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Chapter 12

Biology and Molecular Biology of *Ixodes scapularis*

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Abstract
This chapter describes the biology of the tick *Ixodes scapularis* in relation to its role as the vector of the Lyme disease agent, *Borrelia burgdorferi*. Following a review of the internal anatomy of the tick, we review basic molecular processes that contribute to an understanding of the dynamics of the tick's specialized parasitic processes, including attachment behavior, salivation; silencing of host anti-inflammatory responses to enable blood ingestion at the dermal feeding site; hemoglobin digestion and reproduction. The chapter is divided into three parts: 1) systematic and anatomical characteristics of ticks; 2) host finding, attachment, salivary disruption of host defenses, blood feeding and digestion; and 3) molecular regulation of tick bodily functions and reproduction. In the first part, we review the systematics of ticks and the taxonomic position of the vector of Lyme disease, *I. scapularis*, compared to other tick species. Next, we review the general organization of the tick body, including (a) the mouthparts essential for sucking blood, (b) the powerful sucking pharynx, (c) the midgut and its role in blood and hemoglobin digestion, (d) the salivary glands and their complex cellular organization, (e) the synganglion (a fused central nervous system) responsible for controlling all body functions, (f) the reproductive organs, and (g) the tracheal system that facilitates air intake and removal of CO_2. In the second part, we highlight the role of the tick's salivary glands in secreting a remarkably complex array of anti-hemostatic molecules that modulate the bite site in the host skin and how these salivary molecules facilitate the lengthy blood-sucking process. We also describe how ticks capture hemoglobin and internalize it in midgut epithelial cells for intracellular digestion, followed by the sequestration of heme into specialized hemosomes for disposal as hematin. We also will review the neural control of regulation of tick salivary glands, blood uptake, hemoglobin digestion, blood meal concentration, water/salt elimination, vitellogenesis and receptor mediated vitellogenin uptake in the developing oocytes and their oviposition.

Introduction
Ticks represent one of the most successful ectoparasites, and certainly one of the most feared, because of the numerous disease-causing pathogens that they transmit to humans and animals. Ticks transmit a greater variety of pathogens than any other arthropod vector. In addition, they are feared as pests, causing irritation, inflammation and, in some cases, severe toxic reactions, paralysis and even death. The world-wide public health impact due to tick-borne diseases, including the costs of vaccination and treatment for these illnesses, is estimated to be in the billions of dollars. Annual cost estimates for Lyme disease in the U.S. are ~786 million USD per year and tens of millions USD in several European countries (Mac et al., 2019).

Among the factors that explain the success of ticks as parasites and vectors of infectious diseases are their unique feeding style, remarkably long survival periods, high reproductive potential and well-developed innate immune system. In contrast to all other ectoparasitic arthropods that bite quickly and soon flee with small amounts of stolen blood, hard ticks remain attached for long periods and consume large amounts of blood, up to 10- to 100-fold greater than their original body mass. This is especially the case for the hard ticks (family *Ixodidae*) that cement

themselves into the skin of their vertebrate hosts and remain attached for many days, often consuming up 200 – 300 times their pre-feeding body weight (including excess blood meal water that they secrete back into the host) (Sonenshine and Roe, 2014). While feeding, ticks secrete a remarkable cocktail of anti-hemostatic salivary proteins, prostaglandins, and other small molecules that virtually silence the host's awareness of its presence by disabling pain and itch responses, preventing coagulation, increasing blood supply and other actions that disable the host's normal responses to injury (Nuttall, 2019). Nevertheless, silencing host defenses against tick bites does not always succeed, especially after repeated tick attacks on the same hosts (e.g., laboratory mice) or when ticks attempt to feed on non-permissive, e.g., guinea pigs, or other non-native host species.

Over the course of thousands or even millions of years, evolutionary pressures have led to increasing degrees of host specificity among ticks for birds, bats, other small mammals, and so on. *I. scapularis* is an outlier--feeding on diverse vertebrate hosts that include a wide range of reptiles, birds, and mammals. Incidentally, the ways in which tick saliva alters the host skin surrounding the feeding site enhances the survival and invasion of tick-borne pathogens, a form of saliva-assisted pathogen transmission (Bonnet et al., 2018; Šimo et al., 2017). Following successful blood feeding, ticks drop off their hosts and survive in leaf litter or other ground cover in woodland habitats, rotting vegetation and soil in meadows (non-nidicolous species) or in caves or nests of their hosts (nidicolous species) (Gray et al., 2014; Randolph, 2014). Ticks are remarkable in their ability to survive for long periods without fresh nutrients. Some species, especially among the soft ticks (family Argasidae), can survive for many months or even years between blood meals (Sonenshine and Roe, 2014). Finally, ticks hold the record among blood-feeding arthropods for the numbers of eggs produced. An *I. scapularis* adult female typically produces 1,000 – 1500 eggs after engorgement. Other ixodid ticks produce many more (e.g., ~ 5,400 in the case of the American dog tick *Dermacentor variabilis*) (Sonenshine, 1970). Although much smaller egg yields are produced by soft ticks following each blood meal, these species may feed and lay eggs multiple times, rather than all at once, as with ixodid ticks. Finally, the tick's natural immune system, controlled by the Toll and Imd pathways, ensures that most microbes harbored by ticks do not harm their tick hosts, although some tick-borne pathogens, e.g., *Anaplasma* sp., may affect tick fitness, either negatively or positively (Dumler, 2010; Harris et al., 2017). Following thousands and perhaps even millions of years of tick-pathogen association, a balance has evolved such that these pathogens are unlikely to disrupt the tick's physiological and biochemical processes even though these same microbes are pathogenic once transmitted to their reservoir competent vertebrate hosts.

This chapter will review the many unique physical, biological, and genetic attributes of ticks, primarily the black-legged tick, *I. scapularis*, to provide a framework for understanding their role in the transmission of disease-causing microorganisms to humans and animals.

Part I: Systematic and anatomical characteristics of ticks

Types of ticks (systematics)

Ticks are chelicerate arthropods (phylum Chelicerata), which includes animals that have chelicerae, rather than mandibles, as their cutting and biting appendages. Ticks are arachnids (class Arachnida), the same subphylum that includes spiders, scorpions, and other similar taxa, and are grouped with mites in the subclass Acari. They are classified in their own distinctive order, the order Ixodida, within the superorder Parasitiformes (Keirans, 2009). Their evolutionary origin remains controversial, with recent studies based on molecular systematics only partially supporting earlier hypotheses based on morphology. The chelicerates are believed to have diverged from the Pancrustacea, the group that eventually evolved into the insects, crustaceans and myriapods as early as the Cambrian period (Dunlop, 2010). Ticks are now believed to have evolved in a region of the ancient supercontinent Gondwana land (in the area which eventually became Antarctica and Australia) sometime during the Paleozoic era (Beati and Klompen, 2019; Beati et al., 2008; Jeyaprakash and Hoy, 2009), likely during the Carboniferous period (Beati and Klompen, 2019; Mans et al., 2016), although others predict a more recent origin, sometime during the early Cretaceous period (Klompen et al., 2007). The genus *Ixodes* is believed to have evolved early during this period, placing it close to the base of the ixodid phylogenetic tree (Klompen et al., 2000). Hypotheses concerning tick

evolution, including *I. scapularis,* the focus of our book, are important since they may help to explain how *I. scapularis* remains a non-host-specific species, feeding on an exceptionally diverse array of vertebrate hosts including amphibians, reptiles, birds and mammals. They may also contribute to a better appreciation of why *I. scapularis* is so remarkably permissive for diverse microbial symbionts, including prokaryotic and eukaryotic symbionts pathogenic for humans and other animals.

Characteristics of ticks: external body features and similarities with other acarines

Knowledge of the external appearance of the black-legged tick, *I. scapularis,* is important, since only this tick species transmits the microbial pathogens that cause Lyme disease in humans. It is also a vector of human granulocytic anaplasmosis, human babesiosis and several microbes that cause other lesser known maladies. People, including many scientists, often mistake other human biting ticks, e.g., lone star ticks or American dog ticks as vectors of *B. burgdorferi*, leading to unnecessary expenditure of time and resources.

The tick body plan is similar to that found in many other parasitiform acarines. The body is organized into three major regions: the anterior capitulum, (gnathosoma) which bears the chelicerae, palps and hypostome; the idiosoma, or body region, and the walking legs. The idiosoma is divided further into the anterior podosoma, which bears the four pairs of walking legs (three pairs in larvae), and the posterior opisthosoma, which bears the paired respiratory spiracles and anal aperture. Some authors also combine the capitulum and podosoma into the "prosoma" to distinguish this region from the opisthosoma. Like all other acarines, the ancestral segmentation is obscured by the extreme fusion that occurred during tick evolution. As exemplified by *I. scapularis*, , on the ventral side, adult ixodid ticks have a genital pore located between the first and second pairs of legs, crescent-shaped in females, subtriangular in males, but absent in juveniles; the genital pore is open females but covered with a movable plate in males. A large subtriangular plate, the scutum, is located on the dorsal side of the body in females, nymphs and larvae; the scutum covers the entire body in males (and, therefore, limits expansion of the body during blood feeding). In ticks of the genus *Ixodes,* including *I. scapularis*, sclerotized plates cover the ventral part of the body in males but is absent in females. The ventral plates of the male include the large sternal plate, posterior to the genital pore, the lateral epimeral plates and the adanal plates adjacent to the anus. A large spiracular plate, containing the spiracular aperture, occurs on either side of the body just posterior to the coxae of the fourth pair of legs. The anal aperture is located near the posterior end of the opisthosoma. These anatomical features are shown in Figure 1.

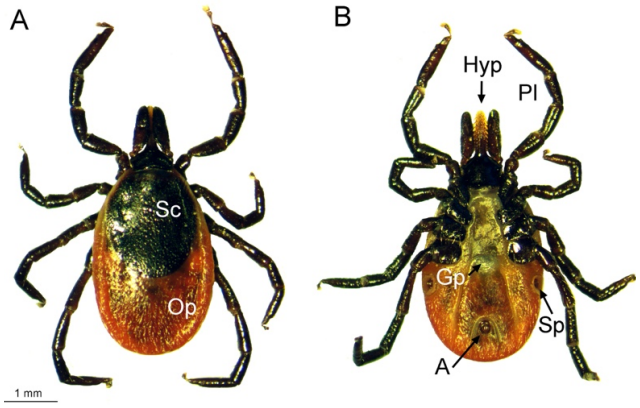

Figure 1. Photomicrographs of an unfed female *Ixodes ricinus* (castor bean ticks), a species closely related to *Ixodes scapularis* and the vector of Lyme disease in Europe. (A) Dorsal view, showing the black-colored scutum (Sc) and the reddish-brown opisthosomal region (Op). (B) Ventral view showing the mouthparts, including the hypostome (Hyp) with its rows of recurved denticles, palps (Pl), female genital pore (Gp) between the hindlegs and the spiracular plates (Sp) on the lateral margins of the body and the anus (A). (Photo authors: D. E. Sonenshine and L. Šimo).

The tick mouthparts (Figure 2), located on the capitulum, comprise the four-segmented palps, chelicerae with sharp cutting teeth, and the elongated, central toothed hypostome which bears the food canal for sucking blood. It is important to recognize that the capitulum is not a "head", since the synganglion, the tick's fused brain and central nerve chord, are located deep inside the body. The capitulum comprises the basis capitulum, the large, flexible structure which contains the pharynx. A pair of ring-like depressions containing numerous tiny pores occurs on the dorsal surface of females. The chelicerae on the dorsal side of the capitulum can be everted or retracted as needed. At their anterior ends, each chelicera has a set of cheliceral digits, bearing sharp spines for cutting into host skin. Instead of cutting like a pair of scissors, the digits move from side to side, ripping and tearing the skin surface. Extending anteriorly from the sides of the basis capituli are the 4-segemented palps which

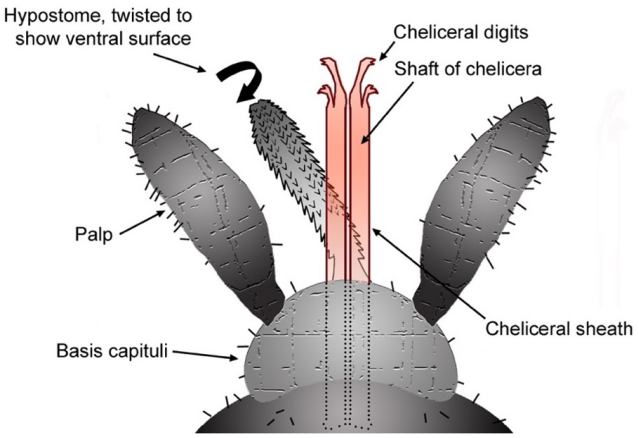

Figure 2. Diagrammatic representation of the mouthparts of an ixodid tick illustrating the locations of the major structures that form the capitulum (gnathosoma). The hypostome is shown teased out of its normal location between palps and the chelicerae. (Graphic authors: D. E. Sonenshine and L. Šimo).

provide gustatory (touch) sensory information that contributes to decisions the tick can make about whether to attach and where.

Finally, the elongated hypostome extending from the center of the basis capitula serves as the anchoring structure, penetrating deep into the host skin. Numerous sharp recurved denticles located all across the ventral side of the hypostome (Figure 2) help hold the tick in place during the initial period of attachment until sufficient cement is secreted (Sonenshine and Anderson, 2014). A deep groove (not shown in the figure) on the dorsal surface of the hypostome serves as both the food channel and the conduit for delivering saliva. During feeding, blood is sucked into this hypostomal food channel by the pumping action of the pharynx, alternating with periods of salivary secretion (3D animations are available in Vancová et al., 2020).

Internal body organization

The internal anatomy of ticks is similar to that of all other parasitiform acarines (Alberti and Coons, 1999) but very different from those of insects and other mandibulate arthropods. In hard ticks, such as *I. scapularis,* it comprises 1) the alimentary canal, beginning with the food canal in the hypostome, pharynx, esophagus, midgut and the hindgut; 2) paired salivary glands; 3) the synganglion and peripheral nerves; 4) heart; 5) Malpighian tubules; and, finally, 6) the male or female reproductive organs. Knowledge of the tick's internal anatomy is essential to understanding how ticks feed, reproduce and survive in their various natural habitats. In this review of the tick's internal anatomy, the major emphasis is directed to the salivary glands and the midgut. Images of the tick's body organs and tissues can be found online by going to the website www.vectorbase.org, selecting tools, controlled vocabulary, and then selecting TADS from the drop down list. This anatomical study is based on *D. variabilis*, but most features are similar to those found in *I. scapularis*.

