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# Functional Anatomy of the External and Internal Reproductive Structures in Insect Vectors of Chagas Disease with Particular Reference to *Rhodnius prolixus*

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Additional information is available at the end of the chapter

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## Abstract

The insect vector of Chagas disease, *Rhodnius prolixus*, has become a very popular model organism for exploring, among other things, the physiology of insects. Its ability to remain in a state of stasis until after engorging a blood meal has focussed most studies on those physiological and developmental processes triggered by the blood meal leaving the details of its sexual physiology vague. This chapter summarizes the relationship between the male and female by describing their respective reproductive systems and genitalia, and how they function during and after copulation. A number of novel processes are noted, such as the transfer of male secretions without the formation of a spermatophore, pump/valve mechanism in the male aedeagus, sensory and a chemical means by which copulation may be facilitated, and the possible mechanism by which adhesive protein is applied to an egg during ovipositioning. Combined with knowledge of its genome, further studies into the functional anatomy of reproduction in this insect have the potential to increase our understanding of sexual reproduction in Reduviidae bugs, and to suggest new ways to control their population growth and the spread of Chagas disease.

**Keywords:** *Rhodnius prolixus*, sexual physiology, male genitalia, female genitalia, copulation, Chagas disease, Reduviidae, Triatominae, aedeagus, spermatophore, accessory reproductive glands

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## 1. Introduction

This chapter describes the anatomy and physiology of internal and external reproductive structures in Reduviidae bugs, the blood-feeding insect vectors of Chagas disease. Chagas disease is endemic to Central and South America, and is also known as American trypanosomiasis [1]. The

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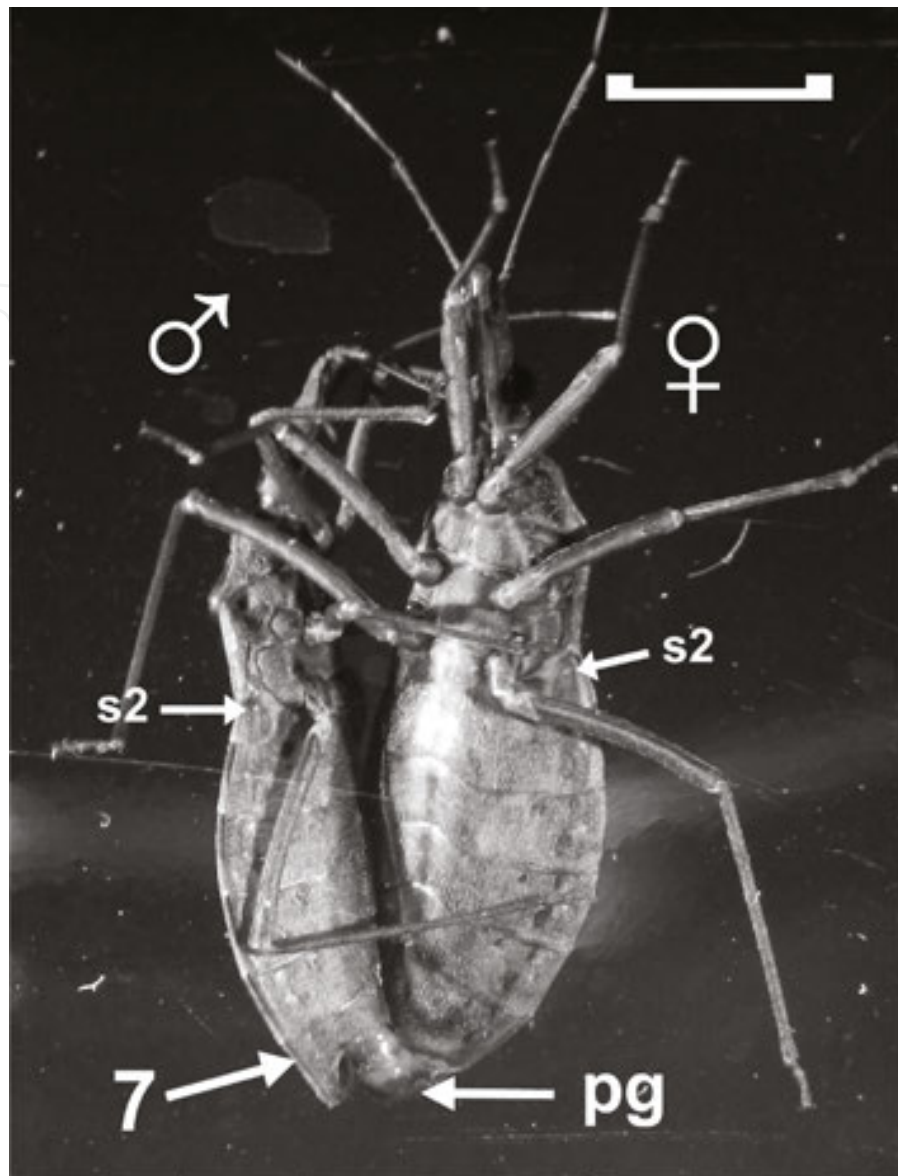
disease is caused by a protozoan parasite, *Trypanosoma cruzi*, which completes one part of its life cycle in the digestive system of the Reduviidae bug, and the other part in the tissues of warm-blooded animals. Animal tissues are infected by the amastigote stage of this flagellate, which multiplies by binary fission, and transforms into trypomastigotes. These trypomastigotes burst from the cell, and enter the blood stream to infect other cells within the host. The trypomastigote is also the stage ingested by the insect during a blood meal. Once in the insect midgut, the trypomastigote transforms into the epimastigote and proliferates. The epimastigotes then enter the hindgut and transform into the metacyclic trypomastigote. When the infected insect takes its next blood meal it also defecates leaving the metacyclic trypomastigote, once in its hindgut, on the host. The metacyclic trypomastigote can enter the blood stream directly through the bite or through mucous membranes to find tissues to infect, and the cycle starts over again. Additionally, the mode of host infection may occur by ingesting food contaminated by the infected faeces of these bugs [2].

The causative agent of Chagas disease and its mode of transmission by Reduviidae bugs was discovered by Carlos Chagas in 1909 [3]. Although transmitted by several species of Reduviidae, one species, *Rhodnius prolixus*, has become the single most significant insect for advancing our understanding of insect physiology. Indeed, outside the endemic regions of the world, *R. prolixus* is known for being an ideal insect model for studying insect physiology rather than for its role in spreading Chagas disease. The events leading to this status include the discovery by Vincent B. Wigglesworth (1899–1994) that *R. prolixus* is able to survive considerable experimental manipulation, and will remain in a state of physiological stasis until it ingests a blood meal of sufficient size [4, 5]. These factors combined with the ease of raising them in the laboratory, has made *R. prolixus* a popular insect model in teaching and research.

