Dissection and internal observation of the giant tropical bont tick *Amblyomma variegatum*

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Abstract

Ticks are ectoparasites that feed on the blood of animals and humans and are among the arthropods responsible for numerous human and animal diseases worldwide. The tropical bont tick, *Amblyomma variegatum*, is a significant natural vector of *Ehrlichia ruminantium*, the causative agent of heartwater. This disease is endemic in many countries, including those in sub-Saharan Africa and certain Caribbean islands. Due to the potential of heartwater and its vector to pose an economic and health threats to non-endemic regions, they have been designated as high-consequence foreign animal diseases and pests by the United States Department of Agriculture's Animal and Plant Health Inspection Service. Understanding the ecology and biology of *A. variegatum* is critical for developing effective control methods to limit its spread to other geographic regions. A key step in this process is the isolation of tick organs and further elucidation of their functions (i.e. vector competence). However, the literature contains limited documentation of the complete internal structure of *Amblyomma* tick species. In this study, we describe the internal structure of unfed nymphs, partially engorged males, and unfed and partially engorged

females of the tick species *A. variegatum*. The objective is to facilitate the identification and the localization organs of interest while highlighting the morphological differences between the tropical bont tick and other tick species. This foundational work will support the development of various applications following the isolation of tick organs. These findings not only deepen our understanding of *Amblyomma variegatum* anatomy but also provide a critical foundation for tackling the broader challenges of tick-borne diseases and advancing vector control strategies in both endemic and non-endemic regions.

Keywords: *Amblyomma variegatum*, heartwater, dissection, tick organs, anatomy, hard-bodied ticks

Introduction

The earliest known study of insects was initiated by the Greek naturalist and philosopher Aristotle, who emphasized their insignificance (Friedrich et al., 2014). The internal anatomy of arthropods became the subject of extensive study for many centuries (Kennedy et al., 2021). Indeed, the study of insects advanced with Galileo's invention of the telescope in the early 17th century, which was adapted for observing insects. This progress was followed by the work of Antonie van Leeuwenhoek, considered the father of microscopy, whose studies included dissection of spiders (Cocquyt, 2022).

The process of dissection under a microscope can be as straightforward as exploring the internal phenome of arthropods (Deans et al., 2012). This was illustrated by Zacaï *et al*., who utilized microscopy to visualize an arthropod fossil, *Sidneyia inexpectans*, dating back several million years ago (Zacaï et al., 2016). They emphasized the internal digestive system associated with the fossilized gut contents and compared it to that of modern chelicerates. Since then, a variety of dissection techniques have been developed for use with diverse arthropods species. Primarily, the dissection of larger arthropods offers a rapid approach to conducting internal investigations. Identifying the internal organs of hard ticks can be achieved through dissection and subsequent removal of internal organs. The use of simple tools such as fine tweezers and sharpened blades is beneficial during the dissection process. Similarly, the application of strong tape or wax facilitates the dissection process.

Even with the advent of several powerful imaging techniques such as X-ray, microtomography (µCT), or confocal laser scanning microscopy (CLSM), light-optical microscopy remains the best method for analyzing morphological diversity (Sasso Porto et al., 2016). To date, the fragility of the internal structure of arthropods has meant that microscopic dissection involves a risk of tissue damage. Consequently, the manipulator's skill in performing dissections is of paramount importance to prevent the destruction of organs, particularly in morphological studies. To address this limitation, a recent study has focused on the development of spectral-domain optical coherence tomography (SD-OCT), which enables non-invasive imaging while preserving the anatomical features of tick specimens (Lee et al., 2021). However, this method does not permit the collection of arthropod organs of interest for interaction studies with pathogens (Deans et al., 2012).

The tropical bont tick, *Amblyomma variegatum* (Fabricius, 1794), is an obligate hematophagous arthropod belonging to the phylum Arthropoda, subphylum Chelicerata, Class Arachnida, subclass Acari, and order Ixodida (Camargo-Mathias, 2018). This tropical tick is also referred to as the Senegalese tick in the Guadeloupe archipelago and the Antigua gold tick in Antigua (Pegram et al., 2004). It belongs to the family Ixodidae, characterized by a hard body with a dorsal shield (scutum) that may be ornamented in the case of males, or may not be ornamented for females.

The tick is the vector for heartwater, a major disease in the Caribbean region and is frequently associated with dermatophilosis (Prine and Hodges, 2012). Furthermore, this tick contributes significantly to lameness and the formation of deep abscesses, which can ultimately lead to immunosuppression and metabolic disorders in small ruminants (Barré, 1989). Consequently, the presence of *A. variegatum* results in considerable economic and health losses. More than ten species of *Amblyomma* ticks transmit *E. ruminantium* in Africa. Of these, *A. variegatum* and *A. hebraeum* are identified as the main vectors (Bezuidenhout, 1987). The majority of heartwater-endemic areas overlap with the geographic distribution of *Amblyomma* ticks. The vector potential of these ticks depends on factors such as their abundance, activity, and adaptability to their environment (Uilenberg, 1983).

The tropical bont tick, *A. variegatum*, is not only a significant vector of pathogens in endemic regions but also poses a potential threat to non-endemic areas due to livestock movement and climate change (Estrada-Pena et al., 2012). These phenomena may play an indirect role in the abundance and distribution of ticks via changes in hosts. (Gilbert, 2021). Knowing its anatomy is crucial for studying the tick pathogen interactions, which are important part of potential vector control strategies addressing the socioeconomic and health impacts of tick-borne diseases, which disproportionately affect resource-limited regions. While studies on *Rhipicephalus microplus* and *Ixodes scapularis* have advanced our understanding of tick physiology, the anatomical and functional adaptations of *A. variegatum* remain understudied, necessitating detailed investigation. These adaptations include reproductive efficiency, feeding behavior, and ecological resilience, all of which contribute to its status as a major vector.

The morphological identification of the internal organs of the ticks is of significant value in entomology and parasitology, as it facilitates the development of effective control methods, whether natural or chemical. To further develop control technologies, it is necessary to anatomize the internal structure of ticks. The study of the internal anatomy of arthropods is of great interest due to the remarkable diversity observed, particularly regarding the specific barriers such as target organs they possess. Given the widespread distribution of *A. variegatum*, particularly in some Caribbean islands, there is a clear need to study the tick and its role as a pathogen vector. The impetus for composing this article was the desire to produce a paper identifying the organs of the *A. variegatum* tick and determining the optimal dissection procedures.

Despite the numerous dissection documents available for accurately identifying the internal organs of ticks, particularly those in the genus *Rhipicephalus*, there remains a pressing need for greater emphasis on the internal observations of *Amblyomma* ticks (Cossio-Bayugar et al., 2023; Tidwell et al., 2021). High-quality histologic images of the ixodid tick reproductive and digestive systems are available online (Camargo-Mathias, 2018; de la Vega et al., 2012; Mathias, 2013). Additionally, excellent scanning electron microscopy images of male *Amblyomma* testes and female *Amblyomma* intestines are available (Anholeto et al., 2015; Caperucci et al., 2009). However, apart from the 2009 article by Edwards *et al.*, there are few studies based on the dissection of *Amblyomma* ticks (Edwards, 2009). Despite the pictorial instructions provided in this guide, two different *Amblyomma* species had to be used to visualize the entirety of the tick's internal organs.

Here, we provide an anatomical observation of the internal cavity of *A. variegatum*, from nymphs to adult ticks at different stages of engorgement. The dissected organs are described and illustrated in detail in this document. This report will facilitate the accurate and reproductible sampling of *A. variegatum* tick tissues, enabling highly precise studies of tick pathogen interactions, and, *in fine*, the development of new tick control technologies.