Pharynx

Ticks are pool feeders. They suck up extravasated blood that oozes from the damaged dermal blood vessels into the tissues around their mouthparts. Since such fluids have low hydrostatic pressure compared to intact blood vessels, imbibing these fluids requires a powerful sucking pharynx. In *ixodid* ticks, the pharynx is an elongated organ located inside the base of the capitulum on its ventral side. The interior of the pharynx is lined with a delicate, thin cuticle. Externally, rings of constrictor muscles are wrapped around the pharynx alternate with dilator muscles that extend from the pharynx to the capitulum wall (Figure 3). Contractions of the dilator muscles pull the pharynx open, allowing blood sucked into the food channel to flow into the pharynx. Contractions of the constrictor muscles, coordinated with relaxation of the dilator muscles (3D animation available in Vancová et al., 2020), constricts the pharynx and forces imbibed blood posteriorly into the esophagus and midgut. Opening and closing of tiny valves at either end of the pharynx synchronizes with the contractions and relaxations of the pharyngeal muscles; the anterior valve closes when the constrictor muscles contract, while the posterior valve opens, insuring that blood flows posteriorly and does not regurgitate (for a more detailed review see (Sonenshine and Anderson, 2014; Vancová et al., 2020).

Esophagus and midgut

Blood imbibed from the host is pumped out of the pharynx into the esophagus, an elongated narrow tube that passes through the synganglion to the midgut. At the esophageal-midgut junction, fluid entry is regulated by the proventricular valve, which allows blood to pass into the midgut (Sonenshine and Anderson, 2014).The midgut is the first body organ in which pathogenic microbes imbibed with the host blood can survive and, in most cases, invade the

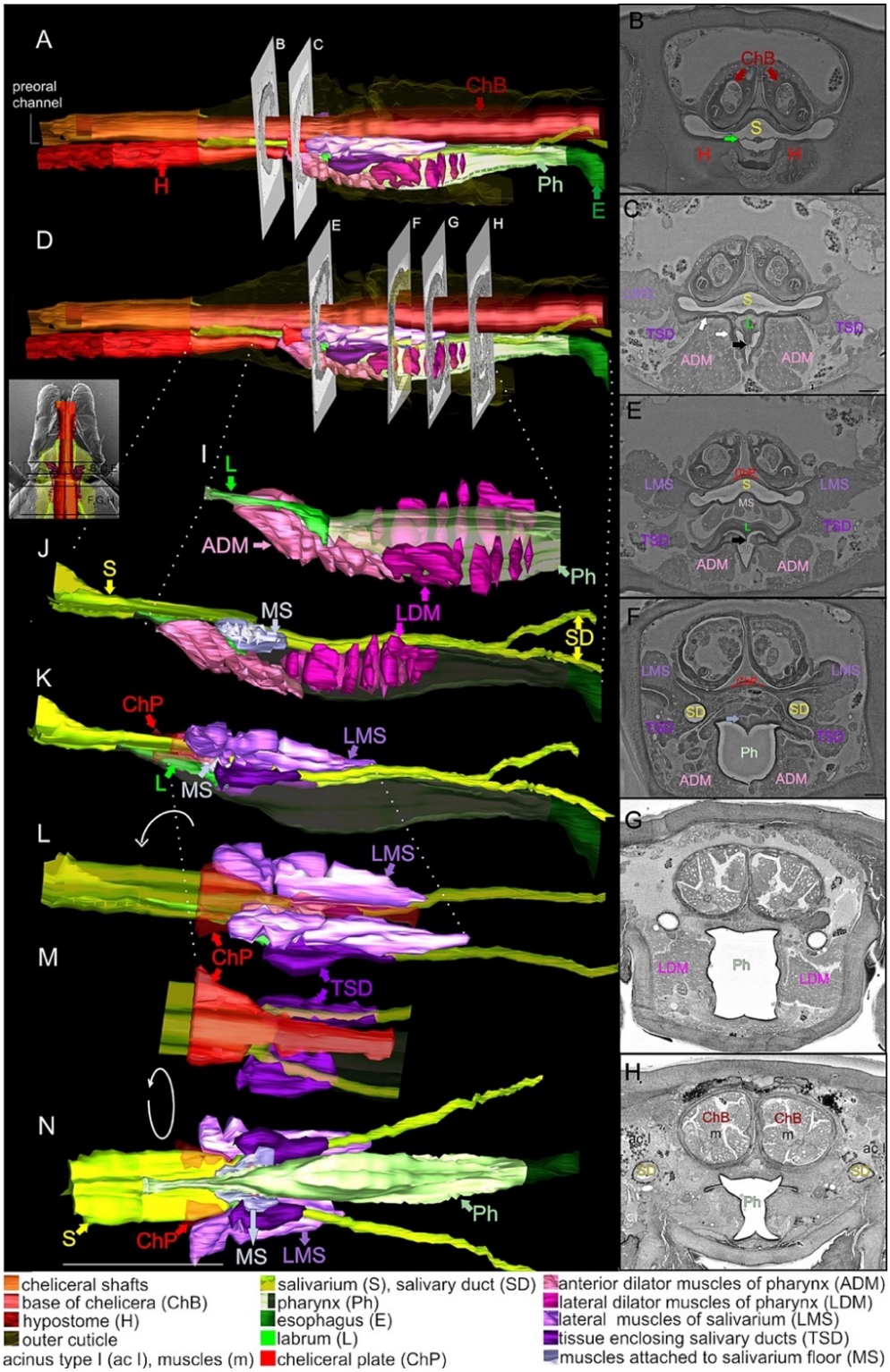

Figure 3. The feeding apparatus of the unfed nymphal stage of the European "castor bean tick", *Ixodes ricinus*. 3D models (A,D,I–N) were reconstructed from serial transverse sections (B,C,E–H) through capitulum part imaged by back-scattered electrons (inset). Bar: 25 µm. The 3D animations are available in original article. From Vancová et al., (2020) Three-dimensional reconstruction of the feeding apparatus of the tick *Ixodes ricinus* (Acari: *Ixodidae*): a new insight into the mechanism of blood-feeding. Sci. Rep. 10:165.

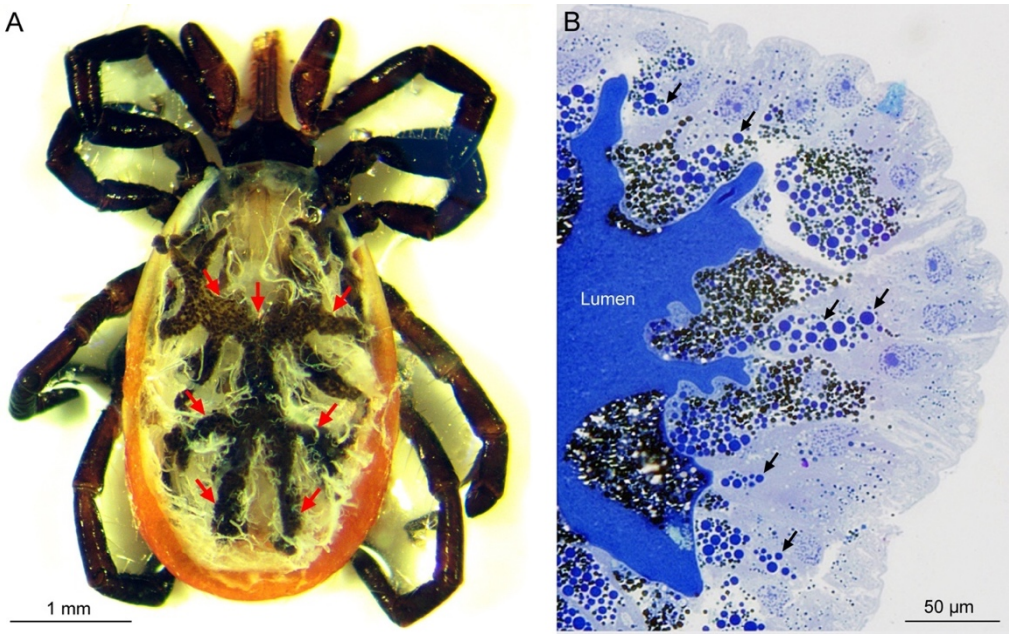

Figure 4. (A) An unfed *Ixodes ricinus* adult female with dorsum removed, highlighting the midgut (red arrows identifying the central ventriculus and lateral diverticulae). (B) Photomicrograph of a histological section of the midgut of a female *Ixodes scapularis* taken on day 5 of blood feeding on its mammalian host (rabbit, *Oryctolagus cuniculus*). Note the masses of hematin-filled vesicles (hemosomes) near the luminal side of many of the midgut cells. The blue-stained vesicles (black arrows in B) are believed to be phagolysosomes containing hemoglobin in various stages of digestion. (Photo authors: D. E. Sonenshine and L. Šimo).

tick's internal tissues. Consequently, knowledge of the morphology and function of the midgut is essential for readers to understand and appreciate its unusual method of intracellular hemoglobin digestion, heme sequestration and detoxification, as well as its role as a long-term food storage organ. These features have profound implications for the survival and development of tick-borne pathogens (Sonenshine and Macaluso, 2017).

The midgut is the largest organ in the tick's body. In *I. scapularis*, it consists of multiple pouch-like diverticula that extend outward from the central portion (stomach) and fill all the available spaces (Figure 4A). During blood feeding by the attached tick, the midgut fills with blood, and the diverticulae enlarge enormously, displacing other internal organs or compressing them (e.g., trachea which must stretch in order to continue to support gaseous exchanges). Digestion of all blood cells takes place in the midgut lumen (Figure 4B). However, except for hemoglobin and serum albumin, which are digested inside the midgut cells (discussed below), no further digestion takes place and serum proteins remain intact and are eventually expelled in the feces (see below) (Lara et al., 2005). Even some hemoglobin, acquired during the larval blood meal, can be found in *I. scapularis* nymphs more than 300 days later (Laskay et al., 2013). These findings show that blood digestion is a gradual process, often requiring weeks or even months, depending on environmental temperatures.

The midgut consists of a simple epithelium surrounded by a single layer of smooth muscle cells. Although previous studies have described different midgut epithelial cell types specialized for ingestion, digestion, secretion and elimination of waste, it is now generally accepted that these specialized cells from a single population of progenitor stem cells stimulated to divide and differentiate by the influx of fresh blood (Starck et al., 2018). The lining epithelial cells vary greatly in size, with some reaching up to 100 µm from the basal lamina to the midgut lumen. A thin semipermeable matrix, the peritrophic matrix, forms adjacent to the midgut cells within the lumen during blood feeding. This membrane protects the delicate epithelial cells from harsh materials (e.g., particulates) and also excludes Lyme disease spirochetes on the luminal side. On their luminal

sides, midgut epithelial cells have numerous microvilli which greatly expand their surface area. Small subcellular pockets form at the cell surfaces, likely sites of hemoglobin uptake where these molecules bind to specific receptors in minute clathrin-coated pits and then are internalized by receptor mediated endocytosis. The pinocytotic vesicles are internalized (endosomes) and fuse with lysosomes to form digestive vesicles. Hemoglobin digestion is intracellular; many cells are filled with digestion vesicles, while other vesicles, termed hemosomes, are filled with hematin resulting from the detoxification of hemoglobin (Gulia-Nuss et al., 2016). Thus, the reader should recognize that one of the unique features of tick blood digestion is that, in contrast to all other blood-feeding arthropods, ticks are mostly hemoglobin feeders, although host serum albumin also is digested by the same or similar multienzyme pathway (Sojka et al., 2016). Also, in contrast to all other blood-feeders, digestion in ticks proceeds slowly, so that the midgut serves as a long-term storage organ, enabling these ectoparasites to survive for many months or even years between blood meals.

During engorgement, the midgut swells enormously. Feeding female ixodid ticks may increase their body size over 100 times or more as the blood meal progresses. During the rapid phase, the cuticle is stretched and thins considerably. However, this phenomenon results in tremendous hydrostatic pressure. One wonders how the internal body organs, especially the air-filled tracheal trunks and tracheae, can avoid displacement and compression. According to Kaufman *et al.* (Kaufman et al., 2016), ticks have evolved a process of pulsatile blood ingestion, “using pulses of high pressure rather than a continuous “squeeze”, to maintain respiratory function. “The pulses of pressure are not highly regular, but average about 50 Hz…”. These authors believe that this is a higher frequency than the “snap back” feature of the cuticle, so that stretch can be achieved by pulses without recovery (contractions) between pulses. These pulses are believed to reflect the periodic contractions and relaxations of the dorso-ventral muscles, since these large and numerous muscles are the ones most likely to produce pressures in the range of 55-60 kPa and frequencies in the range of 30–80 Hz. The frequent pulsations also have the advantage of enabling periodic air flow through the spiracles and tracheal system.

Salivary glands

The salivary glands are the primary organs responsible for transmission of most tick-borne pathogens (Šimo et al., 2017), although exceptions occur in argasid ticks in which pathogens disseminate via excretion of excess body water from their coxal glands (Sonenshine, 2014). Consequently, knowledge of the anatomy and physiology of these organs is essential for readers to understand how they function in support of the tick’s feeding and long-term survival. Tick saliva is undoubtedly one of the most important reasons for their evolutionary success as ectoparasites, enabling them to consume enormous quantities of blood while remaining inconspicuous until they complete their bloodmeals.

In *I. scapularis,* the salivary glands are complex multi-functional organs. They secrete cement (Gulia-Nuss et al., 2016; Kim et al., 2016b; Suppan et al., 2018), essential for attaching the tick to its vertebrate host, as well as a complex assortment of anti-hemostatic proteins, prostaglandins and other agents that enable the tick to suck blood while the host remains unaware of its presence (Nuttall, 2019; Šimo et al., 2017). They also excrete excess water consumed from the blood, estimated to be as high as 70% of the blood meal volume (Bowman et al., 2008; Kaufman and Phillips, 1973; Kaufman and Sauer, 1982), concentrating the blood meal while the tick remains attached, thereby greatly increasing the volume of blood engorged. Exactly how they accomplish these remarkable functions, unique among all blood-feeding arthropods, is the subject of this and later sections (see *Regulation of salivary glands* and *Osmoregulation during the blood meal* in Part III) of this chapter.

The *I. scapularis* salivary gland is a multi-lobed organ (Figure 5), resembling a cluster of grapes, located in the anterior portion of the body (Alarcon-Chaidez, 2014; Bowman and Sauer, 2004; Fawcett et al., 1981). There are three types of alveoli, known as acini, namely, I, II and III (Figures 5 and 6A) in *ixodid* tick female salivary gland (Binnington, 1978).