In his studies on insect physiology, for which he was knighted in 1964, Wigglesworth concentrated on growth, development and metamorphosis in *R. prolixus* [6–8]. Other researchers have examined physiological processes associated with reproduction [9, 10], feeding [11–14], digestion [15, 16] and behaviour [17]. A wealth of knowledge has been gained in close to 100 years of research on this species and with the recent completion of the *Rhodnius* genome project [18], research on this bug should continue long into the future. To help set the stage on which to explore more fully the functional anatomy of both the external and internal features of the reproductive system, this chapter summarizes and clarifies our current understanding of the mechanics of egg-laying, copulation and the formation of the spermatophore. This work is specific to *R. prolixus*, but as noted with a few comparisons, it appears to be applicable to the Reduviidae as a whole.

## 2. Overall design of the adult abdomen

The adult abdomen in *R. prolixus* is flattened dorso-ventrally and in both the male and female, it is divided into seven full-sized segments on the dorsal side. On the ventral side, the female has six recognizable full size segments and the male has five. This number differs because of the way the first three ventral segments merge. Although there are a different number of



**Figure 1.** Ventral view of a copulating pair of *Rhodnius prolixus* seen through the side of a glass jar. Male is to left in picture holding onto the female and the female is standing on the glass surface. s2, second abdominal spiracle; 7, seventh full-sized abdominal segment; pg, posterior genital segment of male. The aedeagus, the male penis, extends from the pg into the female during copulation. Genitalia in both sexes are attached to the seventh full-sized abdominal segment. Scale bar: 5 mm.

full-sized dorsal and ventral segments, the corresponding tergal and sternal plates can be recognized in the lateral view and identified by the spiracle number they are associated with (see **Figure 1**). There are at least eight sets of abdominal spiracles and the first and the eighth pairs are not visible along the ventral lateral side of the abdomen. In both sexes, the first abdominal spiracle appears laterally on a sliver of cuticle on the dorsal side of the abdomen close to the thorax, and is partially covered by the first full-sized dorsal abdominal segment, whereas the eighth pair of spiracles is located on the ventral genital segment and becomes exposed when these segments are extended. By numbering the abdominal segments according to the spiracle they are associated with, the first full-sized abdominal dorsal segment is two

and the last is seven (refer to **Figure 1**). The genitalia in both sexes are specializations at the end of the abdomen and are attached to full-sized abdominal segment seven. In morphological studies that compare different species of insects, the segments of the genitalia are numbered according to their relationship to other very diverse species (for example, see Ref. [19]). For this chapter, which focuses on the functional anatomy in one particular species, the genital segments are referred to according to their position within the genitalia.

### 3. The female reproductive system

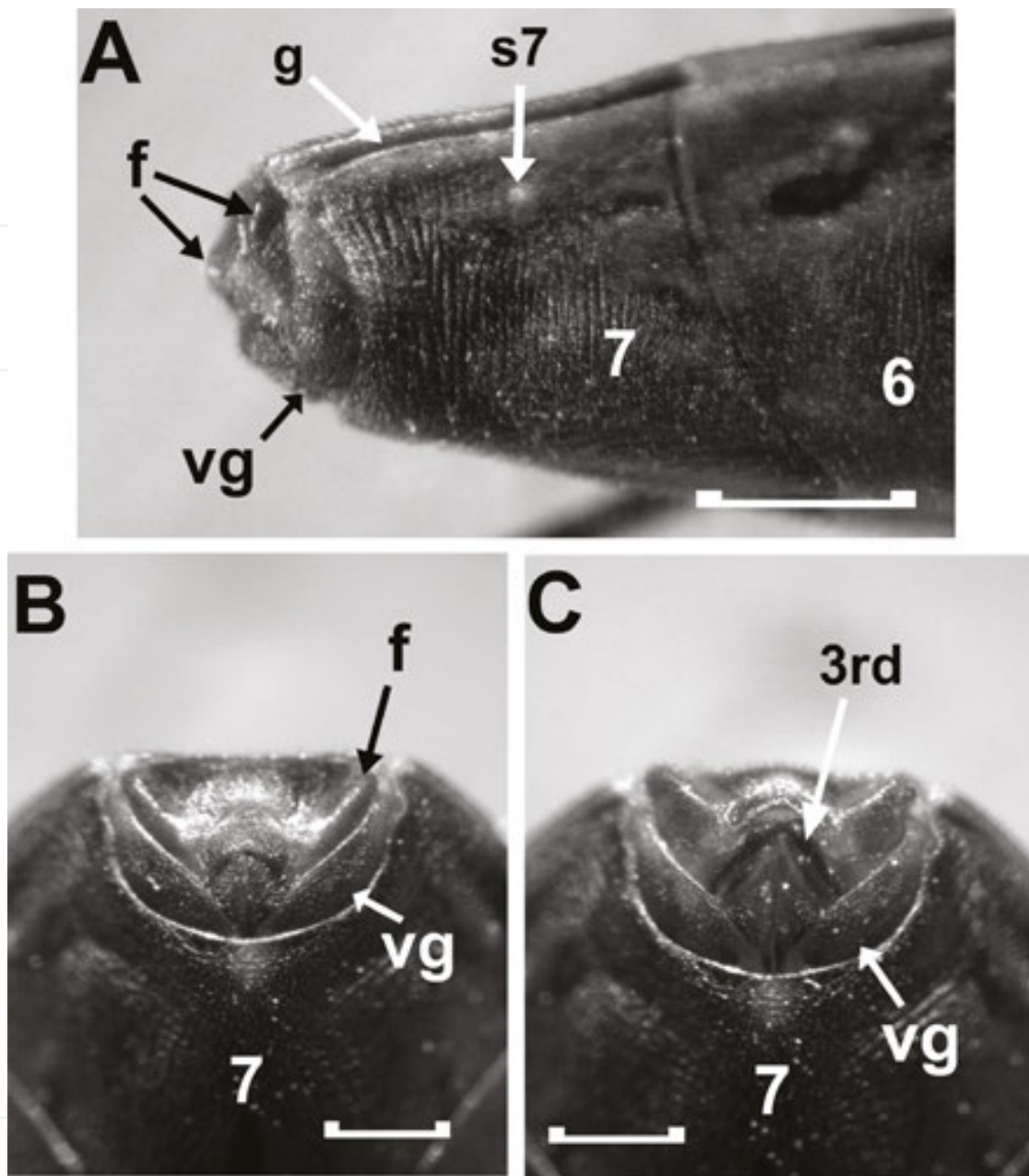
#### 3.1. The dorsal and ventral genital segments of the female

The genitalia of the female are attached to abdominal segment seven, and are equipped with a single dorsal sclerite and a pair of ventral sclerites (see **Figure 2**). When pulled in towards the rear of the animal, these genital sclerites cover the sclerites that surround the opening to the genital chamber. The genital chamber in *R. prolixus* can be referred to as either the bursa copulatrix [20, 21] or the vagina [22, 23]. In keeping with its functional role, this chapter refers to the genital chamber as the vagina.

