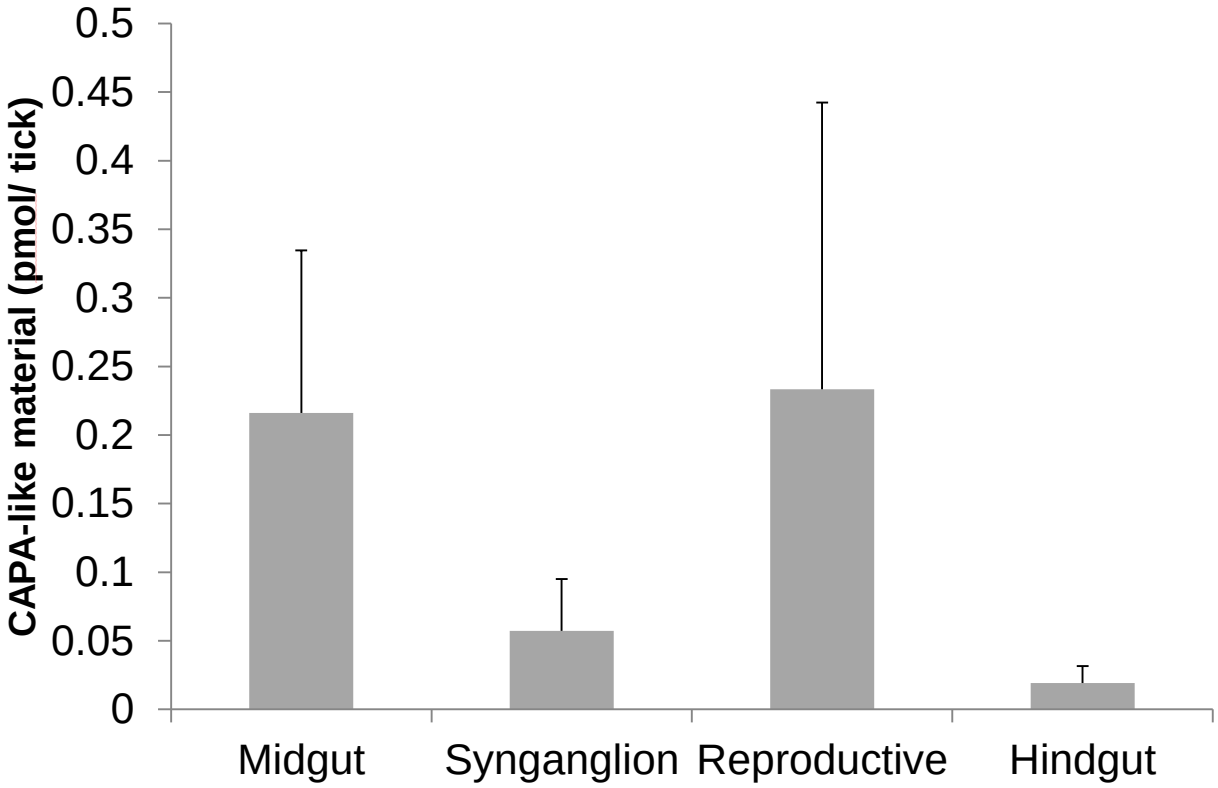

Figure 2.25. Quantification of CAPA-like material in female organs using ELISA. The highest quantity of CAPA-like material was found in the reproductive system with approximately 0.23 pmol/tick, followed by the midgut, synganglion, and minimally in the hindgut. This is an average of 2 biological replicates, with each run consisting of 25 adult ticks ($P>0.05$; one-way ANOVA).