Approach

Materials for the dissection

- Forceps with pointed tips
- Petri dish or glass dissection plate
- Disposable scalpel (No. 3 handle with a No. 11 surgical blade)
- Double-sided tape
- Falcon tubes
- Pins (optional)
- Dulbecco's phosphate buffered saline
- 70% Ethanol
- Sterile ultrapure water
- Microcapillary tubes for hemolymph collection
- Pipette tips

All the dissection tools were treated aseptically before use. Specifically, all the forceps were autoclaved, then immersed in 70% ethanol for 5 minutes and rinsed with sterile ultrapure water. Tools were allowed to air dry on a clean paper towel. All the petri dishes were wiped or sprayed with 70% ethanol and allowed to air dry. In certain cases, glass petri dishes could be used to avoid the damaging of pointed forceps. A LEICA M205FA fluorescence stereomicroscope equipped with Fluocombi (LEICA, Wetzlar, Germany) was used for image acquisition.

Troubleshooting and Practical Notes

During dissections, care must be taken to avoid tissue tearing, especially for fragile organs like ovaries and synganglion. Using fine, aseptic tools and working under a stereomicroscope significantly reduces the risk of damage. Adhesive tapes and clean dissecting surfaces are essential to maintain tissue integrity and minimize contamination. For less experienced researchers, it is advisable to practice dissections on nymphal or male ticks, which have simpler anatomy, before attempting engorged females. If tissues are intended for downstream analyses, such as transcriptomics or proteomics, meticulous handling and immediate storage in appropriate solutions (r.g., PBS or RNAlater) are critical to preserve RNA or protein quality.

Tick general dissection

Tick rearing

For all experiments, batches of unfed *A. variegatum* ticks were reared as a colony for more than 13 years at the CIRAD animal facility in Guadeloupe. Larvae and nymphs were placed on the ear side of a goat in a Mölnlycke Tubifast green line bandage, secured with Kamar adhesive (Vital Concept, Loudeac, France). Following the completion of feeding and subsequent detachment, all the ticks were collected and allowed to molt in a container maintained at room temperature and 70-80% of relative humidity. After ecdysis, nymphs were used for dissections, while adult females were placed on the side of a goat until semi-engorgement for some and detachment for others.

Preparation of the tick

Prior to the dissection, the ticks were washed twice for 30 seconds in ultrapure water and once in PBS in three 0.5 mL Eppendorf microtubes to remove impurities on the tick's cuticle. During the washing process, the tubes containing the ticks were gently inverted by hand. As *A. variegatum* ticks are rapid crawlers, they could optionally be cooled on ice to slow them down. Following the washing step, the ticks were completely dried on Kimtech paper towels (Kimberly-Clark, Nanterre, France) before being immobilize for dissection.

The fixation of the tick was performed by immobilizing a single tick, with its ventral surface facing down, on a piece of double-sided adhesive tape within a Petri dish (Fig. 1). To ensure fixation of the tick, all four legs were extended and secured to the double-sided tape with tweezers. For nymphs and unfed adults, multiple ticks could be fixed in a single Petri dish. Once immobilized, a few drops of sterile, filtered Dulbecco's Phosphate Buffered Saline (PBS) 1X were applied to cover the entire tick body. This prevented desiccation of the tick's tissues and facilitated dissection by swelling the internal organs.

Hemolymph and saliva collection

For hemolymph collection, the same procedure was used to wash the external structure of the tick. The ticks were then fixed on a double-sided tape in the same manner as for the dissection. To collect the hemolymph, the first two legs of the tick were fully extended. Then, the most distal joint of the tick's tarsus was cut with a scalpel, and the drop was collected with a micropipette through a microcapillary tube (Fig. 1B). For larger volumes of hemolymph, several legs may be amputated and the tick's body was gently squeezed to promote drainage.

To collect saliva, semi-engorged ticks that had been removed from animals or artificially fed were used to obtain larger amounts of saliva. After washing the tick as described above, it was immobilized on double-sided tape placed in the upper part of a petri dish, with its ventral surface facing up to expose its mouthparts, including the rostrum and palps. Another piece of double-sided tape was placed near the tick's basis capitula on the edge of the petri dish. A capillary tube was pressed between the hypostome and palps, ensuring the pedipalps remained outside the capillary tube.

A solution of pilocarpine (20mg/ml) was prepared, and 50 µL was gently injected into the tick's stigmata or spiracles (Sp) (Fig 10A) using a Hamilton syringe. Salivation was observed, and the saliva was collected using a pipette tip inserted into a micropipette. If the tick did not salivate after 10 minutes, an additional 20 µL of pilocarpine was injected. The saliva was stored at -80°C for further analysis or use.

Dissecting the external structure: Scutum/alloscutum/conscutum removal

To properly dissect the tropical bont tick, *A. variegatum*, it is fundamental to know the external appearance of the tick, which is the natural vector of the bacterium that causes heartwater in ruminants. The overall appearance of the tick body is similar to that of parasitiform acarines, such as mites.

The tick body is divided into two main regions: the gnathosoma (capitulum) and the idiosoma (body region (Fig. 1). The capitulum, considered as the "head", is located in the anterior part and bears the mouthparts, including the chelicerae, hypostome, and palps. However, this designation requires caution because the eyes and brain are situated inside the body region. Additionally, the basis capituli, located in the capitulum, bears two paired structures known as porose areas.

The idiosoma is divided into the anterior podosoma, containing the genital gonopore and the four pairs of legs, and the posterior opisthosoma, which includes the spiracular plates and the anal pore (Sonenshine and Simo, 2021). Additionally, the idiosoma includes the walking legs. Ixodid ticks, often referred as "hard-bodied ticks", are characterized by their teardrop shape and a dorsal shield or scutum (Cupp, 1991).

In male ixodid ticks, the scutum, known as the conscutum, covers the entire dorsum, preventing the ingestion of large quantities of blood during blood feeding. *A. variegatum* males are unique in having an ornate conscutum with bright orange colors and a dark lateral background (Fig. 1B, 6A).

In contrast, the scutum of adult females, nymphs and larvae, does not cover the entire dorsum. The female scutum consists of a large subtriangular plate near to the capitulum, while the remaining two-thirds of the idiosoma is made up of a less sclerotized plate, the alloscutum (Fig 1A, 4A). During feeding, this part of the female tick expands, allowing it to weigh up to 100 times its initial weight.

Furthermore, the alloscutum of larvae and nymphs tends to enlarge as they ingest blood. Lateral to the scutum/ conscutum, two hemispherical, dark-colored orbital eyes can be observed (Fig. 1A, 1B, 4A). The tick's locomotor system consists of four pairs of legs in the adult and nymph stasis, and only three pairs of legs in larvae. Each walking leg is composed of six podomeres: coxa, trochanter, femur, patella, tibia, and tarsus, arranged proximally to distally on the podosoma (Voigt and Gorb, 2017). The legs of ticks are highly flexible, owing to elastically articulated segments with bright colors in *A. variegatum* ticks (Fig. 1). At the posterior end of the opisthosoma of male and female ticks, as well as nymphs, several festoons are present, separated by marginal grooves.

Once the tick was firmly immobilized, the scutum/ alloscutum/ conscutum (Sac) was removed. The Sac was removed with a no. 11 sterile blade. A sterile blade was used to incise the dorsal cuticle at the distal part of the basis capitulum. A microscalpel was required to dissect ticks, particularly nymphs, to avoid damaging internal organs. The cutting step continued around the tick's Sac, passing above the festoons, and reaching the opposite side precisely (Fig. 4A). Special care was taken to keep the internal

cavity intact while cutting the dorsum of the tick by bypassing the spiracular plates, which are connected to the internal organs.