The dorsal genital sclerite is hinged on the posterior edge of full-size abdominal segment seven (**Figure 2A**), and narrows towards its posterior tip to take on a triangular shape. In its retracted position, it sits under the animal extending ventro-anteriorly (**Figure 2B**). It has symmetrically arranged lateral flaps (**Figure 2A**) to which the male can attach his parameres when this genital sclerite is extended during copulation. Its posterior tip has a prominent medial ridge that overlaps sclerites ventral to it when the vulva, the external opening to the vagina, is closed. When the genital segments are relaxed as a result of decapitation of the female, the third valvula becomes visible (**Figure 2C**). A slender branch of cuticle connects the lateral edge of the dorsal genital sclerite to the base of the second valvula which is one of the three pairs of sclerites associated with the vulva.

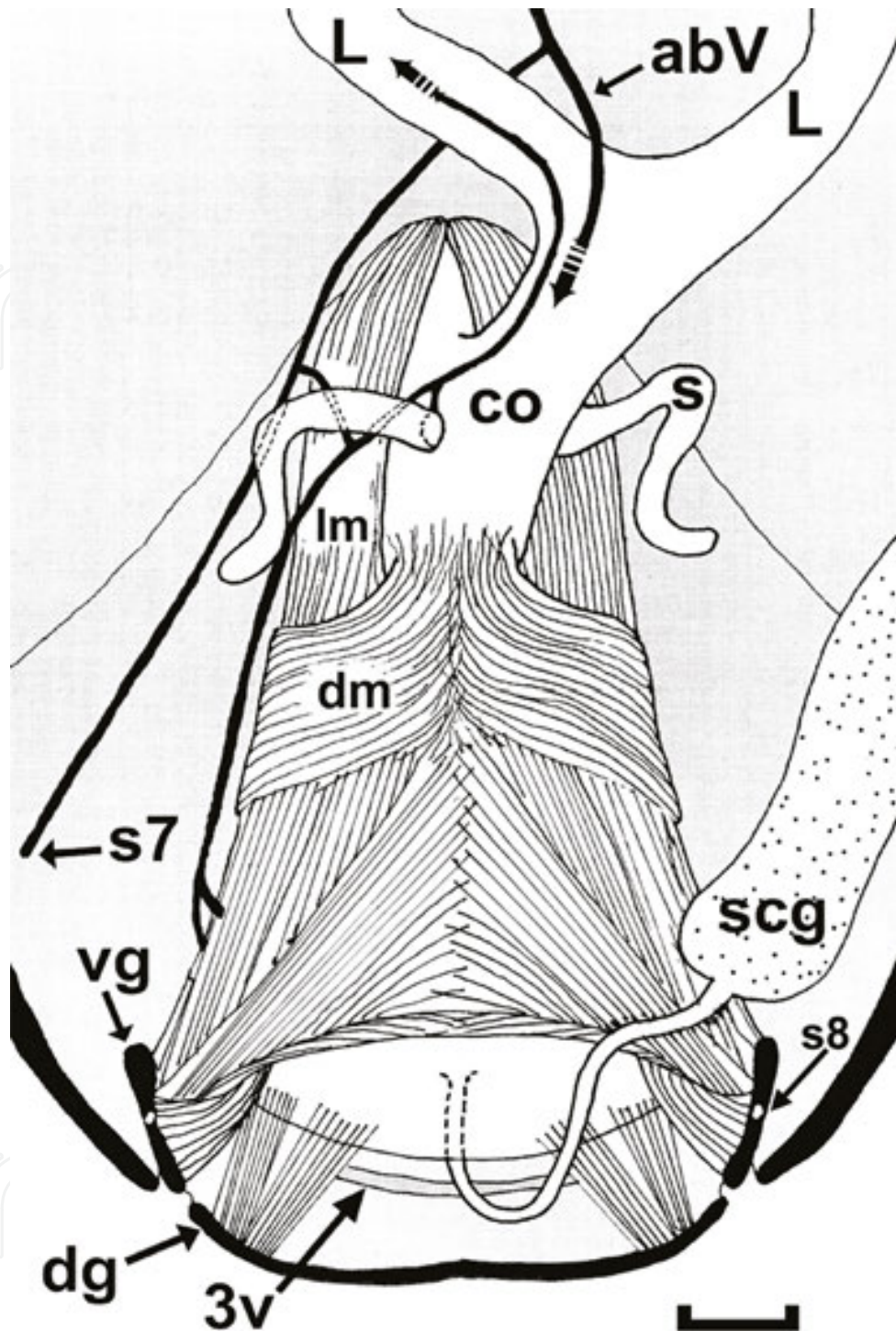
On its interior side, the dorsal genital sclerite has a pair of apodemes with each member of the pair located between the midline and the right or left side of the sclerite. Anchored to these apodemes are a pair of bilaterally symmetrical muscle bundles which fan out a short distance anteriorly to attach to the posterior lining of the vagina. Contractions of these muscles pull the dorsal genital sclerite anteriorly onto the underside of the animal to close off the vulva and the anus. Relaxation of these muscles allows the dorsal genital sclerite to extend exposing the anus during defaecation, or the vulva during copulation, egg-laying or the expulsion of the male secretions after copulation.

While the dorsal genital sclerite covers the dorsal to lateral sides of the rear of the abdomen, the ventral to lateral sides are covered by a pair of ventral genital sclerites. The relationship of these ventral sclerites to the ventral side of abdominal segment 7 is governed by the shape of the abdomen in cross section. Whereas the dorsal abdominal surface is flat, the ventral portion forms a deep trough. The anterior part of each pair of ventral genital sclerites sits in this trough so that they lay over part of the interior side of abdominal segment 7. The eighth abdominal spiracle is located on the ventral genital sclerite, but not on its outer nor inner surface. Instead,



**Figure 2.** External female genitalia of an adult *Rhodnius prolixus*. (A) Lateral view showing the genitalia attached to the full-size abdominal segment seven (7) identified by the seventh spiracle (s7). 6, full-sized abdominal segment 6; f, the lateral flaps on the single dorsal genital segment; vg, the ventral genital segment consisting of a pair of bilaterally symmetrical sclerites; g, the lateral abdominal groove characteristic of adult *Rhodnius*. Scale bar: 2.5 mm. (B) Dorsal and ventral genital segments held close to the body closing off the anus and entry to the genital chamber. Scale bar: 1.0 mm. (C) Dorsal and ventral genital segments are partially relaxed after decapitating the animal, and the third valvula (3rd) becomes visible. Scale bar: 1.0 mm.