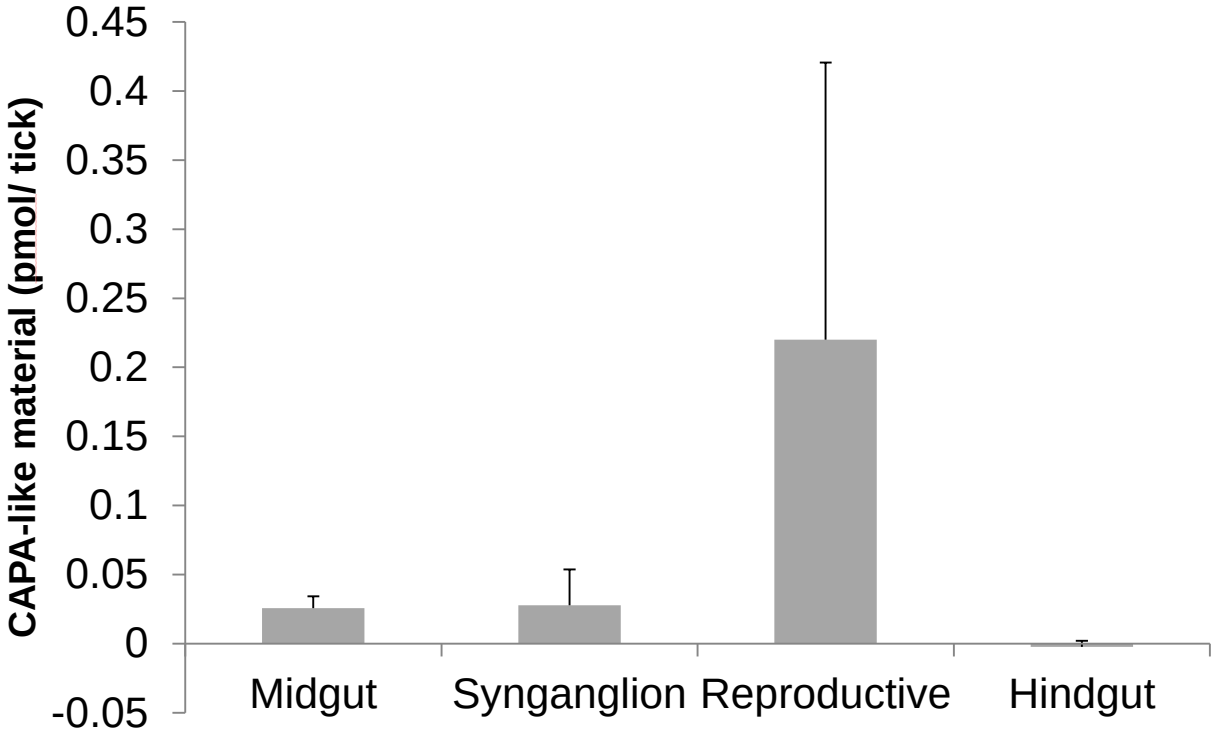

Figure 2.26. Quantification of CAPA-like material in male organs using ELISA. The highest quantity of CAPA-like material was found in the reproductive system with approximately 0.22 pmol/tick, followed by the synganglion, midgut, and no CAPA-like material was detected in the hindgut. This is an average of 2 biological replicates, with each run consisting of 25 adult ticks ($P>0.05$; one-way ANOVA; n=2)