Following the incision of the Sac, two dissecting forceps (Entomopraxis, Barcelona, Spain) were used to remove it. Simply put, one forceps held the tick's body on the double-sided tape, while the other forceps carefully lifted the Sac away from the rest of the body. Once the cuticle was lifted, all of the thin white tubules and attached muscles were removed from the Sac to gain complete access to the tick's internal anatomy (Fig. 2). The dorsum was either glued to the double-sided tape or cut off completely from the tick. At this point, the small white tubes with a spider's web shape are designated as tracheae (Tr). These structures are part of the respiratory system of ticks and facilitate gas exchanges through the body and organs of the tick (Woolley, 1972). The tracheal system of the tick is extensive and opens externally through two lateral spiracular plates (Sp) (Starck et al., 2018). It is noteworthy that there is considerable variation in the structure of the respiratory system among ticks, particularly with regard to the morphology of the spiracles. In *A. variegatum*, the spiracular plates of the female specimen are considerably larger than those of the male specimen. This is due to the fact that females have a larger body size and distinct physiology. These physiological mechanisms, including the digestion of large blood meals, excretion, and egg production, require larger spiracular plates to ensure sufficient surface area for energy exchange via gas exchange (Baker, 1997). Due to their small size, the tracheae of larvae, compared to nymphs and adults, are significantly less developed. They have the peculiarity to facilitate gas exchange directly across the integument (Binnington and Obenchain, 1982).

In ixodid ticks, the pharynx is an elongated organ situated on the ventral surface of the basis capitulum. It facilitate the passage of ingested host blood through the food channel to the esophagus and midgut (Sonenshine and Simo, 2021). The gut extends throughout the entire body region of the tick and represents its largest organ. The entire tick gut includes the midgut, with a central portion, the stomach (St), which is connected to several dark brown, spider-shaped diverticula (Div) (Fig. 2, 4B, 6B). During the tick's engorgement phase, the midgut-diverticula is fill with host blood to be digested. Due to the considerable size of the midgut, other internal organs may be displaced or compressed (Sonenshine and Simo, 2021). This phenomenon is particularly salient for *A. variegatum* females, as they possess a larger body size and consume greater quantities of blood compared to other tick species. The hindgut and the Malpighian tubules (Mt) are key components of the tick's excretory system, playing vital roles in maintaining homeostasis and excreting guanine-rich wastes while transporting them to the rectal sac (Rs) (Šimo and Park, 2014). Two pairs of larger white tubes located laterally to the midgut, represent the Malpighian tubules, which are clearly visible. In ticks, nitrogenous excreta from the Malpighian tubules and undigested waste from the midgut are concentrated in the rectal sac and eliminated through the anus via a short rectum (Fig. 4B) (Šimo and Park, 2014). These connections are situated beneath the intestinal tissue. In Fig. 6D, the excretory system is faintly visible once it has been extracted from the cavity.

Dissecting the respiratory system: tracheae removal

Once the attached muscles and connective tissue were carefully removed from the ventral part of the dorsum, the entire tick cavity became visible (Fig. 2, 4B, 6B). To release the internal organs, two dissecting forceps were gently used to grasp the tracheae at the spiracular plates between the third and fourth pairs of legs on each side of the tick (Fig. 5).

In some instances, when dissecting feeding adult female ticks, the tracheal trunks at the base of the spiracular plates were observed denser. If difficulties arose, the spiracular plates could be incised, and the tracheal trunk carefully extracted with the forceps. After removal of the tracheae, the salivary glands (Sg) and the synganglion (Sy) became more prominent (Fig. 3A).

The Gené's organ (Go), a specialized structure found only in female ticks, is located in the anterior region of the cavity, just above the basis capituli. This structure facilitates coating the eggs with a wax-like substance during oviposition.

Dissecting the digestive system: midgut removal

The midgut stomach was withdrawn from the tick body by grasping the junction of the esophagus and stomach with one dissecting forceps and retracting the midgut with another pair of forceps. The

esophagus is a narrow, elongated tube that runs anteroventrally from the synganglion to the anterior part of the stomach.

Subsequently, the diverticula of the midgut could be gently pulled away from the rest of the organs without damaging them, ensuring that their digestive contents are not discharged (Fig. 2B, 3A, 8B). Since the midgut is elastic, it can be easily removed while ensuring that it remains intact. The midgut was rinsed in three successive pools of sterile PBS with aseptic dissecting forceps if analysis was required (Grabowski and Kissinger, 2020). After removal of the midgut, the salivary glands and the synganglion became more visible (Fig. 3A, 3B, 4C 6E).

Tick salivary glands are complex, multi-lobed organs that serve multiple functions during both the tick's parasitic and non-parasitic periods on the host (Bowman and Sauer, 2004). The salivary glands are paired structures located in the anterolateral regions of the tick's body. The salivary glands of female ixodid ticks consist of clusters of grape-shaped alveoli, called acini, which are categorized into three types (type I, II and III) (Šimo et al., 2017).

The salivary glands of ticks are innervated by branched peripheral nerves originating from the synganglion (Sy), which represents the central nervous system of these arthropods. This organ performs several essential functions in ticks, including movement control and stimulus processing (Rispe et al., 2022). The synganglion is located in the ventro-anterior region, posterior to the midgut stomach, and is composed of multiple ganglia (Obenchain, 1974). The synganglion is a translucent, heart-shaped structure linked to a multitude of peripheral nerves (Pn) that innervate various parts of the tick body (Sonenshine and Simo, 2021).

Dissecting the digestive and the nervous system: salivary glands and synganglion removal

Two dissection forceps were employed to collect the salivary glands. One forceps grasped the principal salivary duct at the base of the chelicerae at the anterior upper end, while the other gently pulled out the salivary glands. As with the midgut, the salivary glands were rinsed in three baths of sterile PBS prior to collection and cleared of any remaining tracheae or connective tissue (Fig. 2C). The same procedure was applied to remove the salivary gland on the opposite side. The size of the salivary glands varies with the feeding status of the ticks, becoming larger in half-fed and fully-fed ticks. The glands could be rinsed in three pools of PBS on either a Petri dish or a sterile glass slide, using aseptic dissecting forceps.

Once the salivary glands have been removed, the heart became visible especially in nymphs (Fig. 4C). As part of an open circulatory system, the pumping action of the heart and the arterial vessels facilitates circulation within the hemocoel (Binnington and Obenchain, 1982). This organ appears pentagonal during systole and more spherical during diastole (Cossio-Bayugar et al., 2023). The heart (H) lies in the dorsal portion along the mid-sagittal axis and is connected anteriorly with the aorta, and leads to the synganglion (Fig. 4C). Given the fragility of the synganglion, its removal requires precision. One forceps can be used to grasp the whitish neuronal extensions located on its lateral side.

Dissecting the reproductive organs: testes and ovaries removal

Following the removal of the synganglion, the tick's reproductive system, particularly in females, becomes more apparent (Fig. 5). It is important to note that the reproductive system only develops at the adult stasis. Consequently, the nymph lacks both reproductive organs and a gonopore, which are essential for the reproduction (Fig. 4C, 4D).

The male reproductive system includes the genital opening (gonopore), testes (T), vasa deferentia (Vd), ejaculatory duct, seminal vesicle (Sv), and accessory gland complex (Arg). The male gonopore is located in the ventroanterior region of the body, ventral to the synganglion, and opposite the anal aperture (Sampieri et al., 2016). Mated male ticks are capable of excreting a sac-like spermatophore that protrudes from their genital opening and delivering it with their mouthparts to the female's gonopore (Fig. 10D)(Kiszewski et al., 2001).

The male posterior region is composed of a pair of tubular, elongated testes, positioned dorsolaterally within the opisthosoma. These are connected to the accessory reproductive glands by the vasa deferentia (Fig 6E, 6F)(Anholeto et al., 2015; Sampieri et al., 2014). The testes serve as the primary site of spermatogenesis, which begins during the nymphal stage. The appearance of tick testes of ticks

may vary slightly even within a single species, depending on the nutritional and reproductive status of the individual (Oliver and Brinton, 1972).

In metastriate ticks, the testes are linked by an extremely thin strand of filamentous tissue and taper into the vasa deferentia. The testes are positioned along both sides of the male body, dorsally to the synganglion and laterally to the rectal sac (Oliver, 1982). A comparison of *R. microplus* males and *A. variegatum* males reveals significant differences in the morphology of their testes. *A. variegatum* males possess testes that are thinner and less pronounced in the opisthosoma. The vasa deferentia represents the narrowing of the paired testes at their anterior ends, joining in the anterior region to form a single duct called the vas deferens or seminal vesicle.