it sits approximately midway along its lateral edge (see **Figure 3**), and this edge becomes exposed to the outside when the ventral genital segments are extended out of abdominal segment 7 to open the vagina. The ventral genital segment is attached to the inside of the ventral side of abdominal segment 7 by at least four sets of skeletal muscles. These muscles, which have yet to be fully documented, provide the female with considerable control of the



**Figure 3.** Line diagram of a dorsal view of the lower reproductive system in a female adult of *Rhodnius prolixus* as revealed by methylene blue staining and electrophysiology. abV, left branch of abdominal nerve 5 which supplies the genitalia. Hashed arrows denote the course of the nerves not included in this diagram. s7, is the seventh abdominal spiracle which is innervated by a nerve which branches off abV and travels under the lateral oviduct (L); s8, the eighth spiracle located on the edge of the ventral genital segment (vg); co, common oviduct to which the spermathecae (s) are attached; lm, longitudinal vagina muscles; dm, dorsal vagina muscles; scg, secretory portion of the cement gland whose excretory duct is attached to the inside of the vagina on the dorsal side of the vulva; dg, dorsal genital segment; 3v, the base of the third valvula which is attached to the lining of the vagina on the dorsal side of the vulva. Scale bar: 0.5 mm. (Adapted with permission from Ref. [22]).

sclerites of the ventral genital segment, a control that would be exercised during copulation and ovipositioning.

On the side facing the vagina, the ventral genital sclerites are directly attached to the bulk of the muscles that overlie the vagina (**Figure 3**). The muscle bundles fan out in three different directions and become intertwined as they proceed over the vagina. The most anteriorly attached muscle bundles extend anteriorly along the ipsilateral side of the vagina, past the common oviduct, to attach to the posterior medial edge of abdominal segment 7 where the muscle bundles associated with the dorsal genital sclerite also attach. The muscle bundles attached to the mid anterior region of the ventral genital sclerite form a distinct twisting pattern, and extend directly across the body over the posterior end of the vagina to the contralateral ventral genital sclerite. The more posteriorly attached muscle bundle extends anteriorly and contralaterally travelling across the top of the vagina around the contralateral side of the common oviduct to attach to the medial posterior edge of abdominal segment 7. The interwoven nature of the muscle fibres and the diagonal pattern assumed by many of them would help to ensure that pressure generated during their contractions would be evenly spread over an exiting egg.

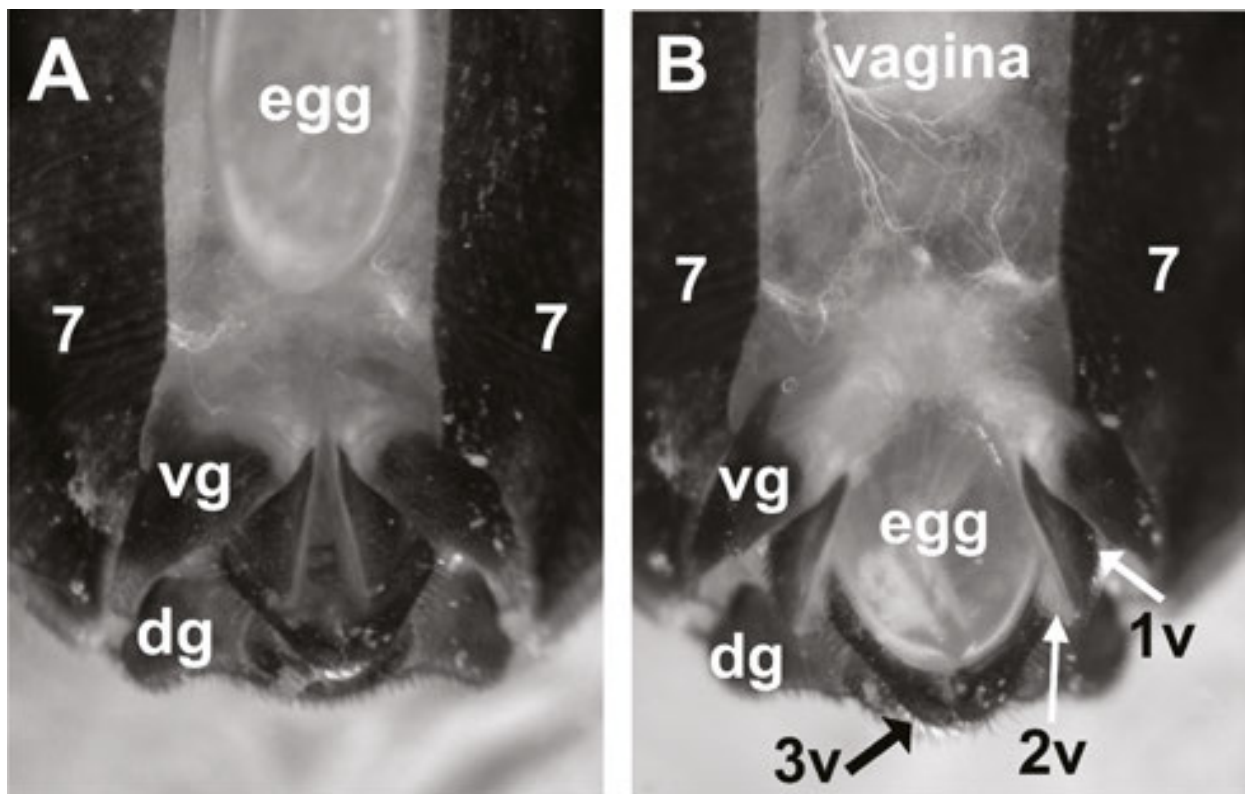
### 3.2. The vulva

The vagina opens to the outside through the vulva, which is surrounded by three sets of sclerites (see **Figure 4**). These consist of a single dorsal sclerite, a pair of lateral sclerites which are attached to the dorsal genital segment by the previously mentioned slender branch of cuticle and a pair of ventral sclerites. The base of each of these sclerites is attached to the soft articulating cuticle that lines the vulva and is continuous with the soft cuticle lining the vagina. When using the scanning electron microscope to compare the external female genitalia in fourteen species of *Rhodnius*, da Rosa et al. [24] refer to the dorsal sclerite as the gonapophyse 9, and the other two sets as the gonapophyse 8. For this chapter, we have adopted the designation which is specific to *R. prolixus*, and refer to the sclerites that surround the vulva as the valvulae (see Text-**Figure 1** in Ref. [25]). The ventral pair of sclerites represents the first valvula, the lateral pair, the second valvula and the single dorsal sclerite is the third valvula (**Figure 4B**). The valvulae are more than simple pieces of cuticle that guide the material through the vulva and out of the vagina. Each set has an anatomical specialization to suggest that they play more than a passive role in sexual physiology.