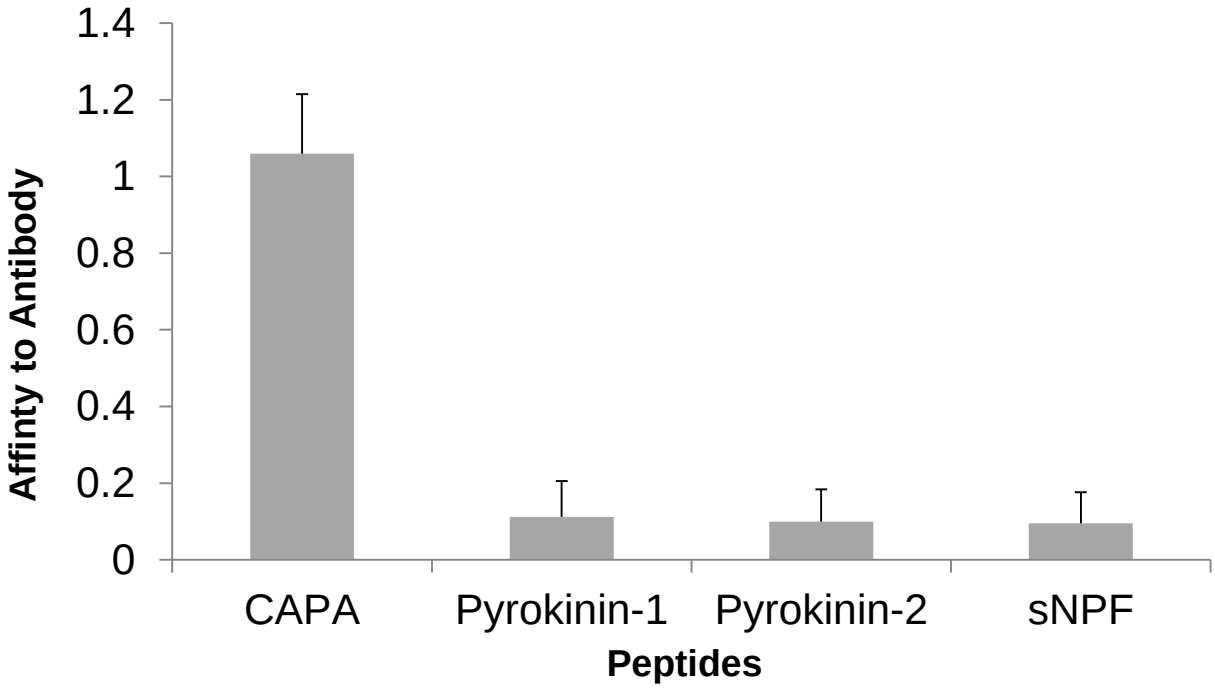

Figure 2.27. *Rhopr*CAPA- α2 affinity to peptides with similar C-terminal structures. The primary antibody had the highest affinity to *Ixosc*CAPA, followed by Pyrokinin-1 and 2, and *Rhopr*sNPF. The primary antibody recognized *Ixosc*CAPA 11.16 fold higher than sNPF.

Hindgut Contraction Assays

In order to determine the regulatory activity of CAPA-PVK in *I. scapularis,* impedance contraction assays were conducted on the various tissues that demonstrated CAPA-like immunoreactivity. Despite testing the various tissues, including the midgut, reproductive, and hindgut, however, only the hindgut for both sexes exhibited noticeably significant effects upon exposure to *Ixosc*CAPA-PVK (data for other tissues not shown). Hindgut tissues in saline undergo spontaneous contractions that occur at a low frequency; however when *Ixosc*CAPA-PVK was applied, there was a dramatic increase in the frequency of these contractions (Figure 2.27). Furthermore, as a positive control, SIFa was applied to the tissues in saline containing the *Ixosc*CAPA-PVK. Despite visually seeing a difference between the contraction frequency of the hindgut when exposed to SIFa in comparison to when it was exposed to IxoscCAPA-PVK, it was not statistically significant.. For both males and females, the treatments with both IxoscCAPA-PVK and SIFa were shown to elicit a significant (repeated measures ANOVA, $p<0.05$) increase in the frequency of hindgut contractions compared to spontaneous contractions in saline alone.