As with the testes, the shape and the size of the vasa deferentia depend on the reproductive state of the tick and can vary from narrow, coiled tubes to enlarged, straight structures (Oliver, 1982). Histologically, the vasa deferentia are indistinguishable from the common vas deferens. The seminal vesicle serves as the point of connection between the vasa derentia and the ejaculatory duct.

The ejaculatory duct is a short, narrow tube located in the ventroanterior region, extending below the synganglion to the external gonopore (El Shoura, 1987). The male accessory reproductive glands (Arg) play critical roles in all stages of the reproductive biology of the mated female, from the sperm deposition to oviposition (Gillott, 2003). For instance, male accessory glands stimulate female engorgement and activate spermatozoa upon arrival at the female seminal receptacle (Reuben Kaufman, 2007). This multi-lobed complex is located ventrally to the opisthosoma and connects to the ejaculatory duct on its ventral face and to the vasa deferentia on the dorso-lateral region (Sampieri et al., 2014).

Dissection of males: testes

Since the internal anatomy of *A. variegatum* males is simpler than that of females, isolating the organs is relatively easier (Fig. 2A, 6B). After removing the trachea and the midgut, the reproductive system becomes exposed, particularly the testes, which occupy significant space (Fig. 6C, 6E). Using dissecting tweezers, the ejaculatory duct can be gently pulled out of the tick's body, bringing the entire reproductive system with it (Fig. 6F, 6G).

During mating, metastriate males insert only their chelicerae into the female's genital pore, leaving the hypostome and palps outside, barely in contact with the female's surface (Fig. 10A) (Kiszewski et al., 2001). This mechanism facilitates the transfer of the sac-like spermatophore that protrudes from the male genital opening (Fig. 10C, 10D). Similar to males, the female genital opening is located ventrally relative to the synganglion (Fig. 10A).

Dissecting females: Gené's organ and ovaries

As ticks are known to have a complex life cycle in the wild, some ixodid ticks exhibit high reproductive potential, with an oviposition capacity of up to 20,000 eggs. This strategy is believed to ensure the survival of their offspring in the wild. The female genital system comprises the gonopore, similar to that in male, ovaries (O), a common oviduct or uterus, a bipartite vagina divided into a cervical and vestibular vagina, and a seminal receptacle (Sr). Additionally, females possess two accessory glands: tubular and lobular.

The ixodid tick ovary is an elongated, U-shaped tube situated in the posterior region of the body, above the rectal sac (Fig. 3C, 5). The tick ovary is a hollow organ filled with oogonia and primary oocytes at various early stages of development. A fold or longitudinal groove extends along the antero-dorsal surface of the ovary , containing primary oocytes internally at their earliest stage of development, and more advanced oocytes externally (Fig 9C) (Diehl et al., 1982). During feeding, the female's oocytes tend to protrude into the hemocel, giving the ovary a grape-like shape (Fig. 8A).

At each end of the ovary are whitish, paired, and folded oviducts (Ovi), which converge to form a common oviduct or uterus (Fig. 3A, 9C). The oviduct tends to stretch considerably during the passage of the eggs due to peristaltic contractions of the oviduct walls (Fig. 7D). The uterus connects to the cervical vagina via a short tube.

The vagina consists of a posterior, broad, and short cervical region, as well as an anterior, narrow, and long vestibular region (El Shoura, 1989). In addition to the uterus, the seminal receptacle, tubular accessory glands and the vestibular vagina converge in the cervical vagina (Fig. 9B) (Vega et al., 2012). The vestibular vagina extends from the gonopore to the cervical vagina and opens externally to the

gonopore via the vulva. In the female *A. variegatum*, the vagina situated in the podosoma is more complex to observe.

The seminal receptacle is a sac-like extension of the cervical region that serves as the storage site for spermatozoa after mating, facilitating the fertilization of eggs (Oliver, 1989). Final stages of spermiogenesis occur in the seminal receptacle of copulated females until full engorgement (Fig. 10C) (Brinton et al., 1974). This structure is positioned above the uterus and opens postero-dorsally into the cervical vagina (Diehl et al., 1982).

The Gené's organ is unique to female ticks. It secretes a waxy substance that covers the oviposited eggs, protecting them from the environment. A peculiarity of this organ is its ability to evert itself from the camerostomal aperture, located between the posterior base of the capitulum and the anterior region of the scutum. Internally, this organ is situated in the anterior body region, below the capitulum, and consists of branched tubular glands that are highly developed in the engorged females (Fig. 9A).

The tubular (Tag) and lobular accessory glands are associated with the Gené's organ and also contribute to the egg waterproofing during the oviposition period of the female. The tubular glands form the bulk of the Gené's organ and are located anteriorly within the hemocoel, in continuity with the horns (Fig. 9B). The paired tubular accessory glands open into the vagina at the dorsal boundaries of the cervical and the vestibular regions (Booth, 1985). During oviposition, the lumen of each gland fills with a secretion that coats the egg surface as it passes through the vagina (Diehl et al., 1982).

The lobular accessory gland is a distinctive feature of ixodid ticks. This gland results from the maturation of the vestibular vaginal epithelium as the entire female reproductive system develops. Two porose areas are located dorsally on the female basis capituli, below the rostrum (Fig. 1A). During oviposition, these areas are covered by the Gené's organ. The porose areas secrete a substance that is incorporated into the waterproofed wax of the organ of Gené as it everts from the camerostomal fold.

The tracheal system of *A. variegatum* females is much denser than that of the males, making its removal more complex (Fig. 2A, 6B). During dissection, with the ventral surface of the tick facing downward, the Gené's organ is located dorsally, above to the synganglion, while the female reproductive apparatus is positioned ventrally to the synganglion. For better access to Gené's organ, the capitulum can be removed with a surgical blade or scissors (Fig. 9A).

An incision should be made horizontally beneath the basis capitulum, just above the camerostome, to avoid damaging the organ. Once the connective tissue and residual tracheae are cleared from the tick's internal cavity, the Gené's organ becomes visible. Using dissecting forceps, the Gené's organ can be extracted from the camerostomal opening by firmly grasping it. It should be noted that the tubular accessory glands of the Gené's organ do not fully develop until the female has completed a blood meal (Fig. 9A, 9B). Following the removal of the trachea and the midgut, the oviducts leading to the ovary in the posterior region, and the vagina, located ventrally to the synganglion, should become visible.

The vagina was then grasped with dissecting forceps, and the entire female reproductive apparatus was carefully removed from the internal cavity (Fig. 9C). This apparatus includes the bipartite vagina, seminal receptacle, uterus, accessory glands, oviducts, and ovary. It should be noted that these components have not been differentiated.

Conclusion

Collecting tick organs is crucial for understanding vector-pathogen interactions *in vivo.* Here, we describe *A. variegatum* anatomy and how to collect several organs of interest, especially the tick midgut, salivary glands, and ovaries, which play crucial role in tick vector competence. Dissection methods are a reliable approach that requires sufficient dissecting skills to enable the efficient observation and harvesting of organs without tissue degradation and subsequent contamination. Given the intricate internal structure of ticks, special care must be taken when dissecting tissues, especially if they are intended for subsequent analysis. The collection of hemolymph, the fluid that fills the tick's hemocoel, may be particularly relevant because of its hemocyte composition and interest of these cells. These components are of fundamental importance to understanding how bacteria can adapt within the tick compartment. As demonstrated in the case of *A. americanum*, the complete depletion of granulocytic