As is the case for the dorsal genital segment above it, the third valvula has an overall triangular shape, but is smaller and displays a medial line that separates the sclerite into two distinct halves (**Figure 5**). The two halves are joined only from the anterior base of the sclerite to approximately 1/3 their length, beyond which they are completely separated. The lateral and distal margins of each half forms a thick rounded edge which possesses several long fine hairs. The similarity of these hairs to tactile sensors on the insect cuticle suggests that they have a sensory function, and the manner by which they line the edge of the third valvula suggests that this structure serves as a sensory organ.

The second valvula consists of a pair of sclerites that line the lateral edges of the vulva. They are bilaterally symmetrical and elongated or laciniate in shape. They are widest at their base

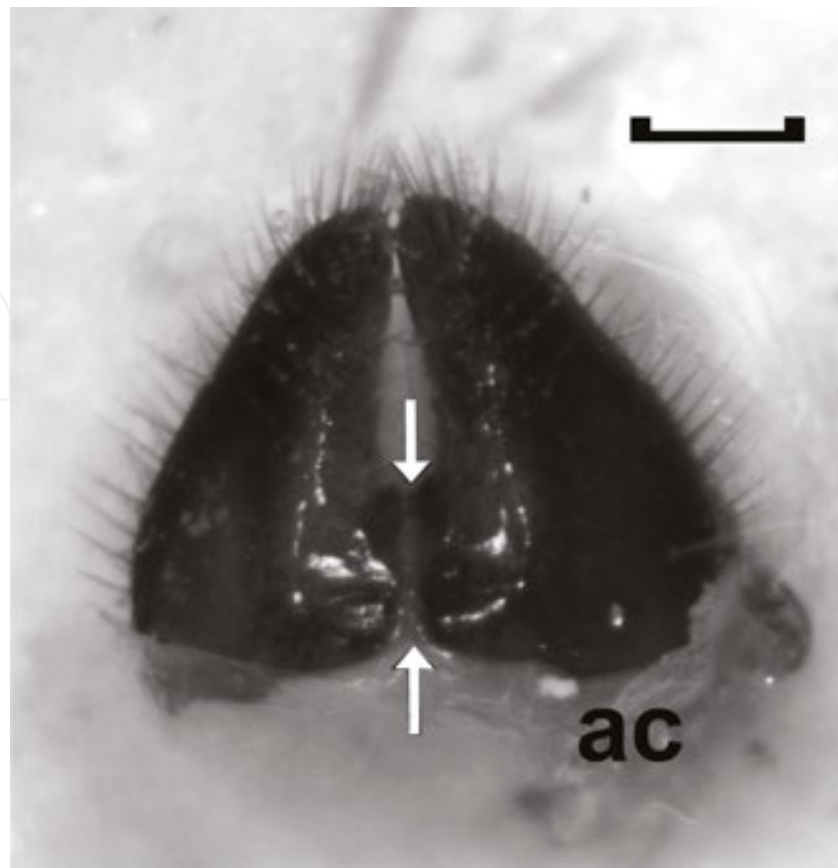




**Figure 4.** Ventral view of the female genital segments with the ventral cuticle of abdominal segment seven (7) partially removed to show the vagina. (A) An already laid egg with a developing embryo is laid over the vagina. vg, ventral genital segment; dg, dorsal genital segment. (B) Egg inserted into the vagina to show that the expansion of vulva occurs mainly from separating the two halves of the vg, the first valvula (1v) and the second valvula (2v). The halves of the third valvula (3v) remain together. The egg is approximately 1.0 mm in width.

where they attach to the soft cuticle lining of the vulva. They also curve along their long axis at their base to form a short tube-like structure, and they narrow posteriorly to a pointed end. The ventral edge of the second valvula forms a ridge along its margin, and this ridge fits into a groove that runs along the dorsal edge of the sclerite in the first valvula. As noted previously, the second valvula is attached at its base to the arm of cuticle that connects to the mid-lateral region of the dorsal genital segment.

The first valvula consists of a bilaterally symmetrical pair of sclerites that are more triangular in shape than the lacinate lateral sclerites of the second valvula (**Figure 4B**). The dorsal edge of the sclerites of the first valvula forms the groove in which the ventral ridge on the sclerites of the second valvula slides. This ridge and groove mechanism allows the second valvula to extend beyond the posterior end of the first valvula while keeping these two sets of sclerites firmly attached. This intricate structural relationship between the first and second valvulae may be an adaptation to serve a physiological role as the egg is passing out of the vagina. For instance, the excretory pore of the cement gland is situated on the dorsal side of the vulva near the tubular bases of the second valvulae (see **Figure 3**), yet on exiting the body, the cement gland secretions appear as dabs of secretions on the ventral, not dorsal, side of the egg [26]. In combination with the tubular nature of the base of the second valvula, and the ridge and



**Figure 5.** Dorsal view of the third valvula removed from the genitalia of the female of *Rhodnius prolixus*. The base of this pair of sclerites is attached to the articulating cuticle (ac) of the vagina on the dorsal side of the vulva. They are firmly joined along the midline between the arrows, and are covered with fine hairs typical for insect tactile receptors. Scale bar: 0.25 mm.

groove mechanism, these valvulae may function to direct cement gland secretions onto the ventral surface of an egg as it passes through the vulva.

The contralateral sclerites of the first valvula are not directly attached to one other, but are connected to each other by the soft cuticle that lines the entrance of the vulva (**Figure 4**). Thus, unlike the fused halves of the third valvula (**Figure 5**), these sclerites can separate, and stretch apart passively, as would be expected when an egg courses through the vulva. They can also be pulled back into the body to close off the vulva when the muscles associated with the vagina contract to eject an egg. Inserting a previously laid egg through the vulva demonstrates how readily the paired sclerites of the third valvula separate to allow the egg to pass (**Figure 4B**).

### 3.3. Female reproductive organs

The structure and function of the internal organs of the adult female reproductive system have been well documented for *R. prolixus* [27, 28]. At its anterior end, this system consists of a pair of ovaries each containing seven ovarioles. Since the developing oocytes in the ovarioles are connected by nutritive cords to the nutritive cells at the anterior end of the ovariole, these ovaries

are classified as telotrophic [29]. At their base, the ovarioles are connected to a calyx which, in turn, is connected to the anterior end of the ipsilateral lateral oviduct. Posteriorly, the lateral oviducts fuse to form a short common oviduct which enters the vagina and terminates in a muscular bulbous structure referred to as the vestibulum [20, 27]. The vestibulum undergoes spontaneous twitch-like contractions which may serve, after copulation, to propel the spermatozoa from the vagina into the common oviduct, then to the spermathecae, the sperm storage organs [20].