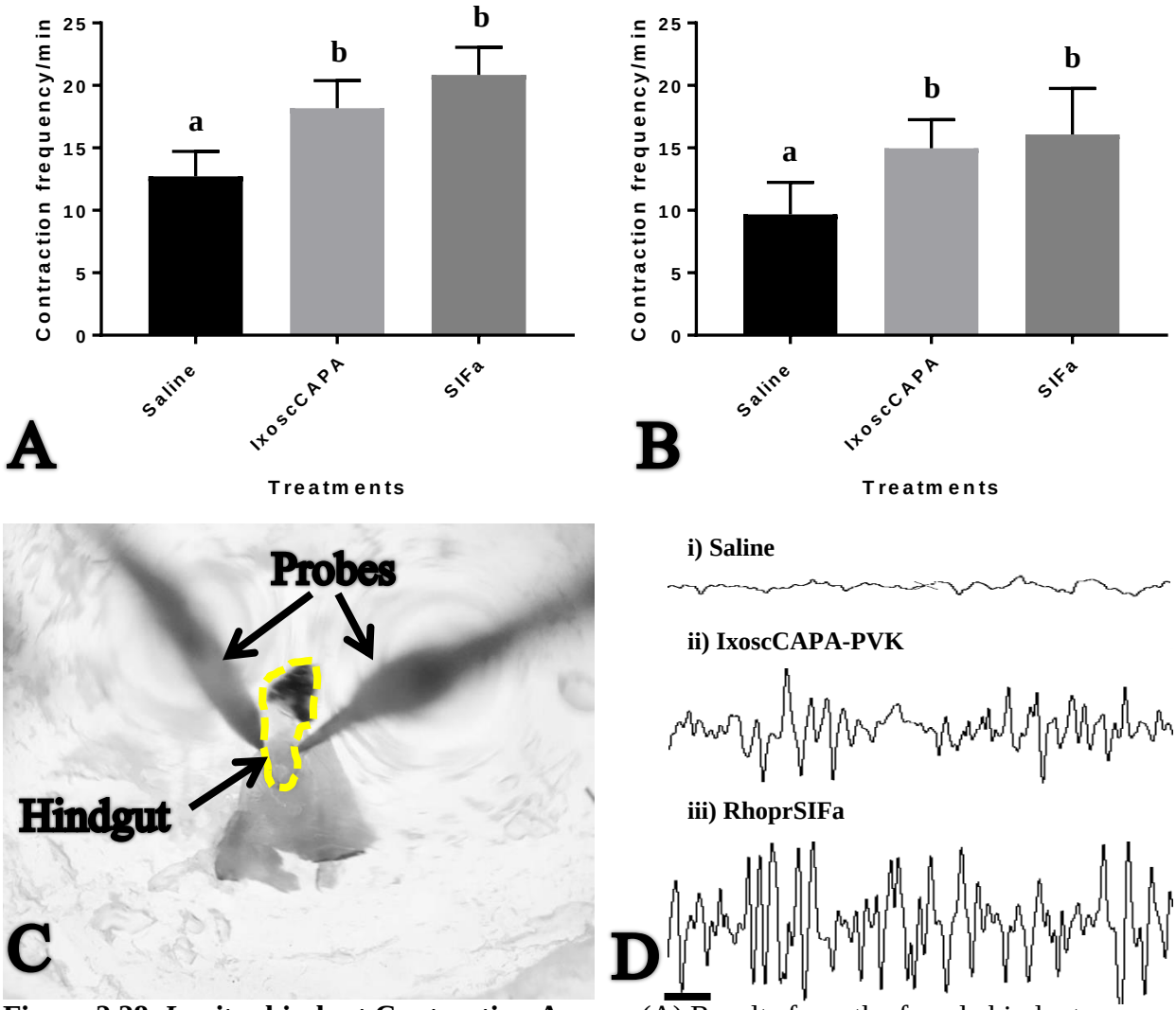

Figure 2.28. *In vitro* hindgut Contraction Assays. (A) Results from the female hindgut contraction assays, showed significant increase in contraction frequencies upon exposing the hindgut to *Ixosc*CAPA-PVK (10^{-6}M), and similar stimulatory activity with *Rhopr*SIFa (10^{-6}M). (B) The contraction assays with the male hindgut, showed similar results. With a significant increase in contractions when *Ixosc*CAPA-PVK (10^{-6}M) was applied, and a more pronounced increase in frequency with the exposure to *Rhopr*SIFa (10^{-6}M) (n=9; $P<0.05$; Repeated-Measure ANOVA). (C) Image demonstrating the experimental setup, with the probes on opposite sides of the hindgut (yellow outline). Mechanical disruptions due to the contraction of the hindgut can be visualized by the readings obtained by LabChart Reader software (D). The top recording is when the hindgut is in saline pre-treatment, followed by exposure to CAPA peptides, and finally at the bottom when the hindgut was exposed to SIFa. Sample recordings from a female hindgut, with each recording spanning 1 minute (scale bar= 10 seconds), showing the general pattern of responses obtained.

Discussion

CAPA (CAPA2b) peptides have been shown to be involved in regulating ionic and osmotic homeostasis in various insects by regulating the activity of MTs. Eliciting diuretic effects in *Drosophila melanogaster*, and anti-diuretic effects in *Rhodnius prolixus*(Davies et al., 1995; Quinlan et al., 1997). Recently, a series of sequencing and peptidomic studies have shown the presence of a CAPA peptide in the synganglion of ticks (Neupert et al., 2005; Christie, 2008; Neupert et al., 2009). Terrestrial arthropods and obligate blood-feeders in particular face substantial environmental and physiological stress, thus it is essential that these organisms employ strict regulatory mechanisms to maintain their hydromineral balance. Furthermore, *I. scapularis* is a well-known vector for the transmission of the Lyme disease- causing agent, *B. burgdorferi*, and with the dramatic increases in Lyme disease cases, it is of importance to further the knowledge pertaining to the physiology of these important human disease vectors (Burgdorfer et al., 1982; Kugeler et al., 2015; Ogden et al., 2009). In this study, it was of interest to characterize the distribution and delineate the role of CAPA peptides in *I. scapularis*, which would provide basic knowledge pertaining to their neuroendocrine system that could be used to develop novel tools to eradicate the transmission of tick-borne diseases. In an attempt to elucidate the function of CAPA peptides in the black-legged tick, immunohistochemistry and FISH was conducted to determine the localization of CAPA at the protein and transcript level, respectively. To support these results, this research also utilized RT-PCR, to confirm the presence or absence of CAPA transcript expression, and also developed an ELISA in order to quantify CAPA-like peptides in different tissues of *I. scapularis*. Lastly, in order to demonstrate a physiological function for CAPA peptides in *I. scapularis*, this study examined the activity of