468 hemocytes resulted in the death of the tick following an *Ehrlichia chaffeensis* infection (Adegoke et al.,
469 2023).
470 Further studies will then be conducted on these specific tissues to elucidate the molecular and
471 cellular processes implemented by the tick in response to the pathogen, at both biological and
472 transcriptomic levels. Performing sex-specific dissections may facilitate more precise differentiation
473 among ticks, particularly in the case of engorged females. Given the established role of male salivary
474 glands in the reproductive process, involving the secretion and subsequent lubrification of the
475 spermatophore, it can be hypothesized that female and male ticks may employ divergent strategies in
476 their developmental processes (Tan et al., 2015). Prior research has indicated that tick sex exert a
477 considerable influence on their microbiome. In ecology, the bacterial richness and species diversity
478 present within a single sample are collectively referred to as "alpha diversity" (Andermann et al., 2022).
479 As demonstrated in the case of *Dermacentor variabilis* male ticks, which exhibit higher alpha diversity
480 measures than females. This phenomenon may be attributed to the reduced quantity of blood ingested
481 by males during feeding and their capacity to feed multiple times on a host, which establish a more
482 diverse bacterial community (Duncan et al., 2022). *Amblyomma variegatum*, the tropical bont tick, has
483 received comparatively less attention in scientific studies than the cattle tick, *Rhipicephalus microplus*.
484 This study represents a significant contribution to the field, as it provides the first microscopic
485 observations and a general description of the internal anatomy of *A. variegatum*.
486
487

488 **Figure 1:** Anatomical description of the dorsal surface of unfed adult *A. variegatum* ticks mounted on
489 double-sided adhesive tape. **(A)** Dorsal view of unfed *A. variegatum* female (0.78x). **(B)** Dorsal view of
490 unfed *A. variegatum* male, with its first leg cut to allow hemolymph collection (0.78x).

491 **Figure 2:** Dorsal view of an unfed *A. variegatum* female and its organs. **(A)** Scutum dissection: dorsal
492 view of an unfed *A. variegatum* female with the scutum removed and immersed in PBS (1.6x). **(B)** Midgut
493 dissection: close up view of the gut of an unfed *A. variegatum* female (1.25x). **(C)** Salivary glands
494 dissection: close-up view of the salivary glands of an unfed *A. variegatum* female (3.2x). Ac: acini ; Div:
495 diverticulum ; Sac: <u>scutum</u>/ alloscutum/conscutum (in the female tick) ; Sd: salivary duct ; Sg: salivary
496 glands ; St: stomach ; Tr: tracheae.
497
498 **Figure 3:** Dorsal view of an unfed *A. variegatum* female with the midgut removed and organs. **(A)**
499 Midgut dissection: dorsal view of unfed an *A. variegatum* female with scutum removed and immersed in
500 PBS (1.6x). **(B)** Synganglion dissection: close-up view of the nervous system of unfed an *A. variegatum*
501 female (5x). **(C)** Reproductive system dissection: close-up of the ovary of an unfed *A. variegatum* female
502 (5x). O: ovary ; Ovi: oviducts ; Pn: peripheral nerves ; Sac: <u>scutum</u>/ alloscutum/conscutum (here, in the
503 female tick) ; Sy: synganglion ; Sg: salivary glands ; Tr: tracheae.
504
505 **Figure 4:** Dorsal view of an unfed *A. variegatum* nymph. **(A)** Dissection of the scutum: dorsal view of
506 an unfed *A. variegatum* nymph immersed in PBS, showing a clean incision (yellow dotted line) around the
507 idiosoma (2.5x). **(B)** Removal of the scutum: dorsal view of an unfed *A. variegatum* nymph immersed in
508 PBS, with the conscutum lifted using forceps (2.5x). **(C)** Dissection of the midgut: *A. variegatum* nymph
509 with the entire gut removed and the conscutum cut off (3.2x). **(D)** Unfed *A. variegatum* nymph with the
510 salivary glands, midgut, tracheae and Malpighian tubules removed (4x). Div: diverticulum ; H: heart ; Mt:
511 Malpighian tubules ; Rs: rectal sac ; Sac: <u>scutum/ alloscutum</u>/conscutum (here, in the nymph tick) ; Sy:
512 synganglion ; pink circle : hematin granules released after gut tear ; black triangle: anal aperture.
513
514 **Figure 5:** Dorsal view of an unfed *A. variegatum* female with the midgut, and salivary glands removed
515 (2x). Div: diverticulum ; O: ovary ; Ovi: oviducts ; Rs: rectal sac ; Sp: spiracular plates ; Tr: tracheae.
516
517 **Figure 6:** Dorsal view of a fed and mated *A. variegatum* male. **(A)** Dorsal view of a fully repleted *A.*
518 *variegatum* male with a rounded conscutum (1.25x). **(B)** Scutum dissection: dorsal view of a fed *A.*
519 *variegatum* male with folded conscutum, immersed in PBS (1.6x). **(C)** Midgut dissection: *A. variegatum*

male with the entire gut removed (1,6x). **(D)** Close up of the excretory system (2.5x). **(E)** Salivary glands dissection: *A. variegatum* male with the paired salivary glands removed (2.5x). **(F, G)** Reproductive organs dissection: highlighting the organs of the male reproductive system (1.6x, 2.5x). Arg: accessory reproductive glands ; Ct: connective tissue ; Div: diverticulum ; Hin: hindgut ; Mro: male reproductive organs excluding testes ; Mt: Malpighian tubules ; Rs: rectal sac ; Sac: scutum/ alloscutum/conscutum (in the male tick) ; Sg: salivary glands ; St: stomach ; Sv: seminal vesicle ; Sy: synganglion ; T: testes ; Tr: tracheae ; Vd: vasa deferentia.

Figure 7. View of a fully repleted *A. variegatum* female. **(A)** Dorsal view of a fully engorged *A. variegatum* female (0.78x). **(B)** Dorsal view of an ovipositing *A. variegatum* female with scutum removed (0.78x). **(C)** Dorsal view of *A. variegatum* with thousands of eggs expelled from the oviducts (0.78x). **(D)** Close up of oviducts full of eggs (0.78x). Cp: capitulum ; Div: diverticulum ; E: eggs ; Ooc: oocytes ; O: ovary ; Tr: tracheae.

Figure 8: Dorsal view of a partially fed *A. variegatum* female with the midgut removed and organs. **(A)** Dorsal view of a semi-engorged female *A. variegatum* female with the midgut removed (1x). **(B)** Close-up of the midgut of a partially fed female *A. variegatum* female (0.78x). Mt: Malpighian tubules ; O: ovary ; Sg: salivary glands ; Sp: spiracular plates.

Figure 9: Dorsal view of a partially fed *A. variegatum* female with the midgut, and salivary glands removed, showing reproductive organs. **(A)** Dorsal view of a female *A. variegatum* with a close-up of reproductive system (2x). **(B)** Close up of reproductive organs with accessory glands (2x). **(C)** Close-up of ovary with oocytes (1,6x). Ch: chelicerae ; Fro: female reproductive organs excluding ovary and oviducts ; Go: Gené's organ ; Mt: Malpighian tubules ; Ooc : oocytes ; Sy: synganglion ; Tg: tubular glands ; Tr: tracheae.

Figure 10. View of a mated *A. variegatum* female. **(A)** Ventral view of a semi-engorged *A. variegatum* female with an ectospermatophore attached near to its gonopore shortly after copulation (0.78x). **(B)** Dorsal view of a partially fed *A. variegatum* female with a round alloscutum (0.78x). **(C)** Dorsal view of an endospermatophore contained in the seminal receptacle of a semi-engorged *A. variegatum* female (2.5x). **(D)** Close-up of the endospermatophore dissected a few minutes after the female mated with a male (2.5x). Cc: cement cone ; O: ovary ; Ooc: oocytes ; Rs: rectal sac ; Sac: scutum/ alloscutum/conscutum (in the female tick) ; Sr: seminal receptacle ; Sp: spiracular plates ; Tr: tracheae ; black triangle: anal pore ; pink triangle: gonopore ; red triangle: ectospermatophore.

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Conflict of interest disclosure

The authors declare that they have no financial conflicts of interest in relation to the content of the article.