The spermathecae are one of the two accessory reproductive glands associated with the female reproductive system of *R. prolixus* (see **Figure 3**). They are a bilaterally symmetrical pair of slender blind-ended tubes arising from the sides of the common oviduct [10]. In *R. prolixus*, they take on a curved to twisted orientation, and when stretched, they can extend from 1/4 to 1/3 down the length of the vagina. After ingesting a blood meal of similar size, mated females make significantly more eggs than unmated females, and this difference can be correlated with the presence of spermatozoa in the lumen of the spermathecae [27]. This observation suggests that the cells lining the spermathecae are triggered by the male secretions into producing an endocrine secretion that increases the ability of the female to convert her blood meal into eggs.

The cement gland, the other accessory reproductive gland in *R. prolixus*, synthesises and releases an adhesive protein used to attach the egg to the substrate [28]. It consists of a cuticle-lined excretory duct which empties the secretions through an excretory pore located in the lining of the vagina on the dorsal side of the vulva. The duct receives secretions from the relatively large secretory portion which is folded over itself or the vagina to accommodate a length that is more than twice that of the vagina. As noted above, a laid egg has a thin layer of this adhesive material on its ventral surface [28] suggesting that the first and second valvulae may serve to position the adhesive protein onto the ventral side of the egg as it is being oviposited.

A comparative work on Reduviidae bugs shows that these blood-feeding insect vectors of Chagas disease can vary with respect to the presence of a cement gland and the morphology of their spermathecae [29]. All Reduviidae examined possess spermathecae that are paired blind ended tubes attached to the side of the common oviduct. However, the shape and location of the distal ends of the spermathecae differ depending on the genus. In *Rhodnius*, the spermathecae extend out from the common oviduct and are free to twist, whereas in *Triatoma*, *Nesotriatoma* and *Panstrongylus*, the ends of the spermathecae are held in place ventral to where the lateral oviducts attach to the common oviduct. In addition, the distal ends of the spermathecae take on the shape of flattened disks in *Triatoma klugi*, *Triatoma sordida*, and *Panstrongylus*, while in *R. prolixus* there appears to be no distal specializations. With respect to the cement gland, of the species examined, *Triatoma dimidiata* has a relatively small cement gland while this structure is absent in *T. klugi*, *T. sordida* and *Nesotriatoma bruneri*. All *Rhodnius* species examined (*R. prolixus*, *Rhodnius brethesi*, *Rhodnius nasutus*, *Rhodnius pictipes*) possess a prominent cement gland. This variability may be related to the ovipositioning behaviour since *Triatoma* tend to scatter their eggs loosely over a substrate whereas *Rhodnius* adheres its eggs to the substrate.

### 3.4. Physiology of muscles associated with the vagina and valvulae

The physiology of the muscles associated with the vagina and valvulae in *R. prolixus* has been studied by attaching a force transducer to a small metal hook inserted through the dorsal side

of this chamber, then raising the hook with the force transducer to apply tension to the muscles [22, 30]. In all preparations set-up in this fashion, there is a slow gradual drop in baseline tension until a steady baseline is reached around the 5-minute mark. Many of the preparations show spontaneous contractions at the onset of recording, and these contractions could either disappear after a few minutes or become synchronised into 10–30 second bursts that occur regularly over the recording period. The ovaries, lateral and common oviducts and spermathecae are also capable of spontaneous contractions [31].

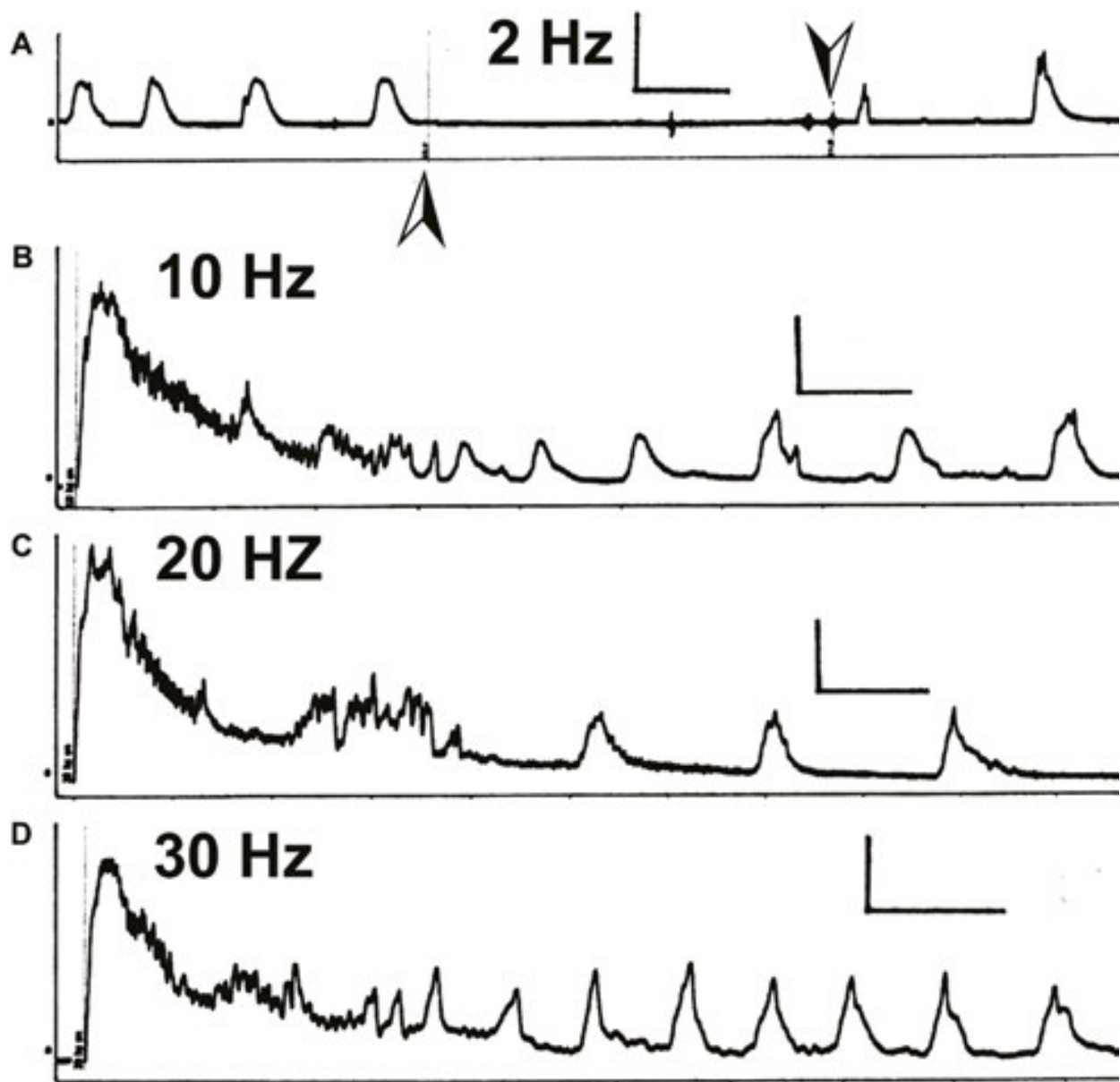
The overall pattern of innervation in the abdomen of *R. prolixus* is bilaterally symmetrical, and evoked contractions of the vagina muscles are elicited by stimulating either one of the paired abdominal nerve V supplying the genital segments. The response is dependent on the rate and duration of stimulation (see **Figure 6**). Any spontaneous contractions that may be present are eliminated with stimulations of 2 Hz or below showing that this system has a prominent inhibitory motor input, whereas slow prolonged contractions, typical of visceral muscle, are elicited at stimulation rates greater than 5–10 Hz. Continual stimulation at 10, 20 or 30 Hz does not maintain the tension suggesting that the excitatory input is not able to completely eliminate the inhibitory input. These physiological studies, combined with methylene blue staining, show that the ipsilateral regions of the vagina are innervated by motor neurons that travel from the thorax to the genitalia through the ipsilateral abdominal nerve V, then along the segmental nerve branch that serves spiracle 7, turning at the level of the common oviduct to travel to the side of the vagina (see **Figure 3**). At the vagina, the nerve divides to course anteriorly serving the ipsilateral spermatheca and lateral oviduct, and posteriorly to serve the vagina muscles. Near the dorsal posterior region of the vagina, the nerve appears to terminate within a nerve network and not on individual muscle fibres. Stimulation of the nerve elicits a relatively large negatively recorded potential at this point indicating the presence of a unique relationship between motor stimulation and smooth muscle contractions, a relationship that warrants further investigation.