IxoscCAPA-PVK on various gut and reproductive tissues, which revealed myostimulatory activity on the tick hindgut.

CAPA-like distribution in the synganglion and peripheral tissues

Results obtained using whole mount immunohistochemistry localized CAPA-like material in several neurons in the synganglion, all of which appear to be on the dorsal surface. These include bilaterally paired neurons localized in the anterior region of the protocerebral lobe, as well as three bilateral pairs of immunoreactive cells localized within the posterior protocerebral lobe. Previously, immunohistochemistry conducted on the *Ixodid* tick *Rhipicephalus appendiculatus* had also shown PRV-amide immunoreactivity in this region, but noted them as putative neurosecretory cells (Simo et al., 2009a). The dorsal protocephalic neurosecretory tracts have been shown to innervate the retrocerebral organ complex (ROC), which is found surrounding the oesophagus at its junction with the midgut on the dorsal side of the supraoesophageal region (Obenchain and Oliver, 1975; Sonenshine and Roe, 2014). This complex was believed to have a putative neurohaemal role, homologous to the corpora cardiac/corpora allata complex in insects, but no neuropeptide has been detected in these organs (Simo et al., 2009a; Sonenshine and Roe, 2014). A cluster of 4 cells were localized in the region of the opisthosomal lobe within the synganglion. As a result of minor variationss of their location, it is hard to define if they are solely in the opisthosomal lobe or in closely positioned lobes, such as pedal lobes 3 and 4. It is possible that a single pair is present in both pedal lobes 3 and 4 with an additional two bilateral pairs present within the anterior region of the opithosomal lobe. Additionally, extensive neuropile staining in the synganglion was evident, which is indicative of the CAPA-like material acting centrally in the nervous system as a neurotransmitter

or neuromodulator. Referring to previous studies on *R. appendiculatus* that found PRV-amide immunoreactivity in the synganglion, they noted that some of the immunoreactive cells were in pedal lobes 3 and 4 (Simo et al., 2009a). Furthermore, neurons in the synganglion of *Ixodes ricinus* that express peptides with sequences related to kinins and periviscerokinins were localized previously (Neupert et al., 2005). The *Ixori*-PVK (PALIPFPRV-NH_2) has high homology to the insect CAP2b (pyroQLYAFPRV-$NH_{2)}$ and the recently sequenced CAPA sequence in *Ixodes scapularis*, pQGLIPFPRV-NH_2 (Cheung et al., 1992; Huesmann et al., 1995; Christie, 2008; Neupert et al., 2009). The CAPA-like immunoreactivity obtained in present study reveals a significant overlap in distribution and number of neurons immunoreactive to *Ixori*-PVK and *Ixosc*CAPA-PVK (Neupert et al., 2005).

There was a difference in the abundance of CAPA-like immuoreactive cells obtained between the midgut of males and females, with females having a far greater number of immunoreactive cells than males. Furthermore, immunoreactivity was noted to be more pronounced in the anterior regions of the midgut, and more so in the midgut diverticula as opposed to the midgut stomach. Since the midgut is the site for blood-meal digestion, it could be suggested that the CAPA-like peptide being detected by immunohistochemistry may be involved in regulating digestion (Balashov, 1972; Sonenshine and Roe, 2014). The midgut endocrine cells in larval *D. melanogaster* have been shown to produce authentic short Neuropeptide-F (sNPF) and Neuropeptide-F (NPF) in the midgut endocrine cells in the adults (Veenstra, 2009; Veenstra et al., 2008). These endocrine cells were localized to the anterior and middle midgut, and the first part of the posterior midgut (Veenstra, 2009; Veenstra et al., 2008). Furthermore, the midgut of adult *Aedes aegypti* has two distinct regions; the anterior thoracic region and posterior abdominal region (Brown et al., 1986). The abdominal region terminates at the MTs and holds the entire