The agranular type I acini are located at the anterior end of the gland and are situated adjacent to the main salivary duct (Figure 5). The cells of these acini are large with a central vacuole and numerous lamellae along their margins. The central lamellate cell abuts the acinus lumen and is believed to secrete directly into the main salivary duct. Other smaller cell types, such as peripheral lamellate cells, peritubular

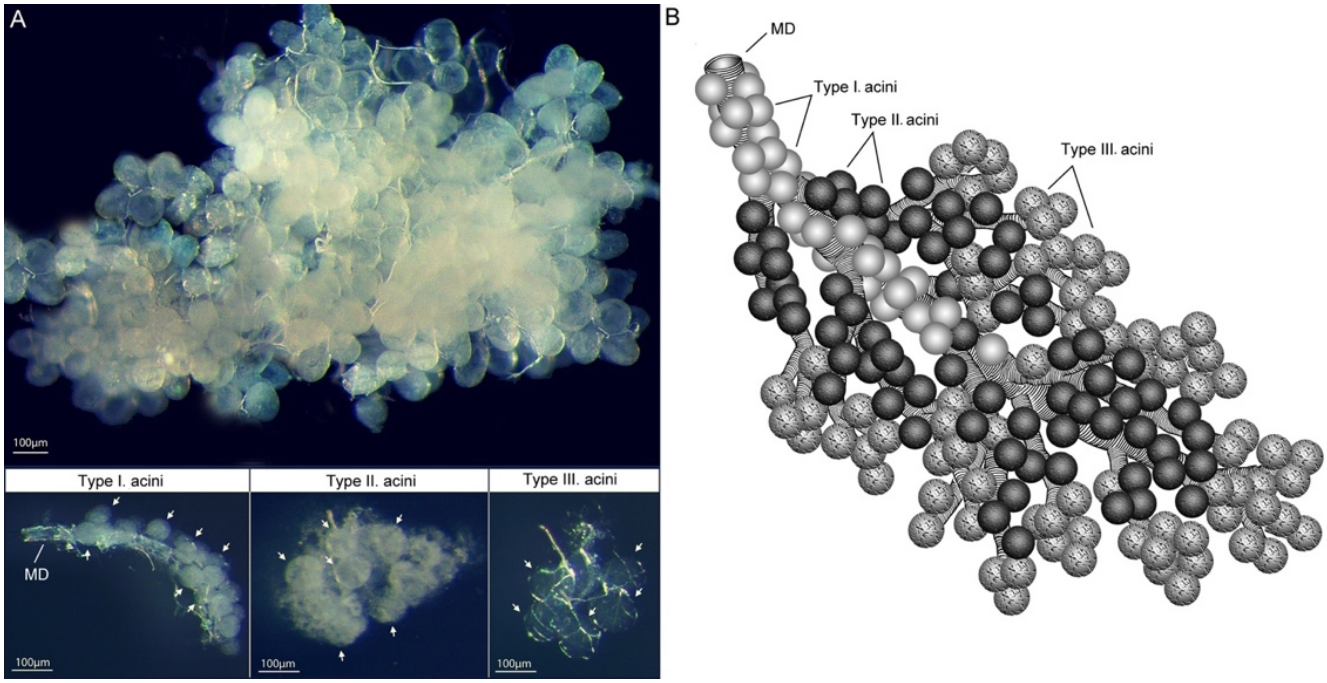

Figure 5. (A) Microphotograph of dissected, intact salivary gland (upper panel) of partially fed *I. scapularis* female, and separated acini types I, II and III (lower panels). (B) Schematic illustration of unfed *Ixodes* female salivary gland highlighting the position of acini types. MD main salivary duct. (Photo and graphic authors: D. E. Sonenshine and L. Šimo).

cells and constrictor cells, are located adjacent to a short lobular duct that directly opens into the main salivary duct. The primary role of the type I acinus is to rehydrate the ticks by uptake of water during periods of fasting (Needham et al., 1990; Rudolph and Knülle, 1974; Rudolph and Knulle, 1978). They accomplish this by absorbing water from hygroscopic saliva secreted by type II and III acini (Figure 7B; Kim et al., 2017, 2019) onto the hypostome, which is subsequently diluted with water from the humid atmosphere of the tick's microenvironment. Typical of cells involved in active transport of water and water regulation, there is a tremendous amount of basal membrane infoldings all along the margin of the acinus (Needham et al., 1990). These salivary acini also appear to be active during tick feeding and in some cases also been implicated in cement secretion (Barker et al., 1984).

The structurally similar type II and III acini comprise the major portions of the salivary glands and lie towards the middle and distal (posterior) regions, respectively (Figure 5). Besides their prominent granular cells (discussed below), types II and III acini contain non-secretory support cells (e.g., basal, adlumenal and ablumenal epithelial (having interstitial projections) and neck cells) (Figures 6B and 12). Contractions of the single adlumenal (= myoepithelial) cell (Figures 6B, C and 12) drives the secretory contents of the acini into the connecting ducts for passage into the main salivary gland ducts (Alarcon-Chaidez, 2014; Vancová et al., 2019). The basal extensions of the myoepithelial cell, along with the apical extension of the basal epithelial cells overlay the acinar valve (Figures 6B, D and 12), serve to control expulsion of the acinar contents to the adjacent duct (Vancová et al., 2019). In the type II and III acini, various granule-rich cells, divided into different cell types within each acinus, namely, types *a-f* (differing in number depending on the tick species) have been recognized (Binnington, 1978). The type II acini have been reported to contain up to six different secretory cell types containing numerous intracellular electron-dense granules (Denardi et al., 2011). Among the most important are 1) the type "*a*" cells, believed to be rich in compounds that contribute to the cement, since they deplete their granules soon after attachment; 2) types "*b*" and "*c*" cells, which contain granules believed to contribute glycoproteins that likely serve in disrupting host

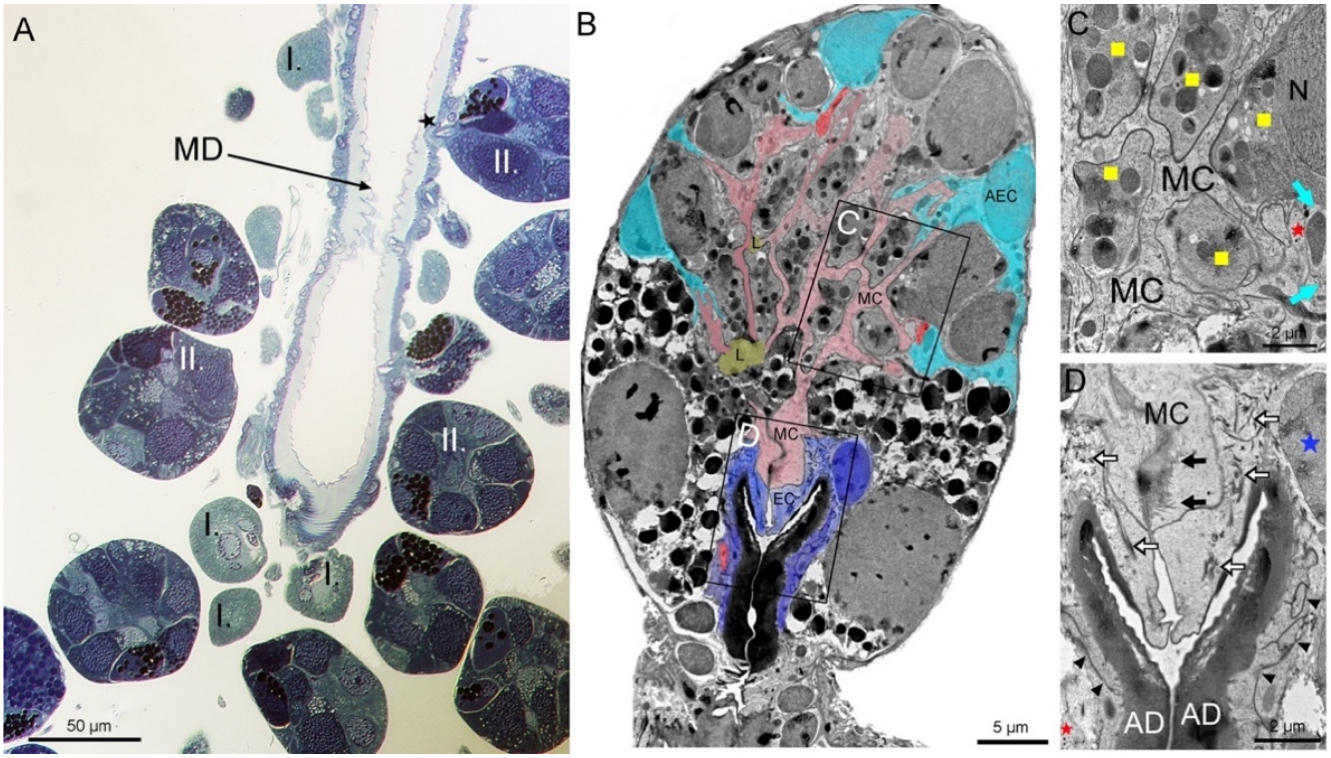

Figure 6. Detailed structure of the salivary gland and the different acinar types. (A) Photomicrographs of histological sections through the type I and type II acini of the salivary gland of a feeding female *Ixodes scapularis*. Image showing the gland acini adjacent to the main salivary duct (MD) in differential interference contrast (Roman number indicate acinus type). Asterisk shows small acinar duct directly attaching an acinus to the main duct. (B) Transmission electron microscopy images of the type III acinus from an unfed *I. ricinus* female salivary gland highlighting its cellular composition and associated axons. Cross-section through the middle of the acinus reveals common axon positions (red), basal epithelial cells (ECs, dark blue), adlumenal myoepithelial cell (MC, pink), ablumenal epithelial cells (AECs, aqua-blue) and lumen (L, yellow). Different granular cells at both basal and apical regions of the acinus are left in black and white. Insets in B are magnified in C and D. (C) The axons (red asterisks) are in direct contact with the MC's finger-like projections which radiate between granular cells (yellow squares) and fine projections of AECs (aqua-blue arrows). (D) The acinar valve region. Apparent microtubules (black arrows) in the MC and (white empty arrows) in ECs are shown. EC labyrinthine junctions (black arrowheads) are closely associated with the acinar duct (AD). The blue asterisk shows the nucleus of the EC. The basal axon in D is indicated by the red asterisk. Panel A photo author: D. E. Sonenshine; panels B – D are from Vancová et al., (2019) Ultrastructural mapping of salivary gland innervation in the tick *Ixodes ricinus*. Sci. Rep. 9:6860.

hemostatic and immune challenges to tick attack. In *Ixodes ricinus*, the secretory granules of the *a*, *b* and *c* cells are glycosylated; most contain mannose, galactose, n-acetyl-D-glucosamine and sialic acid (Vancová et al., 2006). In *D. variabilis* tick, the acinus III-specific "*d*" and "*e*" cells are reported to secrete cement compounds that bind the feeding tick to the host skin (Jaworski et al., 1992). However, it is not known whether the same cells types in *I. scapularis* produce the same compounds. In contrast to the type I acini, these large, complex acini type II and III have their own acinar valves (Figures 6B, D and 12), which open to secondary ducts that collect and transport the protein-rich saliva to the main salivary duct (Binnington, 1978; Vancová et al., 2019). The rich array of salivary proteins secreted by the salivary glands and their roles in tick feeding will be discussed below in *the tick sialome* in Part II of this chapter.

The specific activities of individual acini of tick salivary glands mediate diverse functions that ensure the tick's biological success during both on- and off-host periods (Figure 7)(Bowman et al., 2008; Kim et al., 2019). Studies examining the physiology of tick salivary glands have focused primarily on the on-host tick feeding period, during which the secretory activities of the types I and II acini are most apparent. When feeding, these structures produce hyperosmolar primary saliva that passes via ducts to

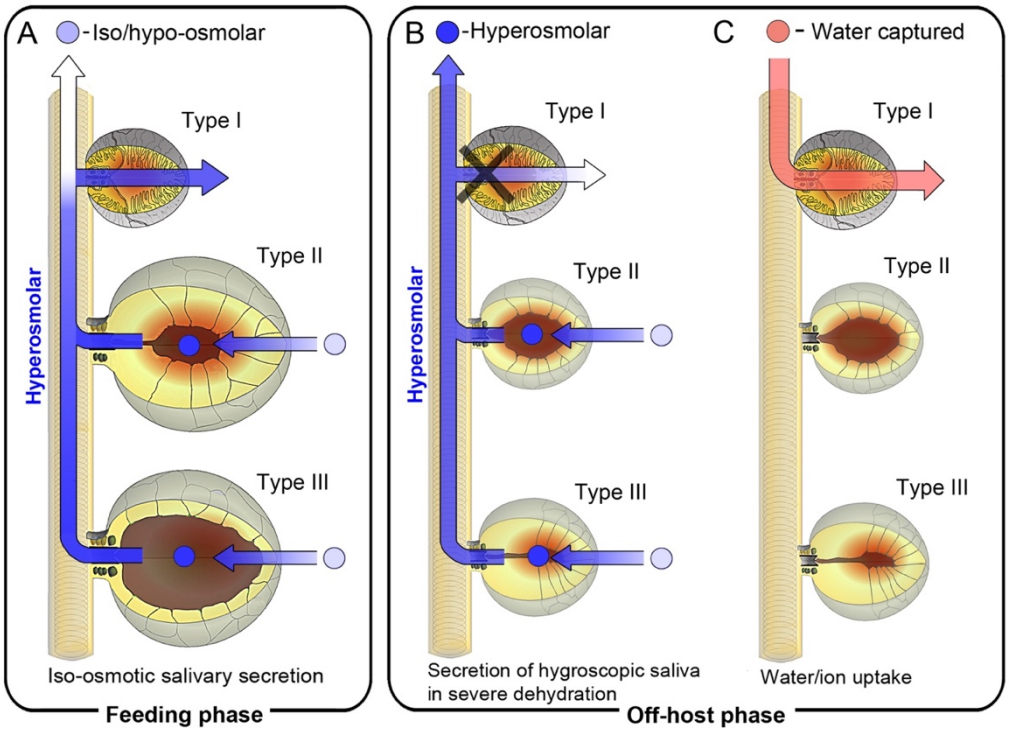

Figure 7. A model explaining roles of specific acini types in *Ixodes scapularis*. (A) The process for iso/hypo osmolar salivary excretion in feeding ticks. (B) Production of hyperosmolar saliva for capturing water molecules. (C) Uptake of water captured in the hygroscopic salivary secretion. From Kim et al., 2018. Neural and endocrine regulation of osmoregulatory organs in tick: Recent discoveries and implications. Gen. Comp. Endocrinol. 278: 42-49, with permission from Elsevier.

the anterior part of the gland where type I acini absorb ions resulting in iso-osmolar saliva (Figure 7A). During the fasting period, when the tick undergoes severe stress due to desiccation, the absorptive function of type I acini is shut down and hygroscopic hyperosmolar saliva is secreted (Figure 7B) onto the hypostome surface to from a crystalized matrix. In a more favorable environment, this deposit is deliquesced by atmospheric water, and the type I acini absorb water from this diluted saliva (Figure 7C), thereby keeping the ticks rehydrated (Kim et al., 2016a, 2017, 2019). The regulation mechanism of tick salivary gland activities seems to be unique among the invertebrate taxa. We discuss its characteristics in the *Regulation of salivary glands* in Part III of this chapter.

The tick bite site has often been viewed as a closed environment with a defined volume. However, as Mans (2019) noted, “the feeding site itself may be dynamic with constant changes in concentration of both host and tick proteins….since ticks alternately salivate and ingest blood meal”. These repetitive salivating and sucking activities, as well as protein dispersal into the general systemic system, make it difficult to estimate the concentrations of the anti-hemostatic molecules active during the feeding process. The bite site is subject to host inflammatory responses, including edema, while salivary secretion of excess blood meal water back into the wound site all contribute to a changing volume. Estimates of the tick bite site (also described, inappropriately, as a feeding pool), range from 10 – 50 µl (Mans, 2019). However, more research is needed on this topic, especially how it differs between different animal hosts.