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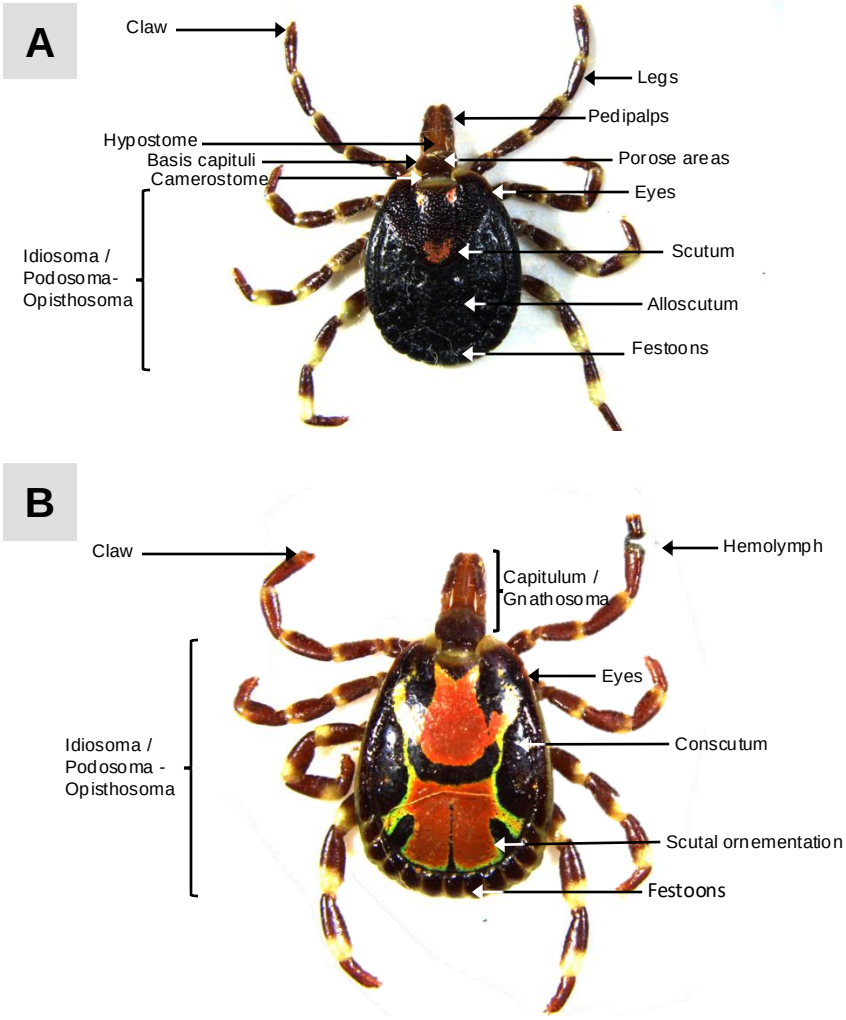
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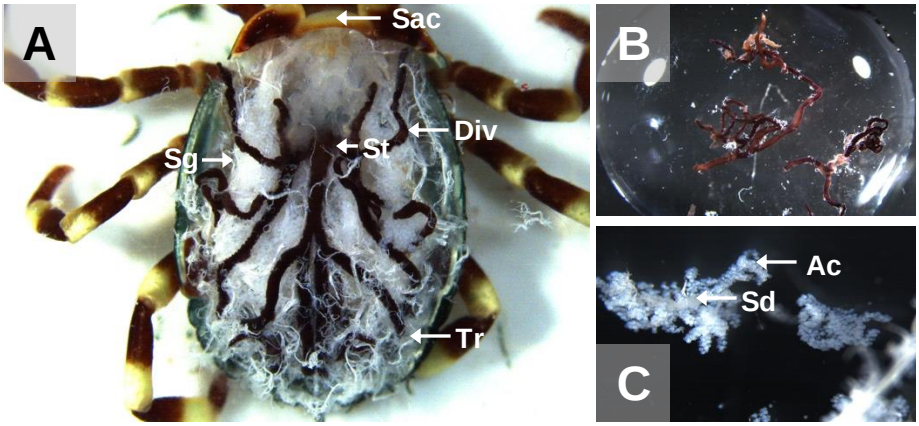
Figure 1.



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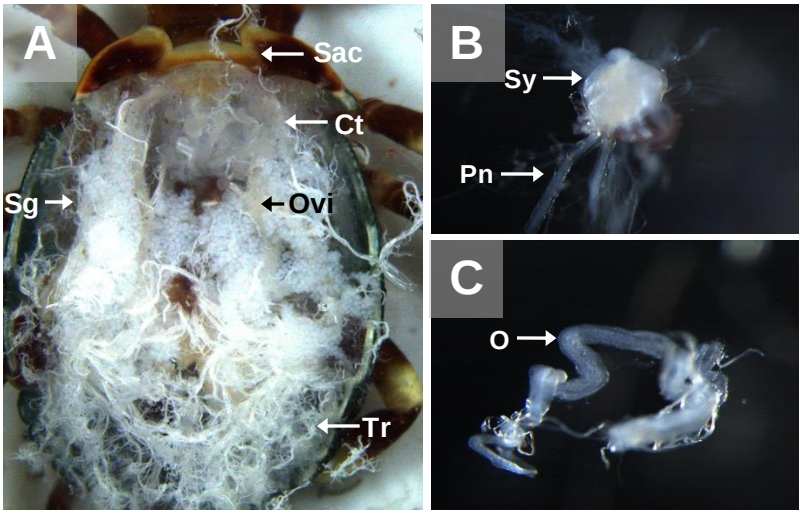

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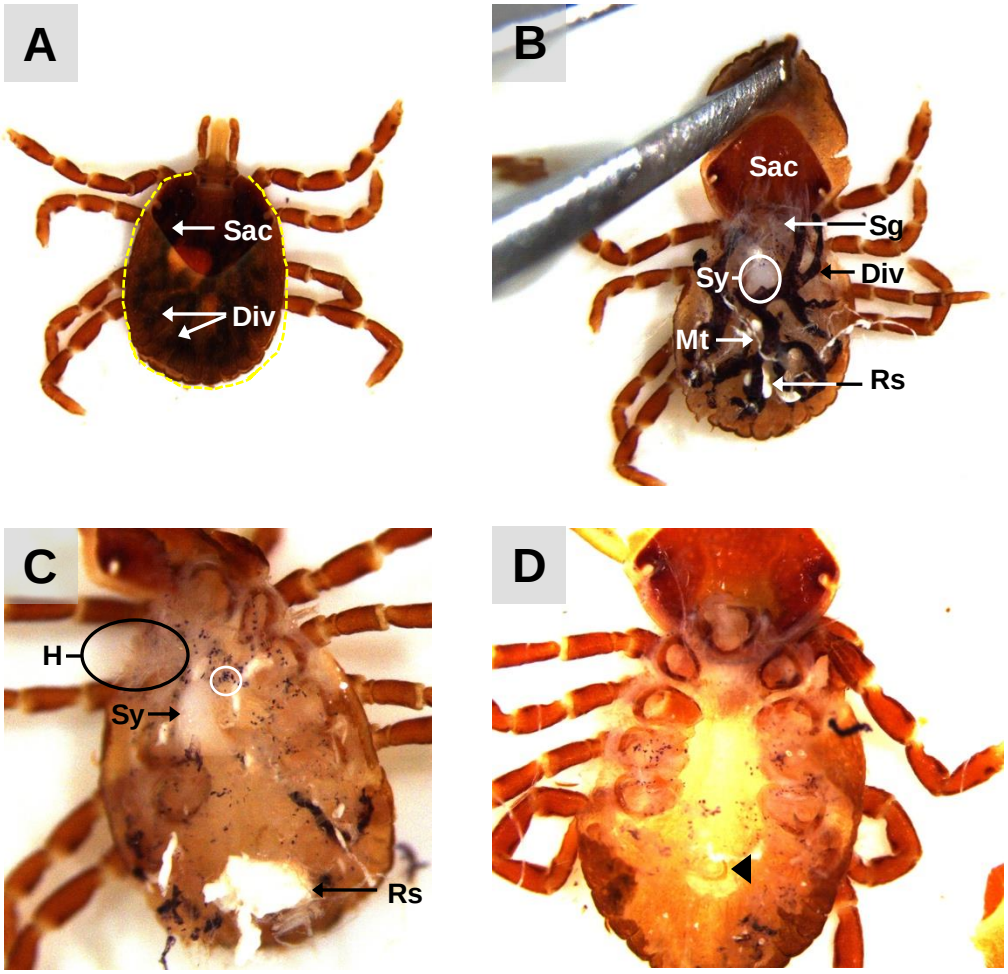