blood meal during digestion (Brown et al., 1986). The epithelium of both regions is composed of digestive cells, endocrine cells, and regenerative cells; however, FMRFamides were solely localized to the abdominal midgut region (Brown et al., 1986). In *argasid* ticks, or soft ticks, digestive activity starts from the midgut stomach and eventually moves to the diverticula, where the slow digestion phase initiates (Sonenshine and Roe, 2014). *Argasids* can survive longer periods on a single meal. In *Ixodid* ticks, however, digestion processes are more or less uniform between the midgut stomach and diverticula, and survival is not as prolonged on a single meal once digestion has commenced (Sonenshine and Roe, 2014). This is one of the important differences between the two tick families; *Ixodid* (hard) and *Argasid* (soft) ticks. Moreover, since over 70% of excess water and over 90% of excess sodium ions are excreted by the salivary glands following entry into the haemolymph through the gut epithelium (Tatchell, 1967; Kaufman and Philips, 1973), it could also be suggested that sNPF may have a role in regulating ionic osmotic homeostasis through regulating the permeability of the gut epithelium to the water and sodium ions.

CAPA peptides are known to be involved in regulating ionic and osmotic homeostasis via osmoregulatory organs, which in insects are mainly the MTs and rectum(Beyenbach 2003; Coast, 2007; Beyenbach et al., 2010). As the salivary glands are the main excretory organ in the tick (Tatchell, 1967; Kaufman and Philips, 1973), it was predicted that there would be CAPA-like immunoreactivity localized there. However, there was no CAPA-like immunoreactivity detected in the salivary glands. This is in contrast with previous studies that suggest axonal projections from cells in the postero-medial region of protocerebral lobe that innervate acini types II and III, thus regulating their function (Simo et al., 2009a and 2009b). More specifically,

axonal projections associated with myoinhibitory peptide (MIP; also called allatostatin B) and SIFamide have been shown to innervate acini types II and III (Simo et al., 2009a and 2009b).

The tick heart was also shown to contain CAPA-like immunoreactivity. The heart lies in a sinus formed by the pericardial septum which is an extension of the connective tissue processes of dorso and ventro-lateral suspensory muscles of the heart (Obenchain and Oliver, 1976). Haemolymph flows from the pericardial sinus, through two pairs of ostia, and finally into two segmental cardiac cavities. Radiating muscle bands encase the pulsating region of the heart. Haemolymph reaches peripheral and anterior regions of the tick body via arterial vessels and the perioesophageal sinus, respectively (Obenchain and Oliver, 1976). As a result of the challenges of accurately dissecting the heart due its small size, it was not used for the RT-PCR, ELISA, and contraction assays.

Notably, this study identified CAPA-like immunoreactivity in the reproductive system of both males and females. Specifically, the accessory glands in both sexes and minor areas in the oviduct, common oviduct and vaginal region of the uterus showed immunoreactivity. This suggests that CAPA-like peptide may have a role in regulating reproductive processes in *I. scapularis.* As a result of C-terminal sequence similarity to pyrokinins and FaRPs, the antibody used in this study could be cross reacting with members of these peptide families, including sNPF and sulfakinin (Christie, 2008; Neupert et al., 2009). In support of this finding, RT-PCR analysis confirmed the presence of the sNPF transcript in various peripheral tissues in addition to the synganglion, including for example, the midgut and reproductive tissues. Thus, members of these peptide families, such as pyrokinins and FaRPs (including sNPF) may have roles in regulating reproductive functions, which is in alignment with previous studies where, for example, reproductive tissues of *R. prolixus* were identified to express the pyrokinin-1 receptor

(Paluzzi and O'Donnell, 2012). Similarly, a pyrokinin receptor was recently shown to be expressed in reproductive tissues of the tick, *I. scapularis* (Gondalia et al., 2016), suggesting a potential role in reproductive biology might be conserved in ticks and insects.