Reproductive system

Reproduction in females among the hard ticks is of the univoltine type (i.e., mating, insemination, engorgement, and ovary development occur with a single feeding). This results in deposition of thousands of eggs in a single oviposition cycle. The female dies following oviposition. This contrasts with the soft ticks, in which feeding, mating, ovary development and oviposition occur multiple times (multivoltine reproduction). As a result, hard ticks are among the most prolific egg layers of all blood

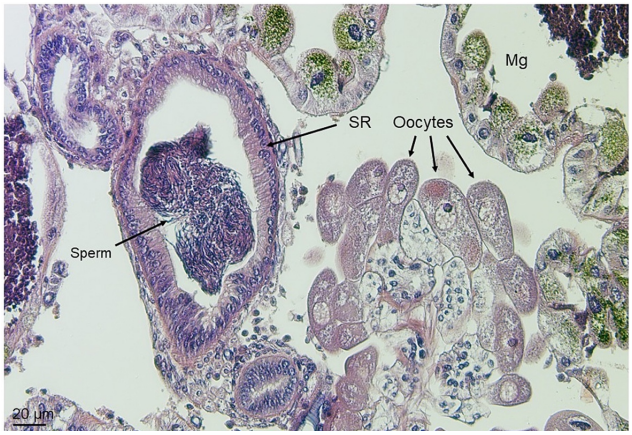

Figure 8. Photomicrograph of the feeding-mated female, *Ixodes scapularis*, fed 5 days, showing a mass of spermatozoa in the seminal receptacle, adjacent to oocytes in the ovary. Mg = midgut; Sperm = spermatozoa; SR = seminal receptacle. (Photo author: D. E. Sonenshine).

feeding arthropods, with some species laying up to 20,000 eggs in a single ovipositing cycle (Arthur, 1962). The biological significance of this phenomenon for ticks and parasites and vectors of infectious diseases will be considered later in the *Reproduction* section in Part III of this chapter.

In *I. scapularis*, as in all other species of the ixodid family, the female reproductive system comprises the U-shaped ovary located across the opisthosomal region of the body; a pair of narrow, coiled oviducts, each extending from either side of the ovary; a single uterus; a muscular vagina, and the cuticle-lined vestibular vagina which opens into the genital pore. As described by Ogihara and Taylor (Ogihara and Taylor, 2014), the ovary is panoistic, producing ova without nutritive cells (i.e., without nurse or follicle cells). In the *I. scapularis* nymphal ovary, the ovary wall consists of a simple epithelium and occasional elongated smooth muscle cells studded with oogonia and interstitial cells. Following the molt from nymph to adult female, many primary oocytes have developed, and ovary maturation progresses, especially after mating and insemination during feeding and engorgement. The oocytes occur on the hemocoel side of the ovary wall, connected to the epithelium by pedicels of funicular cells and bathed by hemolymph as they develop. In partially fed females, the oocytes are supported by funicular cells. One section of the ovary contains a longitudinal groove, replete with undifferentiated oocytes, while the developing oocytes occur in other parts of the ovary, attached to the ovary wall. The luminal side of the ovary is lined with interstitial cells (Ogihara and Taylor, 2014). After mating, the female stores the spermatozoa in the seminal receptacle where they remain available to fertilize the eggs following the transition to vitellogenesis and rapid development of the oocytes for oviposition (Figure 8).

The ecdysteroid hormone (20-hydroxyecdysone) stimulates the production of the yolk precursor; vitellogenin (Vg) is essential for ovarian maturation (the sites and molecular biology of vitellogenesis will be discussed in the *Reproduction* section of Part III). Following mating and the onset of vitellogenesis, waves of oocyte growth occur in preparation for and during oogenesis. The vitellogenic ova (i.e., ova that have incorporated Vg) appear brown in color due to the heme component of the Vg molecule. As they reach maturity, including fertilization and shell formation, the mature eggs are ovulated into the ovary lumen (Ogihara et al., 2019). Except for the ovary and parts of the oviducts, the other components of the reproductive system (i.e., uterus, muscular vagina, and vestibular vagina) are located in the podosomal region of the tick body. In addition, a pair of blind-ending tubules, the tubular accessory glands, extends from the vestibular vagina near its junction with the muscular region of the vagina. During blood-feeding, the cells of the vestibular vagina proliferate externally and form a large gland, the lobular accessory gland. A sac-like structure, the seminal receptacle, which stores incoming spermatozoa, is located above the vagina. Lastly, an unusual feature of ticks is the presence of a separate egg waxing organ, the Gené's organ, which extends its arms out in the space (the camerostome) created by downward flexing of the capitulum, enabling these structures to coat the eggs during oviposition. The reproductive organs are not static structures. Rather, they undergo extensive changes during blood-feeding as the oocytes expand and extend into the surrounding hemocoel (Ogihara and Taylor, 2014).

Synganglion

The nervous system consists of a synganglion and the peripheral nerves. In ticks, as well as in all other acarines, the synganglion is the entire central nervous system, the result of fusion of the ancestral "brain" and the ventral nerve cord (Šimo et al., 2014a). It is located in the ventro-anterior region of the body (Figure 9), immediately anterior to the muscular vagina. The synganglion is surrounded by a delicate sheath, the periganglionic membrane,

enclosing the periganglionic sinus containing filtered hemolymph from the dorsal aorta and heart. There are two major regions, supraesophageal and subesophageal, so-called due to their positions in relation to the esophagus, which extends from the pharynx through this organ to the midgut. The tissue structure of the synganglion is divided into two separate zones, the outer cortical zone with numerous neuronal cell bodies, and the interior neuropile, filled with innumerable neural fibers, axons and dendrites that crisscross the synganglion in all directions. The synganglion contains numerous ganglia, named for the body organs with which they are associated (e.g., protocerebrum, cheliceral ganglia, pedal ganglia, neurosecretory centers, etc.) recognized only by special histochemical staining or electron microscopy (Obenchain and Oliver, 1975; Prullage et al., 1992).

The peripheral nervous system is comprised of the numerous nerves that innervate the legs, palps, chelicerae, and internal organs. Four large pedal trunk nerves extend from the lateral edges of the synganglion to the four walking legs. Several smaller nerves extend from the anterior end of the synganglion to innervate the salivary glands, chelicerae, pharynx, eyes, and other body structures in this region. Other nerves, especially the opisthosomal nerves, extend from the posterior end of the synganglion to innervate the internal organs in the posterior region of the body. Peripheral nerves also innervate several sensory organs, including the chemosensory sensilla (Ferreira et al., 2015; Sonenshine et al., 1984) and infrared-sensing sensilla in the Haller's organ on the leg I tarsi; gustatory sensilla on the terminal segments of the palps; gustatory sensilla on the foreleg tarsi (Phillips and Sonenshine, 1993); the chelicerae and cheliceral digits and the ocelli (eyes) on the dorsal body surface. For sensory perception of their external environment, ticks use a unique organ on their forelegs, the Haller's organ. Located on the foreleg tarsi, this organ comprises a cluster of olfactory sensilla in the anterior pit region of the organ (Carr et al., 2017) and a posterior capsule with pleomorphic sensilla-like elements. Transmembrane receptors associated with the Haller's organ sensilla detect animal odors that facilitate host recognition and attachment for feeding. The role of the posterior capsule is not clearly known, although a recent study in the American dog tick, *D. variabilis* demonstrated it functions as an infrared receptor (Mitchell et al., 2017). Whether it performs a similar function in *I. scapularis* is unknown.

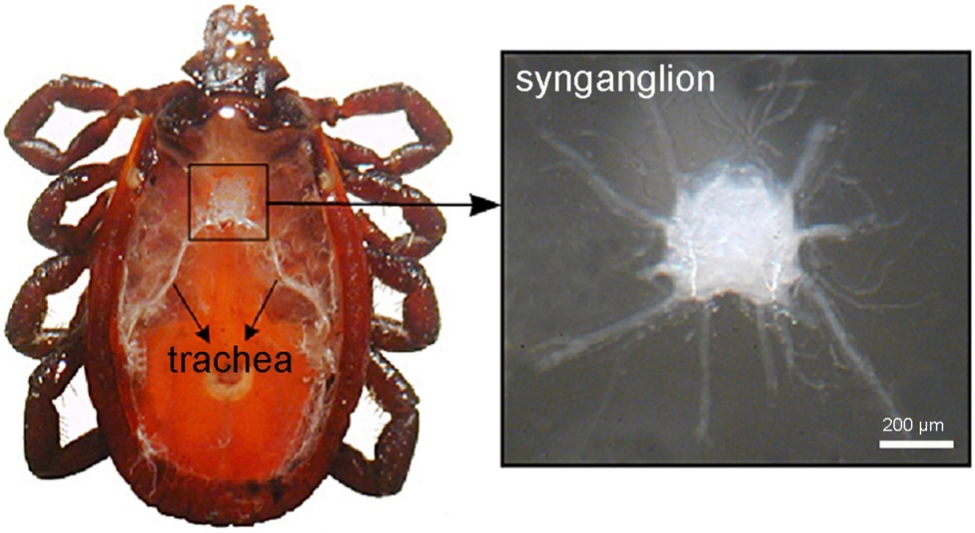

Figure 9. Tick central nervous system, showing the synganglion in the unfed female of the tick *Rhipicephalus appendiculatus*. Left - whole body image, with dorsal cuticle removed, showing the position of the synganglion, and tracheae leading to the spiracles on the lateral margins of the body. Right – enlargement of the ventral body region showing the synganglion and associated nerves. From Šimo et al., (2009) Identification of a complex peptidergic neuroendocrine network in the hard tick, *Rhipicephalus appendiculatus*, Cell Tissue Res. 335(3):639-55 with permission from Springer Nature.

Tracheal system

Respiration in adult and nymphal ticks, including *I. scapularis,* is accomplished via a system of tracheal tubes emanating from a pair of spiracles located on the lateral sides of the opisthosoma just behind the legs. Tracheal tubes are absent in tick larvae, which exchange oxygen and waste gases via the body cuticle. Each spiracle is surrounded by a large, multi-porous spiracular plate containing numerous aeropyles. The ostium of each spiracle abuts a ridge, the macula. Although one would assume that the spiracles can open or close, in ixodid ticks, the macula is immovable and does not seal the ostium. Instead, air exchange occurs via the aeropyles, not the spiracles. A large tracheal atrium is located internally below each spiracular plate and joins several large tracheal trunks. Increases in hemolymph hydrostatic pressure (due to contractions and relaxations of the dorso-ventral body wall muscles) can invert the wall of the atrium, forming a plug that blocks the opening between the ostium and the adjacent tracheal trunks, sealing the space between the ostium and the adjacent tracheal trunks, effectively closing the spiracle. Contraction of muscles attached to the outer surface of the atrium opens the chamber and draws air into the respiratory system. This mechanism acts like a bellows, driving air into the connecting tracheae or expelling waste gases to the exterior (Fielden and Duncan, 2014).

Numerous tracheae extend from the several large tracheal trunks and anastomose throughout the body, reaching into every organ and tissue, eventually differentiating into tiny tracheoles that carry air to individual cells. Each trachea is an elongated tube comprised of a simple epithelium surrounded by a thin layer of folded cuticle, forming the distinctive taenidia, structures that enable one to easily distinguish the tracheae (Fielden and Duncan, 2014).

Part II. Host finding, attachment, salivary disruption of host defenses, blood feeding and digestion.

Host finding behavior

Ticks, as obligate blood feeding ectoparasites, must find vertebrate hosts for their blood meal (hematophagy). Ticks use a unique organ on the tarsal segments of their forelegs, the Haller's organ, to detect common host-derived gases (e.g., NH_3, CO_2) and odors more characteristic of their preferred hosts (Carr et al., 2017). The Haller's organ (posterior capsule) also serves as a heat detector, capable of detecting heat emitting hosts at distances of at least 1 m (Carr and Salgado, 2019). *I. scapularis* is an "ambush" tick that waits in the vegetation for passing hosts, then sensing odors and minor heat emissions to recognize animals onto which it attaches and feeds. Following physical contact with their hosts, ticks also use sensilla on their palps to detect non-volatile compounds on the host skin to assess suitability for attachment and skin penetration (Foelix and Chu-Wang, 1972).

The tick sialome

Mosquitoes, fleas, bugs, and many other blood-sucking insects feed rapidly, often within seconds or at most a few minutes. These biting insects present the familiar "insect bite", and they secrete saliva to facilitate blood flow (Boulanger, 2018; Ribeiro et al., 2010). Ticks, however, have adopted a long-term feeding strategy, attaching to their hosts and imbibing blood over a lengthy time period. Indeed, hard ticks are unique among the vast array of blood-feeding arthropods by their ability to remain attached to host skin and continue blood feeding for many days (Wikel, 2013). Tick saliva is a veritable pharmacopeia of diverse bioactive molecules, mostly proteins and peptides, but also adenosine, prostaglandins, neurotransmitters, endocannabionoids and even microRNAs (Nuttall, 2019; Šimo et al., 2017). More than 500 proteins and peptides have been reported to occur in the salivary glands. The majority of these molecules are secreted proteins and peptides that are released into the saliva (Ribeiro et al., 2006, 2017).

Production of saliva is orchestrated by dopamine, neuropeptides, and their receptors, via both granular type II and III salivary gland acini (for a better understanding of their roles and control see *Salivary glands* and *Regulation of salivary glands* in Parts II and III, respectively) (Kim et al., 2019; Šimo et al., 2009a, 2011, 2014b, 2014a). Saliva at the stage of tick attachment is rich in cement compounds (including *I. scapularis*, although absent in some other species of this genus) that anchor the mouthparts to the host skin. Much has been learned about the composition of tick cement. Ticks begin secreting cement compounds within a few minutes after they attach to the host skin. The first layer, known as the cortex, forms a "cement cone" around the hypostome. Feeding ticks continue to secrete additional cement compounds to form an additional layer, known as the cortical cement layer, that spreads out and further strengthens the cement

cone. In addition to binding the mouthparts to the host's skin, tick cement functions as a sealing agent, filling in any gaps between the epidermal and dermal layers and the inserted hypostome and chelicerae (Suppan et al., 2018). Several authors who have studied the composition of tick cement report glycine-rich proteins (GRPs) as important elements of its structure. In *A. americanum*, the cement is a complex mixture including GRPs, lipocalins, protease inhibitors, detoxification proteins, mucins, storage proteins and others; almost 50% of the saliva consists of proteins of unknown function (Hollmann et al., 2018). Earlier studies by Jaworski et al. (1992) showed evidence of a highly immunogenic 90 kDa protein secreted by specialized cells of the salivary gland, specifically the type d and "*e*" cells found in the type III acini in *D. variabilis* as well as the "*a*" cells of the type II acini in several other species (Coons and Alberti, 1999). Other workers (Antunes et al., 2018) reported a GRP as a major component of the cement composition and a candidate for a vaccine. A large (> 1700 amino acids) GRP also was found in the *I. scapularis* genome (GenBank: EEC15669) and is likely one of the major components of the salivary gland cement mixture. Another important component, 64TRP, was found to be highly immunogenic and provided cross-protection among diverse ixodid tick species (Trimnell et al., 2005). However, we could not find any evidence of this protein in *I. scapularis,* although it is present in the closely related European tick *I. ricinus* (Trimnel et al 2005).