Methylene blue also stains a network of fibres that extend over the base of the common oviduct and dorsal anterior region of the vagina [22]. This network resembles the nerves stained with an antibody for proctolin [32]. Application of various concentrations of proctolin to the preparation shows that increasing concentrations of proctolin have the same effect on tension generation as increases in electrical stimulation of the motor nerves [30]. Thus, proctolin plays a significant role in regulating contractions of the vagina muscles in *R. prolixus* and may serve as the primary excitatory transmitter. Such motor control over the muscles associated with ovipositioning provides the physiological mechanism enabling these insects to lay their eggs during a specific time of day [33], and to correlate the number of eggs laid with the substrate on which they are laid [34].

### 3.5. Egg laying

According to the structure and function of the genitalia in *R. prolixus*, ovipositioning includes the following steps:

1. A mature chorionated egg is released from the base of the ovariole in the ovary and enters the lateral oviduct;



**Figure 6.** Tension versus time graphs generated by vagina muscles of *Rhodnius prolixus*. (A) Stimulating the motor neurons in abdominal nerve V (up arrowhead) eliminated the spontaneous contractions. Turning off the stimulus (down arrowhead) allowed the spontaneous contractions to return indicating the presence of an inhibitory motor input. (B), (C) and (D) Continual stimulation at 10, 20 and 30 Hz caused an immediate rise in tension at the beginning of the traces with tension gradually dropping which may be as a result of the inhibitory motor input combined with fatigue of the excitatory motor input. Scale bars: vertical, 300 mg; horizontal, 1 min. (Adapted with permission from Ref. [22]).

2. Peristaltic contractions of the lateral oviduct propels the egg into the common oviduct. These contractions may be spontaneous or evoked by a motor input;
3. The egg squeezes through the muscular vestibulum at the end of the common oviduct, and as it stretches the walls of the common oviduct, it stretches the opening of the attached spermathecae. This action allows for the release of some of the stored

spermatozoa onto the egg. Release of spermatozoa may also be enhanced by motor stimulation of the spermathecae;

4. The egg stretches the vagina muscles and the nerve plexus attached to the vagina, and this stretching elicits a contraction of the vagina muscles causing them to shorten and pull the valvulae anteriorly, at which point, the valvulae stretch apart in response to the presence of the egg allowing the egg to start its descent out of the vagina. This step probably involves a stretch reflex which causes contractions of the vagina muscles since eggs are often seen in the lateral oviducts, but seldom lodged within the vagina [10];
5. As the egg exits the vulva, secretions from the cement gland are delivered to the dorsal side of the egg, and the first and second valvulae relocate the cement gland secretion to the ventral side of the egg.
6. As the egg leaves through the vulva, inhibitory input can relax the vagina muscles allowing the valvulae to close off the vulva. This action, in conjunction with active retraction of the dorsal and ventral genital segments, squeezes the egg out of the vagina and onto the substrate. As the dorsal genital segment retracts, it may place pressure on the dorsal surface of the passing egg, and such pressure would ensure that the egg contacts the substrate. Two observations suggest this final action of the dorsal genital segment. First, a mature egg in the reproductive system shows no asymmetry but is equally rounded on all sides, whereas an egg which is laid has a distinct indentation on its dorsal surface as would be expected if pressure were placed on this location during its passage to the substrate. Second, this indentation appears to be directly related to the egg passing through the vulva, and not due to structural changes after being laid. In a SEM image of an egg passing out of the vulva, the exiting egg already shows a distinct indentation under the dorsal genital segment [26].