Lastly, CAPA-like immunoreactivity was detected at the junction between the posterior region of the rectal sac and the anal canal. Previous studies have shown there to be muscle bundles acting as an anal sphincter or valve at the rectal sac/anal canal junction (Balashov, 1972; Simo and Park, 2014). Furthermore, it has also been shown that the processes that innervate this are, originate from the neurons in the opithosomal lobe (Simo and Park, 2014). Thus, the observed CAPA-like immunoreactive cells in the opithosomal lobe of the synganglion observed in this study may have a role or function in regulating the anal sphincter or other activity on the hindgut in the tick *I. scapularis*.

Localizing CAPA transcript using FISH techniques

In order to determine if the CAPA-like immunoreactivity that was detected in the synganglion was due to CAPA peptide or structurally-related peptides, FISH techniques were employed. Despite using different primer combinations, and various CAPA RNA probe concentrations, there was no successful detection of CAPA transcript in the synganglion from unfed adult females. It was expected to at least be localized in the synganglion, as previous studies had shown CAPA peptide to be present (Christie, 2008; Neupert et al., 2009), which would require the expression of the CAPA transcript.

RT-PCR analysis was conducted for CAPA using various primer combinations in conjunction with RP49 as a positive control, which was optimized as a suitable reference gene in a recent study (Gondalia et al., 2016). The CAPA transcript was detected exclusively in the

synganglion and not in any other peripheral tissues examined. Subsequently, sNPF and sulfakinin (members of the FaRPs) were looked at to determine if they were being detected non-specifically by the antibody used in immunohistochemical analysis. RT-PCR was conducted for the sulfakinin transcript, but despite using different primer combination, it was not detected. However, sNPF was successfully amplified and its tissue-specific localization was determined. Unlike the strict neuronal expression of the CAPA transcript to the synganglion, sNPF transcript was detected in all of the tissues that were examined including the synganglion, midgut, salivary glands, reproductive tissues, and the hindgut. Therefore, this suggests that the CAPA-like immunoreactivity being detected in cells associated with the various peripheral tissues could be sNPF. Furthermore, it is of interest to note that detection of sNPF in the synganglion for the RT-PCR results was relatively low when compared to the transcript of sNPF in the midgut, salivary glands, and reproductive tissues. This may suggest that the staining observed in the synganglion is likely mainly CAPA, and the staining observed in the other non-neuronal tissues is most likely sNPF. The immunoreactive staining observed at the junction between the rectal sac and anal canal could be CAPA since transcripts only reside in the soma of cells and would be absent from axonal projections from neurons, which is represented by the absence of the CAPA transcript in the hindgut and relatively low levels observed for the sNPF in the RT-PCR results.

In order to confirm these results, FISH was attempted once more, but this time with a sNPF RNA probe. Unfortunately, the FISH technique was not successful, despite using various primer combinations for different probe target regions and various probe concentrations. To confirm the FISH protocol was indeed functional, FISH was conducted using *Aedes*CAPA RNA probe on the CNS of adult *Aedes aegypti.* As expected, the FISH protocol worked in detecting the pair of medially-localized cells in each of the abdominal ganglia. Thus, possible explanations as to why

the FISH protocol was not effective in *I. scapularis* may be due to further modifications being necessary so that the protocol is optimized for ticks. Further, life-stage-specificdifferences or physiological changes that occur during a blood meal may result in changes in expression of the CAPA transcript, which could have resulted in the RNA probe not detecting the CAPA transcript. For example, perhaps in the unfed adult tick, expression of these two peptide genes is very low and thus not detectable. Additionally, it is important to note that the studies conducted on the CAP2b receptor in the tick, *Rhipicephalus (Boophilus) microplus*, showed the presence of the receptor in various peripheral tissues, including the salivary glands, but these studies were conducted on partially fed ticks (Yang et al., 2013). This may explain the results in our FISH experiments which did not detect the transcripts in any of the tissues examined, as ticks undergo morphological, behavioural and physiological changes post blood meal (Franta et al., 2010; Sonenshine and Roe., 2014).