Once the host's awareness of the biting tick has been compromised, the attached tick, soon to be cemented in the skin, can begin its blood meal. Hematophagy is a remarkably well-orchestrated process, controlled by downregulation of genes coding for molecules no longer needed and upregulation of new genes coding for hemolysis of incoming blood cells, the cascade of hemoglobin digesting enzymes, new cuticle growth to help maximize engorgement and water elimination to help concentrate the blood meal. Beginning with the ground-breaking discoveries by Ribeiro and colleagues (Ribeiro et al., 1985, 2006), investigators have identified approximately 500 peptides and proteins in the tick's salivary gland repertoire, most of which are secreted to facilitate feeding (Nuttall, 2019; Šimo et al., 2017). Among these are molecules that silence the host's itch and pain receptors, prevent blood coagulation, dilate blood vessels, disrupt immune rejection, and prevent tissue repair. A brief synopsis of these salivary molecules is described below.

Insertion of the tick's hypostome and chelicerae damages cells in both the epidermis and dermis. Damaged skin cells release adenosine diphosphate (ADP), activate the factor XII coagulation pathway, and elicit kallikreins and other inflammatory mediators causing release of bradykinin. Bradykinin, in turn, binds to nociceptor neuron that can respond within seconds or minutes to initiate the pain response and alert the host to respond (Pinho-Ribeiro et al., 2017). Other expected responses to the initial tick bite injury include itching, vasoconstriction, blood coagulation and inflammation (Šimo et al., 2017), all of which would alert the host to the presence of a foreign invader. However, tick saliva contains a variety of anti-clotting, anti-platelet, vasodilatory, anti-inflammatory and immuno-modulatory proteins that effectively disarm the host defense, at least in tick-naïve animals, allowing *I. scapularis* and other hard ticks to feed before an effective immune defense can be mounted to reject them.

To prevent pain, ticks secrete a salivary metalloprotease, specifically, an angiotensin converting enzyme (ACE) to block the pain-inducing hormone, bradykinin (Francischetti et al., 2009; Jelinski, 2016; Nuttall, 2019). To silence itching and irritation due to histamine released from basophils and mast cells, tick saliva also contains a histamine-binding protein (lipocalin) secreted soon after the initial attachment phase, which blocks host histamine at the wound site (Dai et al., 2010). Lipocalins have been reported to be among the most abundant salivary proteins, in some cases (e.g., *Ornithodoros* sp.) comprising as much as 20% of the total soluble salivary protein. However, the estimate for hard ticks, including *I. scapularis,* is much less, <1% of the total soluble protein mass. Other possible analgesics secreted in the saliva of some tick species include endocannabinoids and even adenosine (Nuttall, 2019). Collectively, these salivary agents effectively anesthetize the wound site, enabling the tick to continue feeding without alerting its host.

Tick bites damage blood vessels, triggering vasoconstriction and blood coagulation. To continue feeding, the tick's saliva contains compounds that counteract the host response to such injury (i.e., formation of a platelet plug, blood coagulation and vasoconstriction of vessels surrounding the bite site).

Anticoagulants in saliva (e.g., ixolaris and penthalaris) block clotting factor VIIa, disrupting clotting by the extrinsic pathway, while basic tail peptides inhibit clotting factor FXa of the intrinsic pathway. These same salivary proteins also activate plasminogen, a protein acquired from the host (Francischetti et al., 2009). Metalloproteases in saliva breakdown fibrinogen and fibrin, inhibiting clot formation by preventing platelet aggregation (Francischetti et al., 2005). Apyrases are enzymes that hydrolyze ATP, liberating ADP and AMP, which inhibit platelet aggregation and subsequent blood coagulation (I.e., acting as anti-coagulants) (Ribeiro et al., 1985). Serpins (serine protease inhibitors) found in the saliva of *Ixodes ricinus* (Chmelař et al., 2017; Šimo et al., 2017), as well as *I. scapularis*, also are important in inhibiting thrombin (i.e., preventing coagulation). They also are reported to function as vasodilators. Diverse serpins, differentiated by their Kunitz domains, occur in the different tick species and target different components of the coagulation cascade (Blisnick et al., 2017). In *I. scapularis* saliva, the basic tail protein Salp 14 is a specific thrombin inhibitor (Narasimhan et al., 2002), believed to work in conjunction with a different Kunitz domain protein, ixolaris, to prevent blood coagulation. In summary, since blood clotting is a complex process, beginning with release of thrombin from blood platelets, it is not surprising that *I. scapularis* ticks target several different elements of the clotting cascade to ensure continued blood flow.

Ensuring continued blood flow during tick feeding is dependent upon open (i.e., dilated) blood vessels. Prominent among the salivary compounds that function as vasodilators are the lipids prostacyclin and prostaglandin (PGE2) (Ribeiro et al., 1992). Another compound identified for this purpose is the apyrase, ATP-dihydrophosphohydrolase, which blocks vasoconstriction by blocking the agonist ADP (Nuttall, 2019). Other participants in this process are the cystatins, an important class of salivary proteins. Cystatins were shown to be secreted early in the attachment process. They are likely to function as among the earliest anti-hemostatic mediators (Ibelli et al., 2013). Cystatins also are known to affect cytokine production, especially IL-1β, a co-stimulator of the cytokine IL-9 (Nuttall, 2019), suggesting a role in blocking cytokine signaling that leads to early influx of T-lymphocytes into the bite site. Other unknown vasodilators present in tick saliva may be needed in the later phases of the feeding process. Blocking host complement is another important function of tick saliva. *I. scapularis* ticks secrete an anti-complement protein, Isac (*I. scapularis* anti-complement) (Valenzuela et al., 2000) as well as a suite of similar anti-complement proteins (Nuttall, 2019).

Tick salivary secretion is a dynamic process; the composition of salvia changes during the different phases of feeding from attachment to repletion (Nuttall, 2019)(Perner et al., 2018). During the first 24 hours, a large number of proteins are secreted into the host skin, including many of the same proteins described above, but also including a putative Kunitz BPTI protein, ferritin, a hemelipoglycoprotein, a glutathione-S-transferase, heat shock proteins, antimicrobial peptides (including defensin) and a variety of ribosomal proteins. Collectively, they contribute to preparing the feeding site, preventing pain and itch responses, enhancing blood flow, preventing blood coagulation, minimizing oxidative stress and combating reactive oxygen species (ROS) and also preventing bacterial contamination (Lewis et al., 2015). Later in the feeding process, numerous genes upregulated during the early stages (e.g., genes coding for lipocalins, cement compounds, etc.) are downregulated while others (e.g., the gene coding for ticks secrete histamine release factor (tHRF), Isac and others) are upregulated (Nuttall, 2019; Šimo et al., 2017).

When ready to begin the final rapid engorgement phase of blood-feeding, *I. scapularis* tHRF binds to basophils, inducing them to release histamine and induce vasodilation (Dai et al., 2010). This is the opposite effect of the early feeding process when blockade of histamine by lipocalins and other secreted proteins minimizes itching and/or pain that would have alerted the host to the tick's presence.

In addition to the complex array of secreted salivary proteins, tick salivary glands also secrete prostaglandins (PG), as noted previously, including PGE_2, $PGF_{2\alpha}$, and PGD_2. Tick salivary PGs have been reported to inhibit the cytokines interleukin-1 and tumor necrosis factor α (TNFα) by macrophages, interleukin-2 and interferon-γ. In addition, PGs, especially $PGF_{2\alpha}$ were reported to prevent platelet aggregation, increase vasodilation and also inhibit mast cell degranulation and release of mediators of inflammation (Sauer et al., 1995).

Despite the tick's well-defined armamentarium for countering host defenses, some reservoir species e.g., the white-footed mouse *Peromyscus leucopus*,

are well equipped to respond to the tick's parasitic attack. When *I. scapularis* nymphs feed on white-footed mice, a permissive host, they provoke a strong inflammatory response. The skin of the bite site becomes replete with T-cells, macrophages, and neutrophils (but few if any eosinophils and basophils). This response is weakest in tick-naïve animals, typically newborns that have just begun foraging, but increases in severity in mice subjected to frequent tick attacks. Nevertheless, the ticks could continue feeding and engorge fully despite the inflammation, largely because the dermal architecture of the feeding lesion remains intact, appearing only as a broad, flattened (sessile) lesion. This phenomenon allows the ticks to remain anchored while they complete their blood meal. Other, unknown factors may also enable tick feeding despite the host's immune response. In contrast, when guinea pigs (*Cavia porcellus*), a non-permissive host native to South America, are subjected to repeated tick infestations, the inflammation is similar in severity to that seen in *P. leucopus,* although the immune cell population is especially rich in histamine-secreting basophils (Karasuyama et al., 2018). However, the dermis at the bite site degenerates as a large but narrow cavitary lesion mostly filled with leucocytes instead of erythrocytes, compromising the quality of the tick's blood meal. Observations of tick's attempts to blood feed from this unstable, cavity centered lesion and the associated hyperkeratotic epidermis showed that it was more difficult for the ticks to remain attached. Ticks on those animals fed poorly and were rejected before they could engorge (Anderson et al., 2017).

Digestion of the blood meal

Ticks are obligate blood feeding parasites, and hemoglobin is their primary nutritive food source. Ticks are unique among all other blood feeding arthropods in their sequestration and absorption of hemoglobin. When blood enters the midgut, midgut cells begin to proliferate, some differentiating into secretory cells, others into digestive cells (Coons et al., 1886). Subsequently, digestion of the blood meal begins with the lysis of blood cells, especially erythrocytes (hemolysis) by hemolysins (Ribeiro, 1988). In *Haemaphysalis longicornis*, trypsin-like serine proteases secreted by the midgut disrupts the membranes of ingested erythrocytes (Miyoshi et al., 2007), leading to lysis and liberating hemoglobin. Transcripts for serine peptidases and cysteine peptidases also were found in the midgut transcriptomes of *I. scapularis and D. variabilis*, implicating these enzymes in hemolysis of red cells. Instead of a single peptidase, multiple families of peptidases have been found in the midgut transcriptomes of these ticks, including serine peptidases, cysteine peptidases, aspartyl peptidases, and metallopeptidases, although it is likely that not all are involved in blood cell hemolysis (Anderson et al., 2008; Sonenshine and Anderson, 2014). Varying amounts of hemoglobin liberated from the hemolyzed erythrocytes bind to specific receptors in clathrin-coated pits on the luminal side of the midgut digestive cells. Subsequently, hemoglobin is internalized by receptor mediated endocytosis. The resulting endosomes fuse with lysosomes to form heterolysosomes (also known as "phagolysosomes") containing abundant hemoglobinases (i.e., enzymes that digest hemoglobin). Albumin also is absorbed, presumably by other, uncoated membrane pits, but almost all other proteins and potential nutrients in blood are excluded, remaining within the midgut lumen and eventually excreted (Sonenshine and Anderson, 2014).

Over the course of the next several hours, the globin moieties are digested while the highly toxic heme, also liberated during digestion of hemoglobin, is detoxified by heme-binding proteins to hematin-like aggregates, similar to hemozoin of *Plasmodium* sp. (Graça-Souza et al., 2006) or the hematin-like waste products in the blood flukes Schistosoma sp. (Brindley et al., 1997; Koehler et al., 2007), and shuttled to hemosomes that accumulate in the luminal side of the hypertrophied digestive cell for later disposal; this process is known as heterophagy. Small amounts of free heme bind to ferritin and are transcytosed into the hemolymph for eventual incorporation into storage proteins or vitellogenin (this is the reason why tick hemolymph and eggs are brown). Hypertrophied midgut epithelial cells containing hemosomes eventually rupture and dislodge their contents (exocytosis) into the midgut lumen along with other excretory wastes. This intracellular mechanism for detoxification of heme is believed to be unique among blood-feeding parasites (Lara et al., 2005). Hemoglobin digestion is a continuous process that progresses slowly over many days or even weeks, depending upon the life stage. Excess blood meal water accumulated during feeding (note that the tick's osmotic pressure is ~ 1.3 to 1.4 times higher than in typical vertebrate hosts) is collected by specialized cells in the salivary glands and secreted back into the host. The blood meal is digested slowly, often after many months (except in

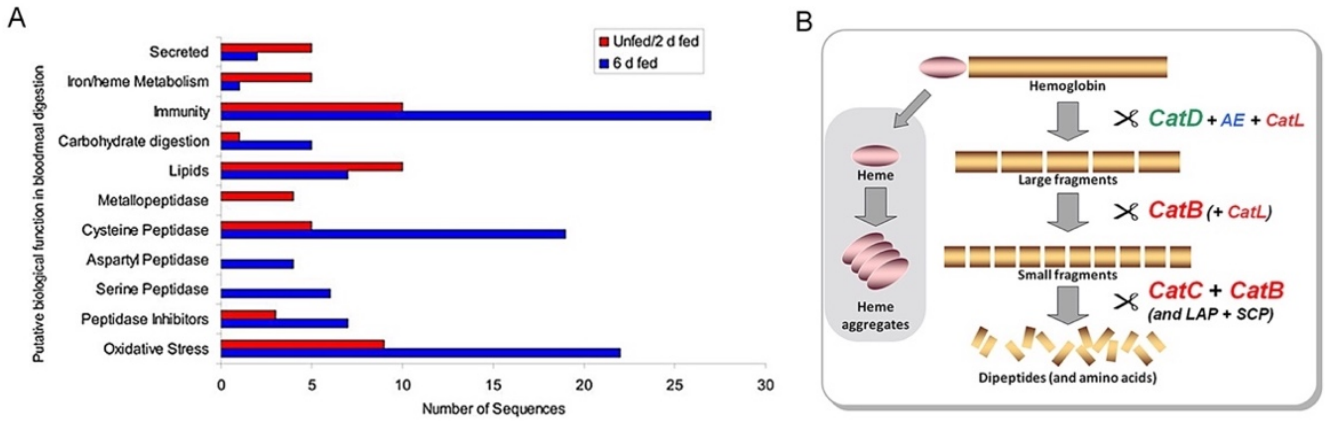

Figure 10. Diagrammatic illustrations of the protein families and/or specific proteins involved in blood meal digestion in feeding female ixodid ticks. (A) Changes in protein families involved in blood meal digestion comparing the early unfed – day 2 attachment period versus the day 6 post-host feeding period in *Dermacentor variabilis.* (B) The hypothetical proteolytic pathway of hemoglobin digestion in heterolysosomes in midgut epithelial cells in *Ixodes ricinus*. The initial breakdown of hemoglobin is done by Cathepsins D and L plus legumain, liberating large fragments, followed in turn by cathepsin B (and secondarily by cathepsin L), liberating even smaller fragments. Finally, cathepsins C and B, with secondary digestion by leucine amino peptidase (LAP) and serine carboxypeptidase (SCP), digest the small fragments into dipeptides and free amino acids. Panel A is from Anderson et al., (2008) Exploring the mialome of ticks: an annotated catalogue of midgut transcripts from the hard tick, *Dermacentor variabilis* (Acari: Ixodidae), BMC Genomics, 9:552. Panel B is from Horn et al. (2009) Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics, Chem. Biol. 16:1053–1063, with permission from Elsevier.

mated, engorged females that begin egg laying). This slow digestion allows the midgut to serve as a food reservoir. However, when the juvenile stage ticks feed again, the remnants of the old blood meal is passed out in the feces while fresh blood is ingested**.** This unique form of blood digestion, with few extracellular enzymes also provides a relatively permissive environment that shelters diverse microbes, including tick-borne pathogens.