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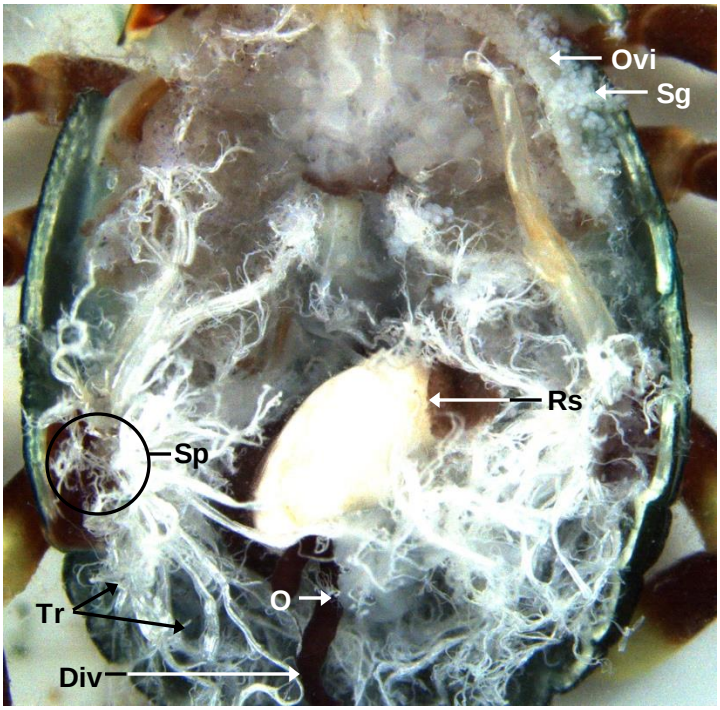

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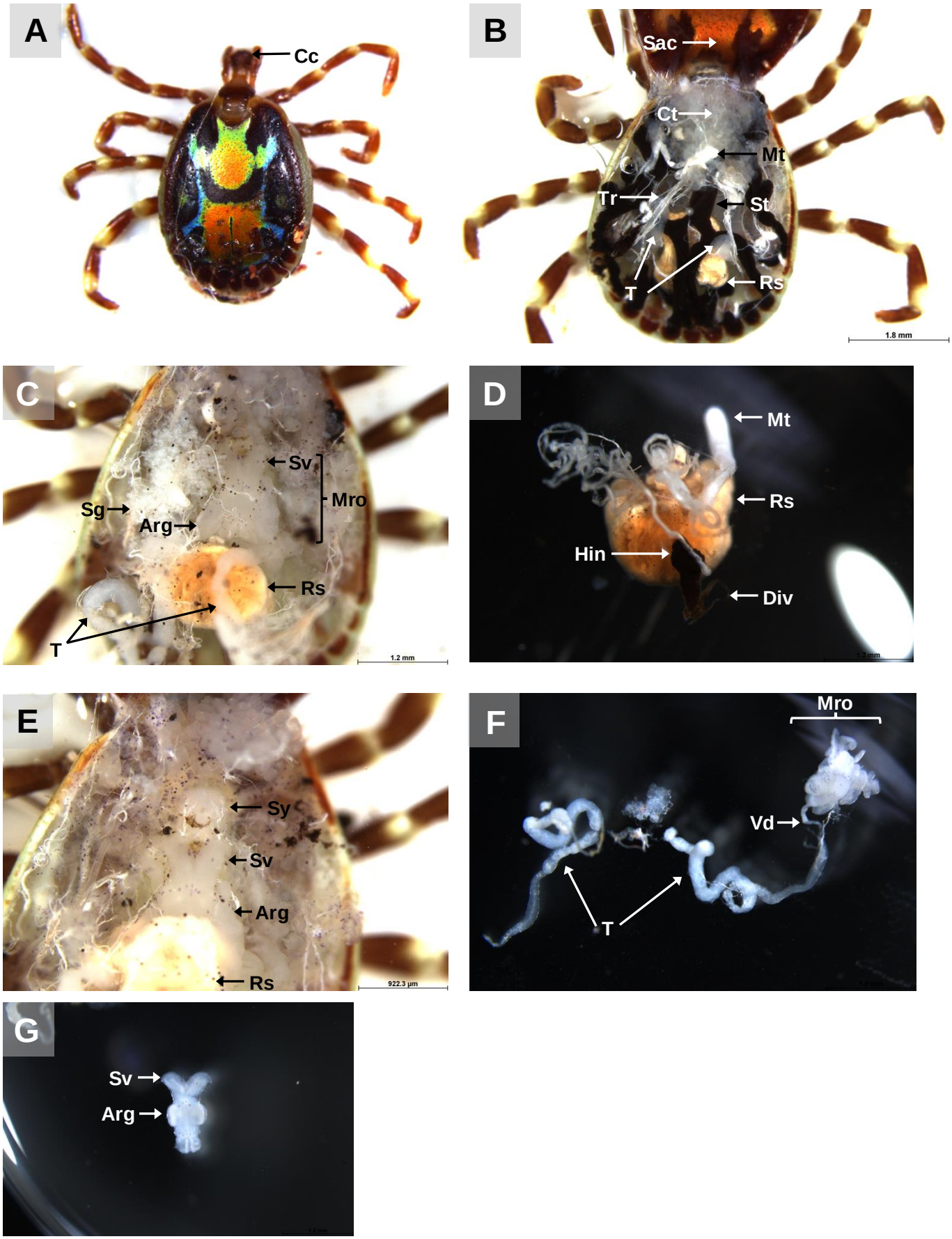
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Sy
Sv
Arg
Rs
922.3 µm
F
Mro
Vd
T
G
Sv
Arg

Figure 6.

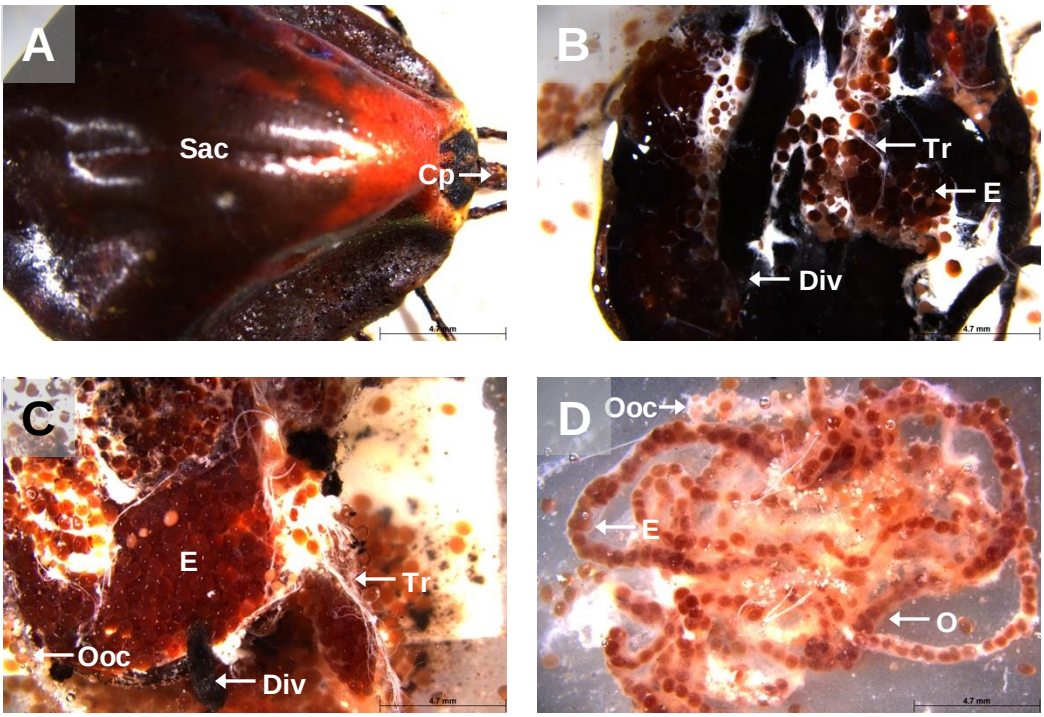

Figure 7.