Quantification of CAPA-like material

The ELISA was successful in quantifying the CAPA-like material, or sNPF, that was detected by immunohistochemistry. The anti-RhoprCAPA α2 used for both the immunohistochemistry and ELISA herein identified to have a binding affinity of at least 11.2 fold greater for the IxoscCAPA-PVK than to the *Rhopr*-sNPF peptide. Thus, the amount of putative sNPF picked up by ELISA in the various tissues is suggested to be ~11.2 fold higher than that measured. The reproductive system for females and males had the highest amount CAPA-like material, 0.23 and 0.22 pmol/ tick, respectively. In females, the midgut had similar amount of CAPA-like material as the reproductive tissues (0.22 pmol/tick), followed by the synganglion (0.06 pmol/tick), and hindgut (0.02pmol/tick). In males, the tissue with the second

highest abundance of CAPA-like material was the synganglion (0.028 pmol/tick), then the midgut (0.026 pmol/tick), and there was none detected in the hindgut. The amount detected, however, was not significant ($P>0.05$; ANOVA).

CAPA-associated stimulatory contractions on the hindgut

Previous studies had shown that axonal projections from neurons in the opisthosomal lobe move posteriorly and innervate the anal sphincter, regulating its contraction and therefore its osmoregulatory function (Simo and Park, 2014). It was of interest to delineate the significance of CAPA-like immunoreactivity observed in the opisthosomal lobe and the staining observed in the hindgut, particularly localized to the rectal sac-anal canal junction. In order to elucidate the function of CAPA peptides in *I. scapularis*, contraction assays were conducted on the hindgut, which revealed a significant increase in the spontaneous contractions when exposed to the CAPA peptide. Furthermore, SIFa was also applied as a positive control, as it was previously shown to promote spontaneous contractions/motility (Simo and Park, 2014). As expected, SIFa showed an excitatory effect with respect to the contractions, but it is of interest to note that it was a more potent stimulator of contractions than CAPA at the same dose (10^{-6}M). The significant change that the hindgut experienced upon exposure to the two peptides ($P<0.05$; repeated-measure ANOVA) provides substantial evidence that the CAPA-like staining in the synganglion may in fact be CAPA. Additionally, since CAPA had myostimulatory activity on the hindgut and CAPA immunoreactivity in neurons of the opithosomal lobe likely innervate the hindgut junction where further immunoreactive staining was observed, it indicates that this study has discovered a novel regulator of gut physiology in this medically-important tick.

Concluding remarks and future directions

The CAPA transcript was exclusively found in the synganglion. The results obtained herein have shown evidence that the CAPA-like immunoreactive cells in the peripheral tissues may in fact be sNPF, since RT-PCR results indicated that the CAPA transcript is solely present in the synganglion. In contrast, sNPF transcript is found in the nervous system and other tissues examined. Thus this reveales the potential distribution of sNPF in various tissues of the black-legged tick, *I. scapularis.* This supports a potential role sNPF has in the nervous system and midgut of *I. scapularis,* as result of the CAPA-like immunoreactive staining observed and RT-PCR results. The current results also suggest a possible role for sNPF in regulating reproductive functions, as a result of the staining observed in the reproductive accessory glands in both sexes, and due to the detection of sNPF by RT-PCR. Moreover, the presence of the sNPF transcript in the salivary glands, a major excretory organ in ticks, is suggestive of a potential role in ionic and osmotic homeostasis.

It was herein identified that CAPA-expressing neurons in the opisthosomal lobe have axonal projections, similar to that of previous studies on SIFa and MIP (Simo and Park, 2014), which project posteriorly and innervate the anal sphincter. The previously tested SIFa and MIP neuropeptides regulate hindgut contractions/motility and similar activity has been shown in the current investigation for IxoscCAPA-PVK; collectively, these neuropeptides regulate hindgut activity and may hold a role in regulating the reabsorption of water by the hindgut (Kaufman and Phillips 1973; Balashov, 1972; Cambell et al., 2010; Simo and Park, 2014; Sonenshine and Roe, 2014). These novel findings will be useful in permitting further discovery of physiological functions for the CAPA-related peptides in this important human disease vector.

Furthermore, given its central role in hydromineral balance in insects, it would be of interest to determine whether IxoscCAPA-PVK regulates the reabsorption of water in the hindgut, as very little is known of the osmoregulatory mechanism in the MTs and hindgut in ticks. Osmoregulatory organs in other organisms have been suggested as targets for controlling the transmission of various pathogens; for example, the MTs in *A. aegypti* have been shown to be targets for mosquitocides (Piermarini et al., 2017). Thus, it is of great importance to further our knowledge of these excretory organs and their control by neuropeptides, which will be useful in future studies aimed at developing novel acaricides.

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