Stages of blood meal and hemoglobin digestion

During the lengthy feeding period, the midgut undergoes extensive changes (Figure 10). Some of the genes coding for proteins associated with the early stages of blood meal digestion and hemoglobin sequestration are down-regulated later in the process, while others are more strongly upregulated or remain unchanged. Figure 10A illustrates midgut transcriptomic changes during the 6-day feeding period in a feeding *I. scapularis* female. During the brief attachment period, when the female tick is cementing itself and establishing the feeding site, genes coding for iron metabolism, immunity, lipids, metalloproteases, cysteine, aspartyl and serine proteases, peptidase inhibitors and oxidative stress are expressed, many rather strongly. However, by day 6, genes coding for immunity, carbohydrate digestion, cysteine peptidase, peptidase inhibitors and oxidative stress are even more strongly expressed while genes coding for iron metabolism and lipid metabolism were down regulated and genes coding for metallopeptidase, aspartyl peptidase and serine peptidase remained the same (Sonenshine and Anderson, 2014).

Once sequestered in the heterolysosomes of the midgut cells, hemoglobin molecules are disrupted so as to liberate the globin for breakdown into smaller peptides and amino acids. The process is carried out as a sequential cascade of different proteolytic events, each by a different proteolytic enzyme targeting different links in the amino acid chain of the globin moieties. These processes are done entirely within the small intracellular vesicles (hemosomes), not in the cellular cytoplasm. In the European sheep tick, *I. ricinus,* two types of proteolytic enzymes, cysteine proteases known as cathepsins D and L and legumain cleave the globins, releasing several large peptide fragments. Next, other cysteine proteases, cathepsin B and cathepsin L, digest the large fragments into smaller ones. Subsequently, cathepsins C and B, leucine amino peptidase (LAP) and serine carboxypeptidase (SCP) complete the process, cleaving the fragments into dipeptides and free amino acids (Figure 10 B). The end products liberating by these enzymatic steps escape into the cytoplasm and are transcytosed into the surrounding hemolymph (Horn et al., 2009; Sojka et al., 2011). It

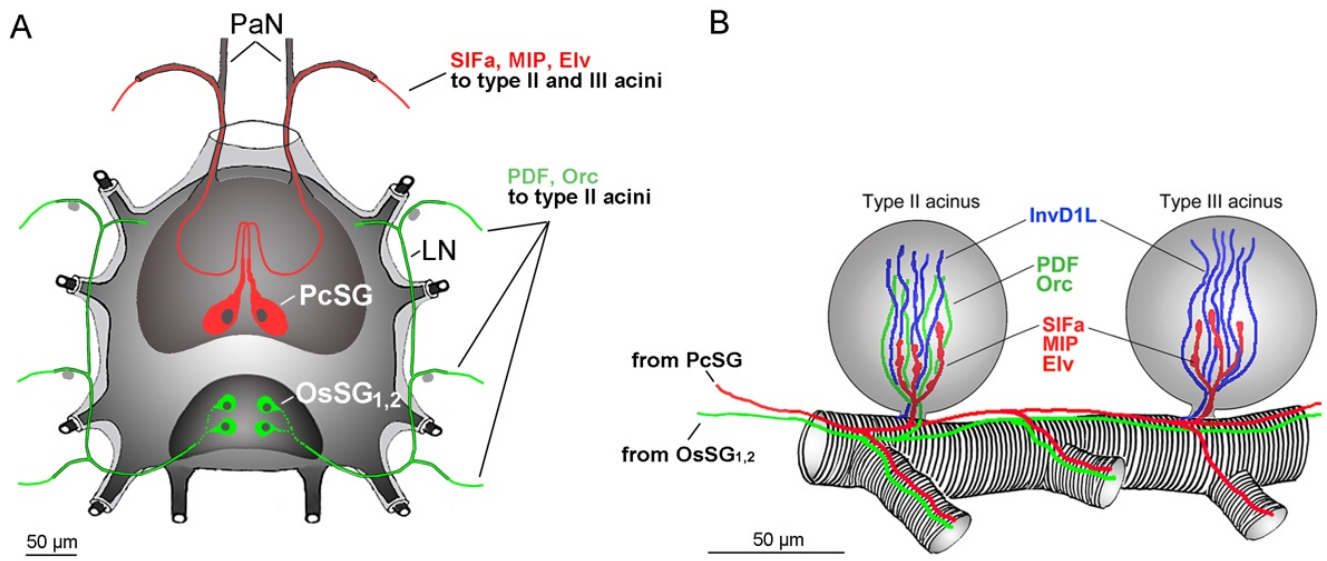

Figure 11. Neuronal control of the *Ixodes scapularis* salivary gland. (A) Tick synganglion with peptidergic neurons identified as sources of salivary gland innervation. Giant protocerebral neurons PcSG (red) expressing three different neuropeptides SIFa (SIFamide), MIP (myoinhibitory peptide) and Elv (elevenin) innervate basal parts of types II and III acini shown in (B). Another set of opisthosomal neurons $OsSG_{1,2}$ (green), reacting with antibodies against PDF (pigment dispersing factor) and Orc (orcokinin) innervate exclusively type II acini shown in (B). Abundant axonal projections expressing InvD1L (invertebrate-specific D1-like dopamine receptor) and innervating (blue in B) both type II and III acini have an unknown origin. Note that image B shows only one single type II and type III acinus attached to the salivary duct. Axons running along the main duct and different axon terminals entering the acini are shown in different colors. PaN – palpal nerve, LN – lateral nerve. Panel A graphic authors: D. E. Sonenshine and L. Šimo. Panel B is modified from Vancová et al., (2019) Ultrastructural mapping of salivary gland innervation in the tick *Ixodes ricinus*. Sci. Rep. 9:6860.

is likely that the same proteolytic cascade occurs in the midgut cells of *I. scapularis*, since homologues of the *I. ricinus* genes have been identified in this tick (Gulia-Nuss et al., 2016, Supplementary Table 21).

Part III. Molecular regulation of tick bodily functions and reproduction

Neuropeptides, neurotransmitters, and their receptors regulating physiologic processes

The *I. scapularis* genome contains at least 39 neuropeptide genes (encoding more than 100 mature neuropeptides), most of which are expressed during different phases of blood feeding and subsequent reproductive events (Gulia-Nuss et al., 2016). In invertebrates, neuropeptides and protein hormones (collectively, neuropeptides), along with their G-protein coupled receptors (GPCRs), are among the most important regulators of biological processes, such as development, behavior, host seeking, reproduction and feeding (Meeusen et al., 2003; Nässel, 2009). Although, within invertebrates, the number of neuropeptide genes is more or less the same, their specific functions may differ among taxa (Schoofs et al., 2017). Alerting the tick's physiological state for successful feeding and subsequent development involves neuropeptides produced primarily by their central nervous system, the synganglion (Christie, 2008; Šimo et al., 2009b). In ticks, functional inferences of neuropeptides has been based, thus far, almost exclusively on the identification of their release sites and/or localization of their receptors in specific target cells/organs (Šimo and Park, 2014; Šimo et al., 2009a; Vancová et al., 2006). In addition speculation about their putative functions has been based on the regulation of neuropeptide and receptor transcripts during feeding (Egekwu et al., 2016). In addition, the GPCRs recognizing a suite of neurotransmitters (e.g., dopamine, γ-aminobutyric acid (GABA), glutamate, etc.) also are expressed during these physiological events (Kim et al., 2014; Šimo et al., 2011, 2012, 2014b). Valuable knowledge of these receptors has been acquired from studies of adult females, although similar gene activities are assumed to apply to larvae, the stage responsible for acquisition of *Borrelia* spirochetes, and nymphs, the life cycle most often responsible for transmission of spirochetes to

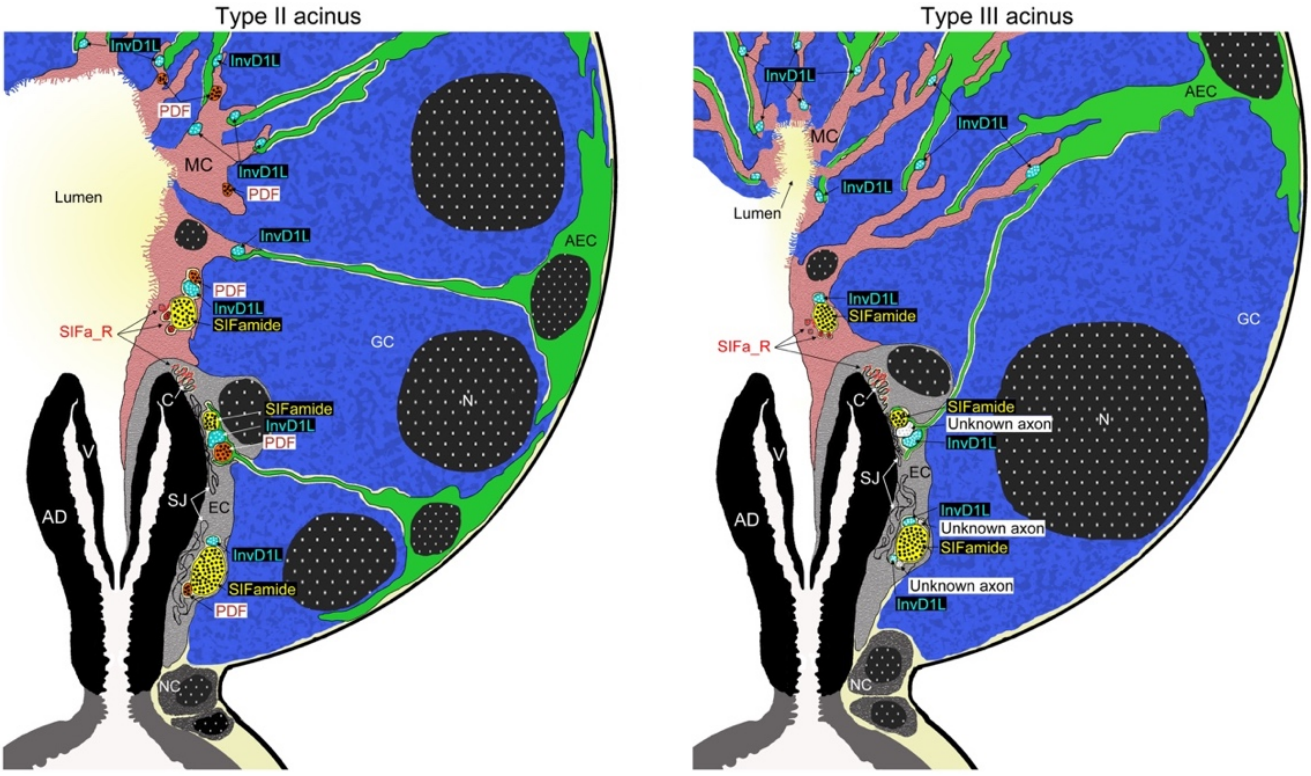

Figure 12. Schematic showing cross sections of type II and III acini from unfed *I. ricinus* female salivary glands highlighting the organization of the acinar cells and their putative associations with different axonal projections. Similar organization is believed to occur in the salivary glands of *Ixodes scapularis*. SIFamide-positive axons (yellow), pigment dispersing factor-positive axons (PDF, brown/orange), invertebrate-specific D1-like dopamine receptor-positive axons (InvD1L, aqua-blue). SIFamide receptor (SIFa_R, red): on plasma membrane and vesicle-like structure (upper arrow) of the myoepithelial cell (MC, pink), axons accompanying the SIFa-axon surrounded by the MC (middle arrow) and canaliculi (lower arrow) of basal epithelial cell (EC, grey). Note that SIFamide-axons also co-express myoinhibitory peptide (MIP) and elevenin (Elv) likely acting in the same acinar region. The acinar valve (V) is covered by EC extensions and overlaid by the MC. AEC, ablumenal epithelial cell: green; GC, granular cell: blue; NC, neck cells: dark grey; N, nucleus: dotted black; AD: acinar duct; C: canaliculi of ECs; SJ: septate junctions. For a complete description of the different granular cell types in hard tick salivary glands see Binnington et al, 1978 and Fawcet et al, 1986. From Vancová et al., (2019) Ultrastructural mapping of salivary gland innervation in the tick Ixodes ricinus. Sci. Rep. 9:6860.

humans and reservoir hosts (Egekwu et al., 2016; Gulia-Nuss et al., 2016). The most recent discoveries in the field of tick neurobiology are discussed below highlighting the unique functions of neuropeptides and neurotransmitters in the tick lineage.

Regulation of salivary glands

Among all of the internal tick organs, the control of salivary glands represents the best-known subject in terms of signaling molecules and their receptors. The activities of this organ are regulated primarily by direct upstream neural signaling pathways arising from the tick synganglion (Figure 11). The post-genomic era provided solid evidence that saliva production is orchestrated by dopamine, as well as neuropeptides and their receptors, via the primary saliva producers, the granular type II and III acini (Kim et al., 2014, 2019; Šimo et al., 2009a, 2011, 2014b). The neural connection of the tick salivary gland with specific cells in the synganglion has been reported in various tick species (Šimo et al., 2012, 2014a), with the most detailed information from the black-legged tick *I. scapularis*.