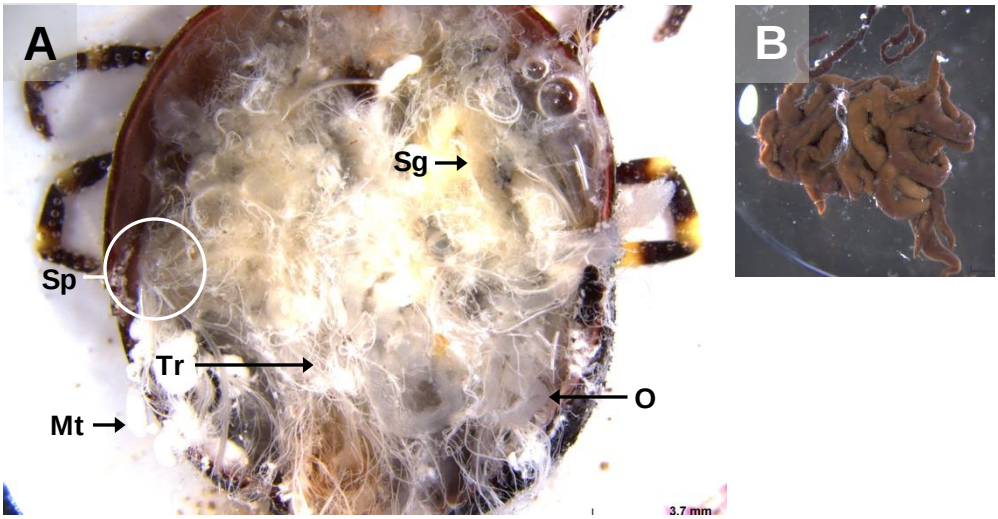

Figure 8.

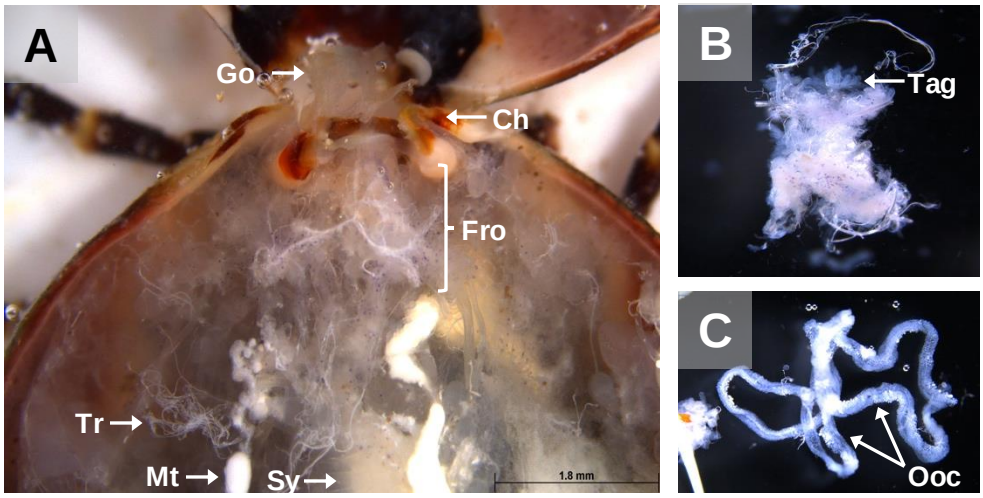

Figure 9.

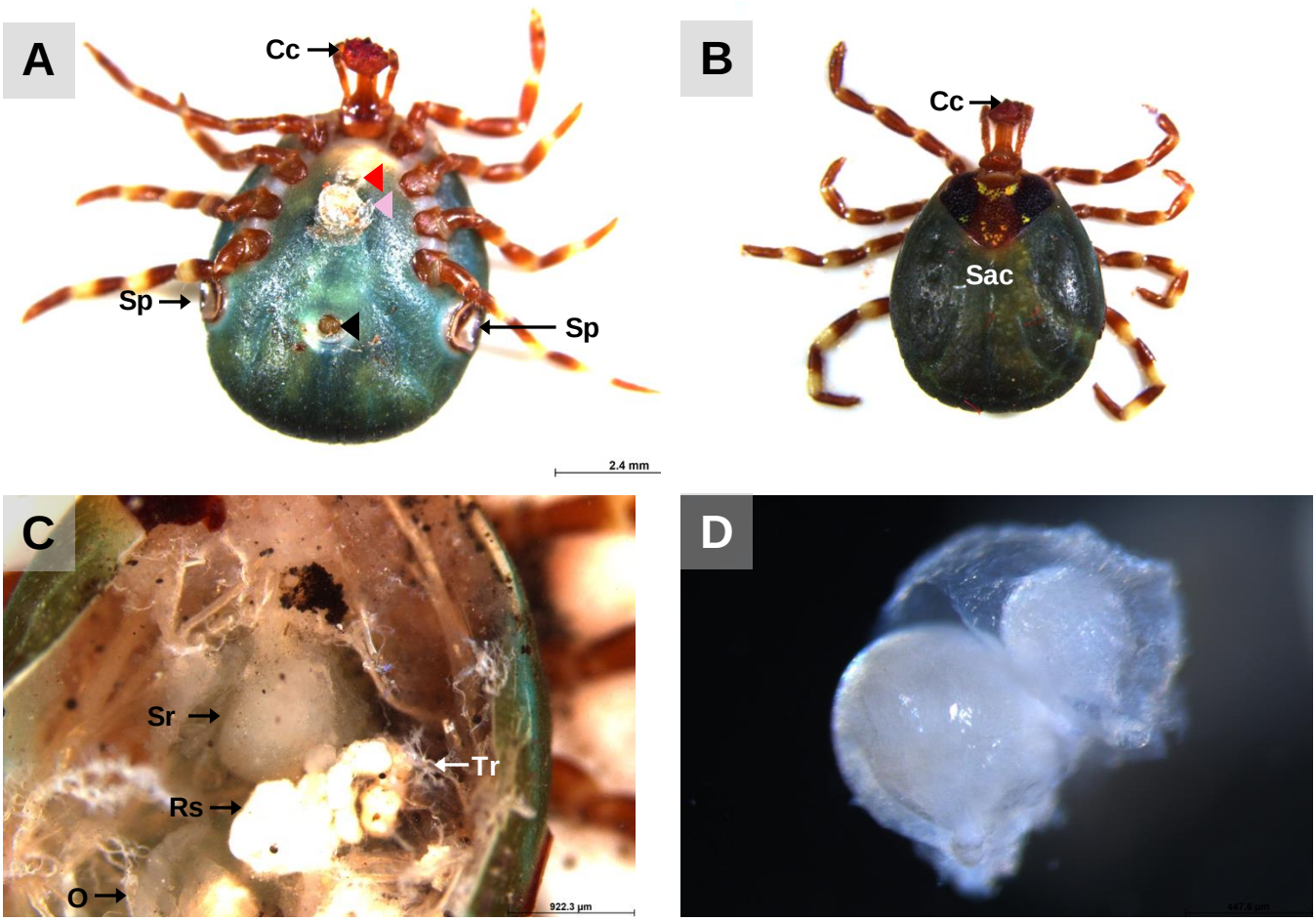

Figure 10.