Specifically, in *Ixodes* females, three different axonal projections, arising from several distinct neurons, extend into the type II and III acini and arborize within them (Figure 11). These projections include a) neuropeptidergic axon terminals arising from giant protocerebral neurons (PcSG) co-expressing myoinhibitory peptide (MIP), SIFamide and elevenin (Elv) terminate at the basal regions in both type II

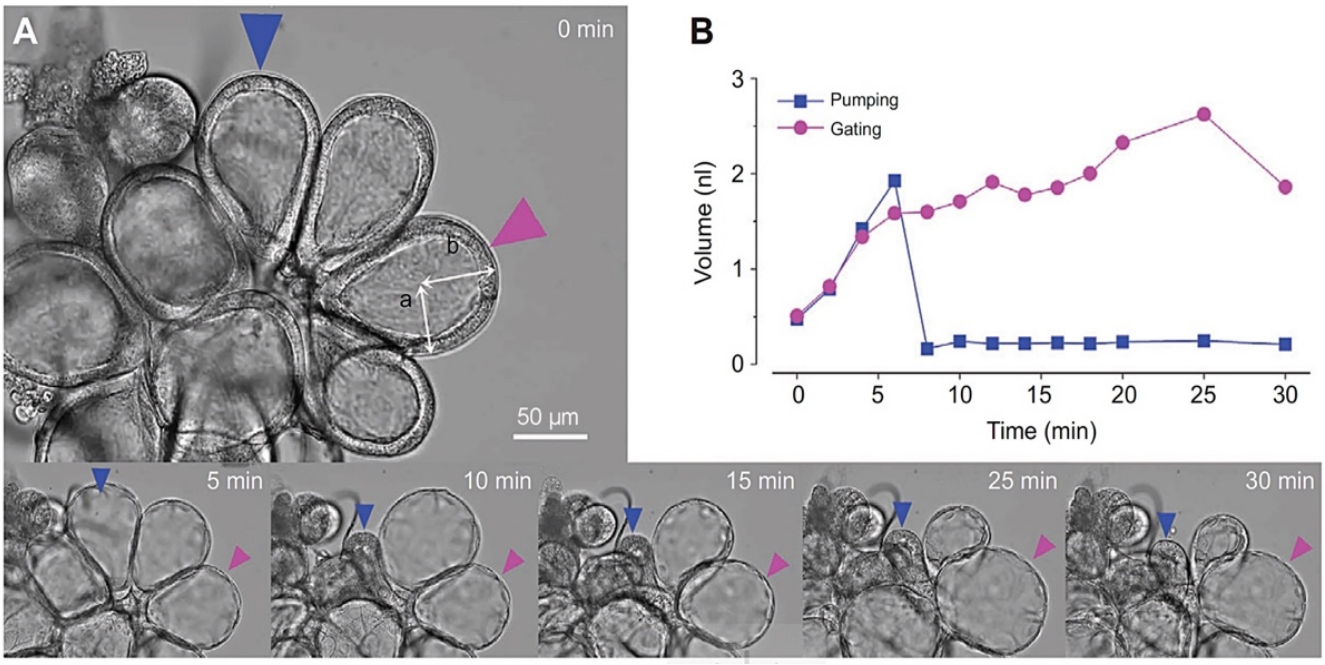

Figure 13. Changes in type III acini size following dopamine treatment, showing pumping and gating actions of individual acini in *I. scapularis* female. (A) Type III acini under the microscope, demonstrating changes in the acini size over time following dopamine treatment. (B) Changes in the acini volume after dopamine treatment. Video available in original article. From Kim et al., (2014) Orchestration of salivary secretion mediated by two different dopamine receptors in the blacklegged tick *Ixodes scapularis*, J. Exp. Biol. 217: 3656-3663.

and III acini (Kim et al., 2018; Šimo et al., 2009a); and b) neuropeptidergic axon terminals arising from two pairs of opisthosomal neurons ($OsSG_{1,2}$) positively immunoreacting to antibodies against both pigment dispersing factor (PDF) and orcokinin (Orc) reach the apical parts of exclusively type II acini (Roller et al., 2015; Šimo et al., 2009b); and c) axonal projections with unknown origin expressing invertebrate - specific D1-like dopamine receptor (InvD1L) reaching the apical regions of both type II and III acini (Šimo et al., 2014b).

The specific role of these molecules in type II and III acini has been inferred from immunogold labeling in *I. ricinus* (Vancová et al., 2019). Specifically, the basal axon terminals co-releasing MIP/SIFamide/Elv are thought to control the activities of the acinar valves via the regulation of myoepithelial and basal epithelial cells in *I. ricinus*, both of which overlay the valvular arms (Figure 12). The exclusive presence of PDF/Orc-axon terminals in type II acini suggests that ticks are capable of selectively controlling particular types of acini via different neuropeptidergic networks (Figures 11B and 12). The direct association of InvD1L - axons with the numerous zones of the myoepithelial cell (MC, Figure 6B, C and 12) supports a proposed model in which activation of InvD1L triggers Ca^{++}-mediated MC contraction and expulsion of acinar contents (Kim et al., 2014; Šimo et al., 2014b). In addition, another dopamine receptor, the epithelial D1, located on the luminal surface of both type II and III acini triggers the cAMP pathway for transport of fluid into the acini (Šimo et al., 2011). Thus, the autocrine/paracrine dopamine (Koči et al., 2014) in type II and III acini orchestrates the influx of fluids and pumping/gating actions (Figure 13) through the D1 and InvD1L dopamine receptors, respectively (Kim et al., 2014). Besides the direct action of dopamine, several other chemical agents, such as octopamine, norepinephrine, GABA, ergot alkaloids and pilocarpine, have been directly or indirectly implicated in salivary gland secretion; their precise mode of action, however, remains enigmatic (Lindsay and Kaufman, 1986; Needham and Pannabecker, 1983; Pannabecker and Needham, 1985).

Osmoregulation during the blood meal

Ixodid ticks, including *I. scapularis,* are unique among blood-feeding arthropods with regard to the

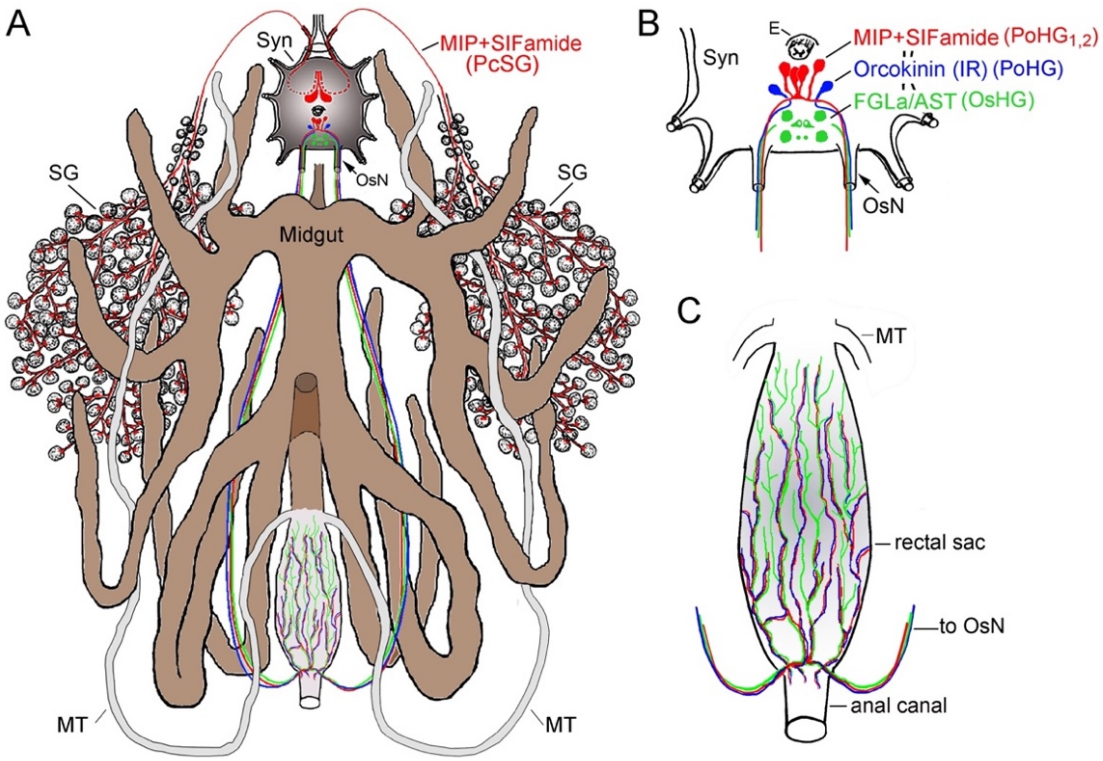

Figure 14. Simplified diagrams showing peptidergic innervation of *Ixodes scapularis* osmoregulatory organs--the salivary glands (SG) and hindgut. (A) Complex view of synganglion (Syn) neurons innervating the hindgut SG. (B) Magnified view of dorsal-post-esophageal region of the synganglion with a focus on putative peptidergic neurons innervating the hindgut. Note that immunoreactive axonal projections from all detected neurons exit the synganglion via the opisthosomal nerves (OsN) and reach the surface of the rectal sac and the anal canal as shown in (C). (C) Immunoreactive axonal projections reaching the surface of the rectal sac and anal canal. Protocerebral salivary gland (PcSG) neurons producing myoinhibitory peptide and SIFamide in innervation of the SG (see Figures 11, 12). E, oesophagus; MT, Malpighian tubule; OsHG, opisthosomal hindgut neurons; PoHG, postoesophageal hindgut neurons. From Šimo et al., (2014) Neuropeptidergic control of the hindgut in the black-legged tick *Ixodes scapularis*, 819-826, Int. J. Parasitol., with the permission from Elsevier.

enormous volumes of blood they consume, as much as 100 times their pre-fed body weight (Tatchell, 1967). Following the attachment phase, a strong pulsing current, achieved by contraction/relaxation of muscles associated with pharynx, drives the ingested blood into the pharynx, then into the esophagus and subsequently fills the central midgut (stomach) and its numerous diverticula (Vancová et al., 2020). To accomplish this remarkable feat, the tick remains attached for many days, while a suite of signaling molecules (neuropeptides and neurotransmitters) along with their receptors are hypothesized to regulate the rate of blood volume intake, periodically accelerating (e.g., FMRFamide neuropeptide and/or metabotropic glutamate receptor, (Audsley and Weaver, 2009; Dillon et al., 2015) or slowing (e.g., sulfakinin neuropeptide and/or GABA) the process (Moran, 2000). Upregulation of certain synganglion neuropeptides, their receptors and neurotransmitter receptors, that are expressed in varying degrees during feeding, is speculated to regulate this process since their activities are consistent with their reported roles in regulating the rates of blood imbibition in other arthropod vectors (Galun, 1987). A major reason for this exquisitely well-regulated feeding process is the tick's need to synthesize new integument as it feeds, effectively enlarging its body to accommodate the enormous fluid volume it will eventually consume. Here too, this unusual process of integument and cuticle growth also is suggested to be regulated by increased expression of genes, e.g., corazonin, eclosion hormone and bursicon, etc. (discussed below and illustrated in Figure 16). The large volume of imbibed blood induces over-hydration and causes an enormous osmoregulatory stress on the ticks due to the significantly lower

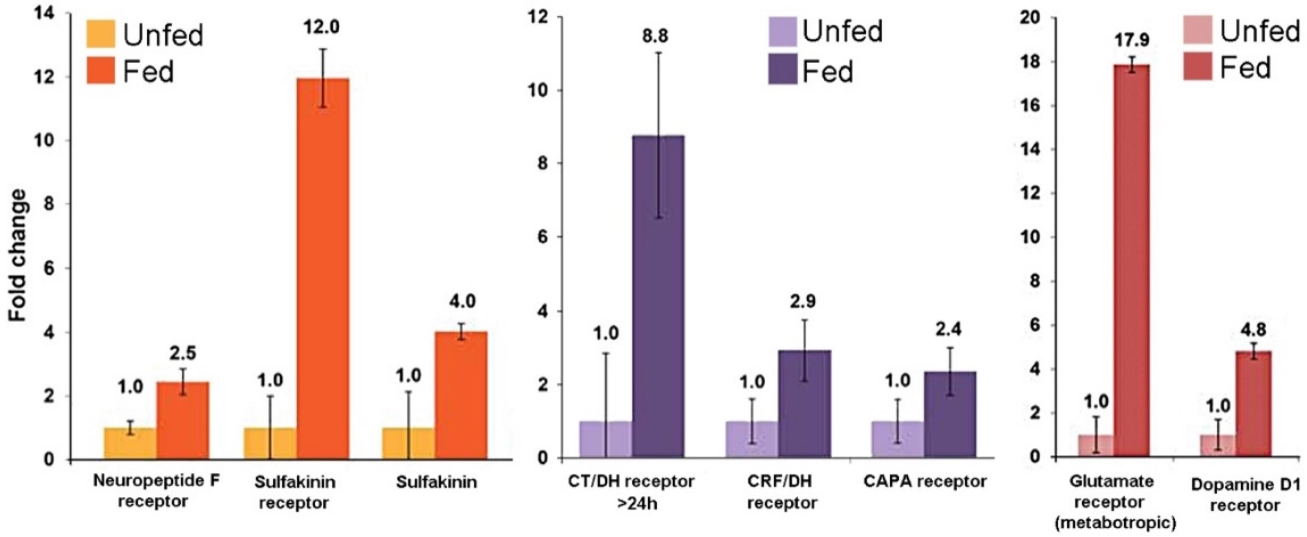

Figure 15. Quantitative PCR (qPCR) assays comparing expression of synganglion neuropeptides, neuropeptides, and neurotransmitters receptors between unfed and partially fed *I. scapularis* females. CT/DH receptor: calcitonin-like diuretic hormone receptor, CRF/DH receptor: corticotropin-releasing-factor related diuretic hormone. From Egekwu et al., (2016) Comparison of synganglion neuropeptides, neuropeptide receptors and neurotransmitter receptors and their gene expression in response to feeding in *Ixodes scapularis* (*Ixodidae*) vs. *Ornithodoros turicata* (*Argasidae*), 25:72-95, Insect Mol. Biol., with permission from Wiley Online Library.

osmotic pressure of vertebrate blood, ~ 300 milliosmoles, versus the much higher concentration of tick hemolymph, ~ 340 milliosmoles (summarized by Egekwu et al. 2016).

Ticks are known to utilize a unique osmoregulatory mechanism for substantial elimination of excess water, ions and metabolic waste essential to maintaining homeostasis during blood feeding (Kaufman and Phillips, 1973; Kim et al., 2019). In contrast to insects, in which the Malpighian tubules carry out these processes (Park, 2012), in ixodid ticks, the salivary glands are the primary osmoregulatory organs (Bowman and Sauer, 2004; Tatchell, 1967). An earlier study in *Dermacentor andersoni* found that 70% of water and 96% of sodium ions from the digested blood meal are transferred via the gut wall into the hemolymph where they are taken up by salivary glands and secreted back to the host through the saliva (Kaufman and Phillips, 1973). The epithelium of the type III acini (and likely type II as well) has been suggested as the specific transport site for water and electrolytes from hemolymph into the salivary glands (Fawcett et al., 1981; Meredith and Kaufman, 1973). Furthermore, it has been proposed that dopaminergic signaling mediates the formation of sodium-rich primary saliva by the potassium pump (Na/K-ATPase) located in the epithelial cells in the types II and III acini (Kim et al., 2016a). These same authors also suggested that, besides the ions transferred via an electrochemical gradient, Na/K-ATPase may activate aquaporins channels to regulate sequestration and elimination of water through the gut and salivary glands and its secretion back into the host (Campbell et al., 2010). By eliminating such a large proportion of the ingested blood meal water during the final phases of blood feeding, ticks are able to concentrate their blood meal by as much 3 times the amount they originally ingested (Koch and Sauer, 1984). In addition, the influx/efflux (Figure 13) of solute by type II and III acini is regulated by autocrine/paracrine dopamine signaling via D1 and InvD1L dopamine receptors respectively (Kim et al., 2014; Šimo et al., 2011, 2014b). Axons co-expressing the neuropeptides SIFamide, MIP and elevenin are thought to be involved in controlling myoepithelial cells in these structures to regulate salivary secretion (for more details see section *Regulation of salivary glands* in Part III of this chapter; also see Figures 11, 13; (Kim et al., 2019; Šimo et al., 2012; Vancová et al., 2019).

Another important excretory/osmoregulatory organ playing a significant physiological role in maintaining homoeostasis and waste elimination is the tick's

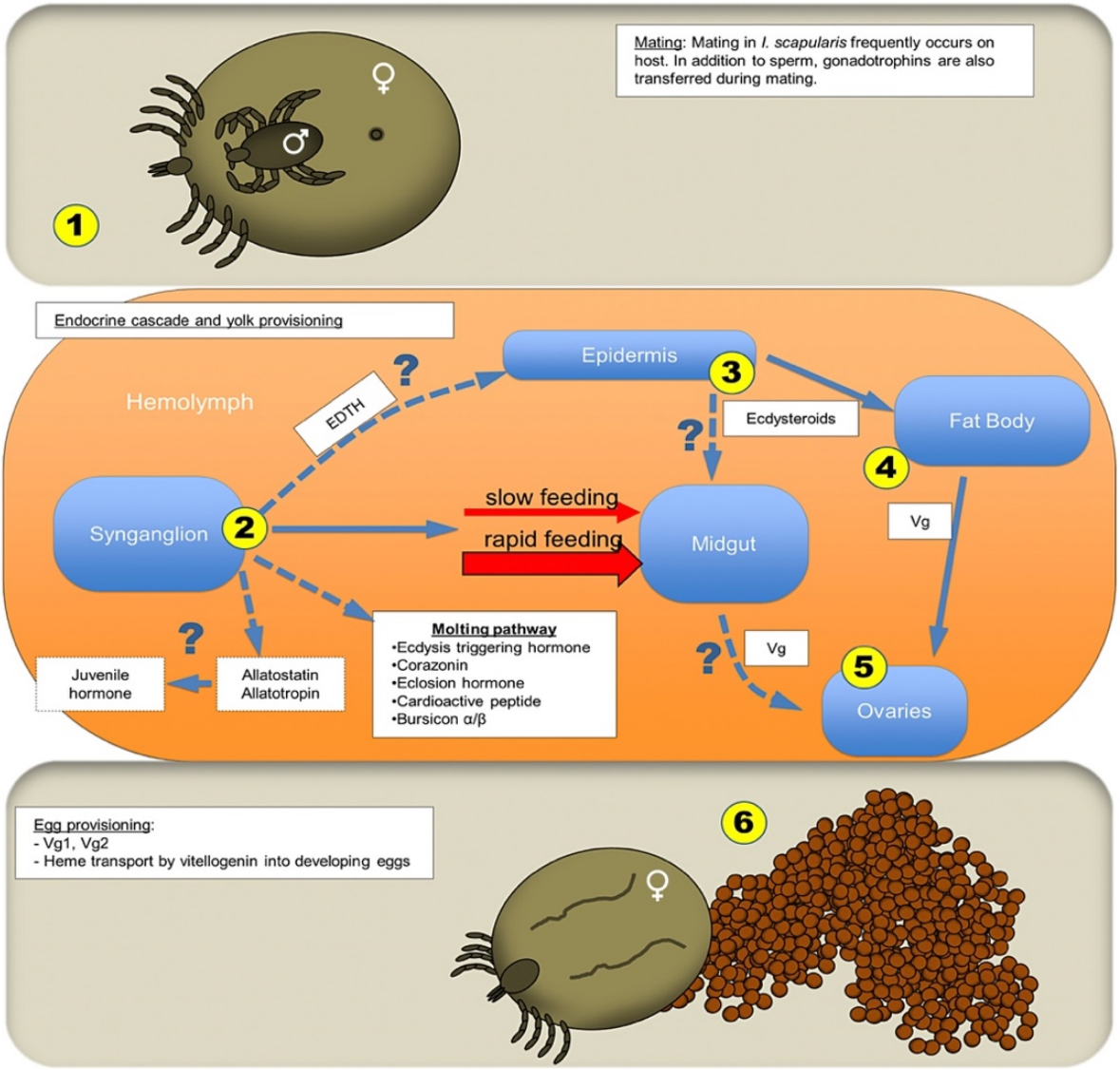

Figure 16. A putative model of neuroendocrine processes controlling mating and egg production in *Ixodes scapularis*. (1) Mating takes place off or on the host, required for rapid blood feeding during which the male transfers sperm and gonadotropins (unidentified at present), among other seminal components, including the spermatophore, (2) Gonadotropins stimulate the synganglion to release EDTH, which in turn stimulates rapid engorgement. The unknown gonadotropins also initiate synthesis and release of allatostatins and allatotropins (which may stimulate or inhibit the mevalonate-farnesal pathway), (3) EDTH initiates production of ecdysteroids by the epidermis, (4) rapidly rising ecdysteroid titers activate transcription factors for vitellogenin synthesis (Vg) in the fat body and specialized cells of the midgut activate transcription factors, as well as vitellogenin receptor (VgR) in the ovaries (5) Vg is taken up via VgR-receptor mediated endocytosis by developing oocytes and incorporated into the yolk as vitellin, and (6) The female produces a single batch of up to 3,000 mature eggs. Dashed lines indicate proposed pathways and factors. 20-E, 20-hydroxyecdysone; CAP, cardioactive peptide; EDTH, hypothesized epidermal trophic hormone; Vg, vitellogenin; VgR, vitellogenin receptor. Modified rom Gulia-Nuss et al., (2016) Genomic insights into the *Ixodes scapularis* tick vector of Lyme disease, Nat. Commun., 7:10507.

hindgut, comprised of Malpighian tubules, the rectal sac and the short anal canal (Sonenshine, 2014). During feeding, up to 74% of potassium and about 25% of water is excreted via tick's hindgut (Kaufman and Phillips, 1973). The feces, or wastes, from the midgut, consists of hematin, other nutrients and undigested hemoglobin. These fecal wastes are passed to the rectal sac. In addition, nitrogenous wastes in the form of guanine crystals accumulate in the Malpighian tubules and also are passed into the

rectal sac (Sonenshine, 2014). During or immediately after feeding (or after molting of juvenile stages), masses of dark fecal material and white powdery-like wastes are passed to the external environment (Sonenshine et al., 2003).

Although the regulation of diuresis has been extensively studied in insects (Park, 2012), little is known about the mechanisms in ticks. In *I. scapularis*, a rich neuropeptidergic axonal network arising from specific neurons in the tick synganglion arborize on the surface of the tick rectal sac and anal canal (Šimo and Park, 2014). Specifically, immunopositive axons reacting with four different neuropeptides were described: FGLamide related allatostatin, MIP and SIFamide (both co-expressed in the same axonal projections) and orcokinin (Figure 14). Receptors for MIP and SIFamide were observed on the visceral muscles of the *Ixodes* rectal sac. These data suggest that these neuropeptides, through their cognate receptors, may regulate ion and water transport via the rectal sac (Šimo and Park, 2014, Campbell et al., 2014). An *in vitro* experimental setting for testing activities of these neuropeptides showed strong myostimulatory effects of SIFamide on hindgut motility, whereas MIP antagonized these actions (Šimo and Park, 2014). The bioactivity of SIFamide/MIP on the dissected hindgut demonstrated the first direct evidence for the role of these tick neuropeptides. Along with their proposed functions in the control of salivary glands (Figures 11 and 12), these two peptides are hypothesized to function as important regulatory factors of both primary osmoregulatory organs in ticks (Kim et al., 2019). A pictorial view of the target organs and the neuropeptides that regulate their function is shown below (Figure 14).

Although, the functions of most of the tick neuropeptides await experimental evidence, sequencing of the *I. scapularis* genome uncovered interesting features in tick neuropeptidergic signaling system and its potential involvement in processes relevant to their feeding biology (Gulia-Nuss et al., 2016; Šimo et al., 2014a). Most of the tick neuropeptides have clear orthologues to those of other invertebrates, including corazonin, eclosion hormone, cardioactive peptide and bursicon α and β (Figure 16), all known to regulate ecdysis, cuticle synthesis, hardening and tanning in insects, respectively (Nässel, 2009). The identification of specific functions of these neuropeptides in adult ticks, which do not molt, may help to uncover novel pathways for regulation of cuticle synthesis during the ingestion of large quantities of blood meal during feeding as well as other developmental processes. Another interesting feature reported in the *Ixodes* genome was the remarkable increase (up to 10-fold) in the number of certain neuropeptide receptors (Gulia-Nuss et al., 2016), among which were insect orthologs known to regulate satiety (sulfakinin) and water balance (kinin, tachykinin, and calcitonin-like diuretic hormone CT/DH, and corticotropin-releasing-factor related diuretic hormone CRF-DH) (Nässel, 2009). Whether all of these receptors are functional genes, rather that pseudogenes, awaits experimental confirmation; however, this finding supports the hypothesis that crucial functions regulating massive body increase size and osmoregulation are accomplished via greatly increased expression of this repertoire of specific neuropeptide receptors (Gulia-Nuss et al., 2016).

An interesting study by Egekwu *et al.* (2016) compared the levels of transcripts encoding neuropeptide/neuropeptide receptors and neuro-transmitter receptors in *I. scapularis* synganglion during blood feeding. Especially noteworthy was that transcripts for neuropeptide F and sulfakinin/ sulfakinin receptor in the *I. scapularis* female synganglion were significantly upregulated in partially fed *I. scapularis* females when compared to unfed female ticks (Figure 15)**.** Neuropeptide F plays a role in *Drosophila* food signaling (Chung et al., 2017), and sulfakinin is known as the satiety gene, slowing or stopping blood ingestion in various arthropods (Nässel and Williams, 2014), a function similar to cholecystokinin, which reduces food intake in vertebrates (Moran, 2000). Whether these neuropeptides play a similar role in ticks remains to be confirmed by further investigations. In the same study, another set of neuropeptides receptors for CT/ DH, CRF/DH and CAPA was significantly upregulated in the synganglion of partially fed *I. scapularis* females. These are well-known for their contributions to water balance and diuresis in insects (Nässel, 2009; Park, 2012), and current data suggests they may control similar physiological processes in ticks. Very strong upregulation of the metabotropic glutamate receptor during feeding was hypothesized to correlate with the activities of the *I. scapularis* pharyngeal pump (Egekwu et al., 2016), but experimental proof is currently lacking (Figure 15). In addition, upregulation of the D1 dopamine receptor supports the involvement of this neurotransmitter in

salivary gland regulation as described in previous studies (Šimo et al., 2011, 2014b).

Extensive additional investigations are required to fully understand the role of these signaling molecules and their receptors in tick biology. Current achievements in genomics, peptidomics, transcriptomics, immunolocalization or direct bioassays represent an excellent starting point to elucidate the different signal transductions at specific body sites. The tick neuropeptidergic system is an ideal target for the development of novel control measures against tick-borne diseases.

Reproduction

In *Ixodes*, mating occurs both on and off the host, but the other hard tick genera mate exclusively on the host. In these other genera, the males and females begin feeding separately from one another and both undergo development of their reproductive organs as they suck blood. Mating normally occurs within several days after slow feeding on their vertebrate hosts. However, mating and insemination are required for the females to commence rapid engorgement, vitellogenesis and oviposition. The transition of the mated adult female to the reproductive phase that occurs soon after blood feeding is a complex process (Figure 16) controlled by the ecdysteroid hormone, 20-hydroxyecdysone (20-E), in contrast to insects, where it is regulated by the juvenile hormone, JHIII (Chang, 1993). Exhaustive studies by (Neese et al., 2000) failed to reveal evidence for the occurrence of any of the known juvenile hormones in any life stages of a tick (*D. variabilis)* or soft tick (*Ornithodoros parkeri).* However, although all of the enzymes for the mevalonate pathway (homologous to the JH pathway) were found in different tick species, the final enzymatic steps that convert farnesoic acid to JH were absent. The authors suggest that this highly conserved pathway may function in regulating reproduction (Zhu et al., 2016). Activation and regulation of the pathway may be controlled by neuropeptides allatotropin and allatostatin (see description below).

In mated *I. scapularis* and most other hard tick females, unknown factors in the male's seminal fluid (gonadotropins noted above) trigger a rapid increase in blood feeding (Donohue et al., 2009; Weiss and Kaufman, 2004). This signaling event stimulates unknown elements in the synganglion that initiate production and secretion of ecdysone in the epidermis (Brown et al., 2009; Zhu et al., 1991), subsequently converted to 20-E in the fat body and tissues. Synthesis of 20-E is regulated by the ecdysiotropic hormone (Roe et al., 2008). Increasing concentrations of 20-E stimulate certain organs, primarily the fat body and midgut, to commence biosynthesis and secretion of vitellogenin (Vg), the precursor of the yolk protein, as well as upregulation of the Vg receptor (VgR) in the ovary. Discovery of the gene for the Vg receptor in the *I. scapularis* genome suggests that a similar 20-E regulated process also occurs in the tick species. However, it is not known precisely when vitellogenesis occurs in *I. scapularis* females (i.e, when insemination occurs) since the males remain in copula with their female partners through the blood feeding period, departing only after female repletion and drop off has occurred. All of these events have been thoroughly documented by numerous studies over many years (reviews by (Cabrera et al., 2009; Ogihara and Taylor, 2014).

It is not known whether *I. scapularis* and other ixodid ticks regulate reproductive activity by means of the same neuropeptides, allatotropin and allatostatin, that regulate it in insects (Klowden, 2013). Both of these neuropeptides, along with their receptors, were identified in the *I. scapularis* genome as well as in the synganglion transcriptome (Egekwu et al., 2016; Gulia-Nuss et al., 2016). In addition, gonadotropin-releasing hormone (GnRH), which is known to stimulate ovary (Zandawala et al., 2018) and prothoracicotropic hormone responsible for secretion of ecdysone (molting hormone) in insects (Schwedes and Carney, 2012), also has been reported in the *I. scapularis* genome (Gulia-Nuss et al., 2016), suggesting a similar pathway. Although the direct action of these neuropeptides in ticks awaits experimental confirmation, Egekwu et al., (2016) reported significant up-regulation of transcripts encoding allatostatin and its receptor in the synganglion of partially fed *I. scapularis* female. They speculated that inhibition of oocyte development by allatostatin signaling prevents oviposition before the full supply of nutrients needed is available. No enlarged oocytes were noted by one of the authors (Sonenshine, unpublished) during the dissections of partially fed *I. scapularis* females, supporting this hypothesis.

Much remains to be discovered regarding these complex biosynthetic pathways and their regulation by synganglion signaling (neuropeptides,

neurotransmitters, and their receptors) that enable blood feeding ticks to produce thousands of eggs to ensure survival of their populations.

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