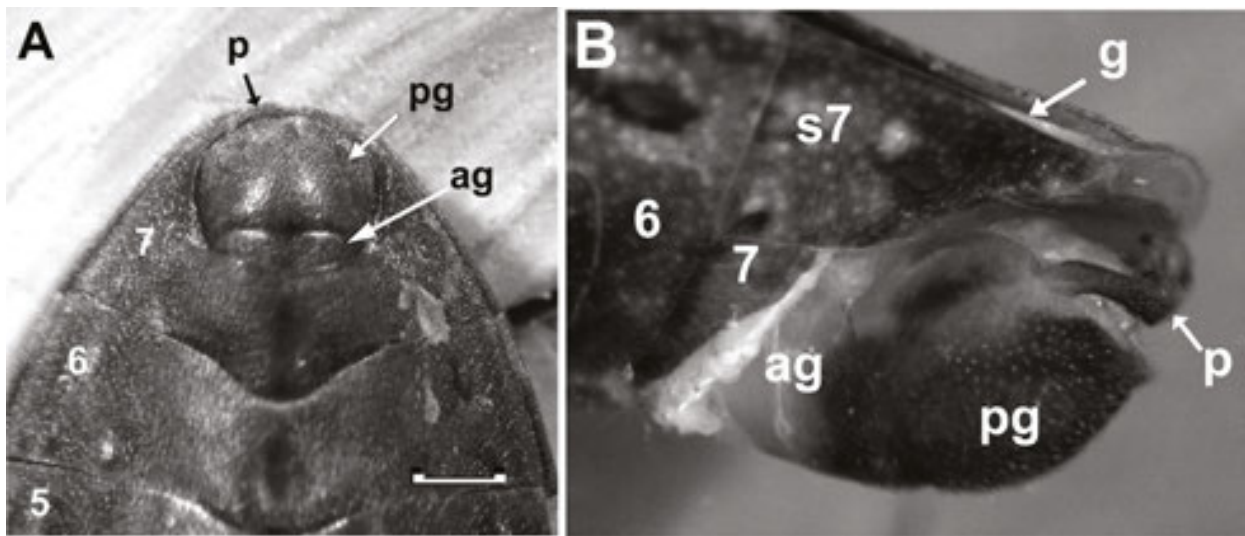
The role of the female reproductive system is also important in copulation and the ejection of male secretions after copulation, and these events will be considered following a description of the male reproductive system.

## 4. The male reproductive system

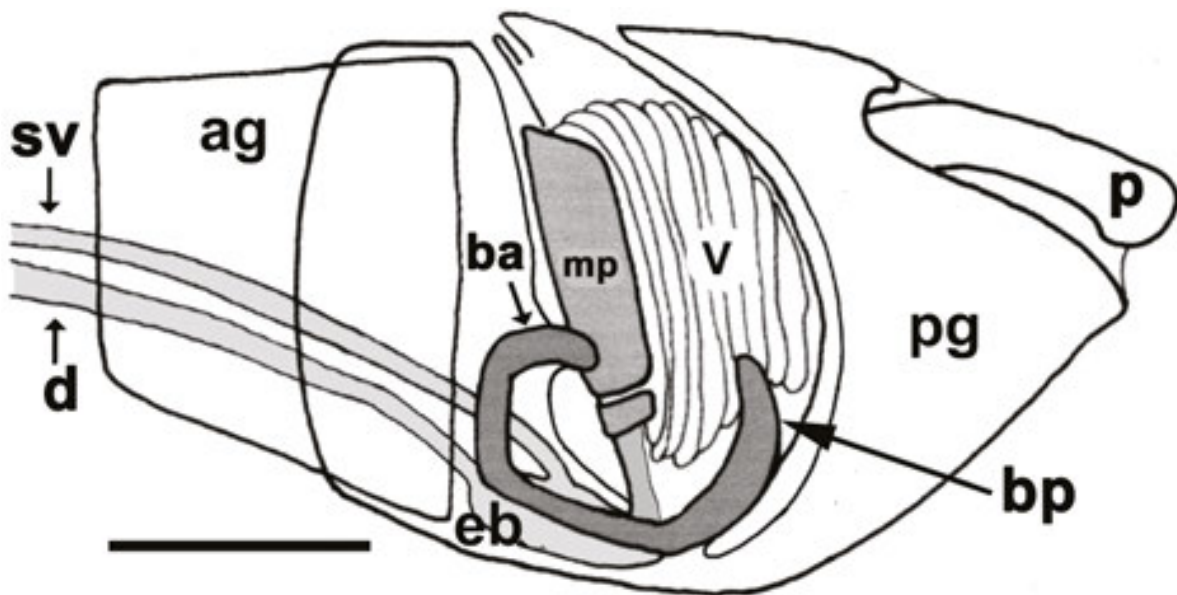
As in the case for the female genitalia this chapter simplifies the nomenclature by identifying the male genital segments according to their structure and function observed in the adult.

### 4.1. The male genitalia

The genitalia of the adult male are positioned on the underside of full-sized abdominal segment 7, and consist of two segments which move together (see **Figures 7** and **8**). The anterior segment is smaller with no cuticle specializations, and serves to attach the larger posterior genital segment to abdominal segment 7. The skeletal muscles extending between the anterior genital segment and abdominal segment 7 move the genitalia enabling the male to



**Figure 7.** External view of the male genital segments in the blood-feeding insect, *Rhodnius prolixus*. (A) Ventral view showing abdominal segments 5, 6 and 7 (5,6,7). The genitals consist of an anterior genital segment (ag) which is attached to abdominal segment 7, and a posterior genital segment (pg) attached to the ag. p, the distal end of the right paramere sitting in a groove along the posterior dorsal edge of the pg. Scale bar: 1.0 mm. (B) Lateral view of the genital segment with the posterior edge of abdominal segment 7 removed to reveal the anterior region of the ag. g, lateral groove in adult abdominal cuticle; s7, seventh abdominal spiracle.



**Figure 8.** Line diagram summarizing the functional anatomy of the male genitalia in the blood-feeding insect, *Rhodnius prolixus*. The aedeagus sits in a pocket in the posterior genital segment (pg) and contains a convoluted bag-like structure which may serve as a valve (v). The aedeagus is anchored to the softer cuticle lining the pocket by the posterior (bp) and anterior (ba) arms of the basiphallus. mp, medial plate under which secretions from the ejaculatory bulb (eb) are delivered. sv, duct from seminal vesicle; d, duct from accessory reproductive glands; ag, anterior genital segment; p, paramere. Scale bar: 0.5 mm.

extend his genitals away from his body and to turn them laterally to face the corresponding female genitalia. It is this anatomical arrangement that determines the side-by-side position copulating pairs assume (**Figure 1**).

The posterior genital segment is twice the size of the anterior genital segment, and is rounded at its posterior end taking on a bulbous shape (**Figure 7**). On this posterior-rounded side, there are two bilaterally symmetrical arms of cuticle, the parameres, which are attached to the posterior lateral edges of the posterior genital segment (p in **Figures 7** and **8**). On their distal ends, the parameres possess fine hairs characteristic of sensory hairs associated with tactile stimulation in insects [35], and when not extended, they fit into a groove on the posterior dorsal edge of the posterior genital segment with their slightly flattened hook-like ends facing one another (**Figure 7A**). During copulation, the male extends his parameres to make contact with the female genitalia, and during insemination, the curved blunt tips of the parameres wrap around the lateral flaps on the dorsal genital segment of the female. These parameres have been considered homologous to claspers in other insect species [36], but they do not appear to firmly latch on to the female [37]. Preliminary results suggest that the parameres serve a sensory function aiding the male to determine the position of the female genitalia before and during copulation. Their position and sensory function suggest that they are homologous sensory organs to the third valvula in the female genitalia.

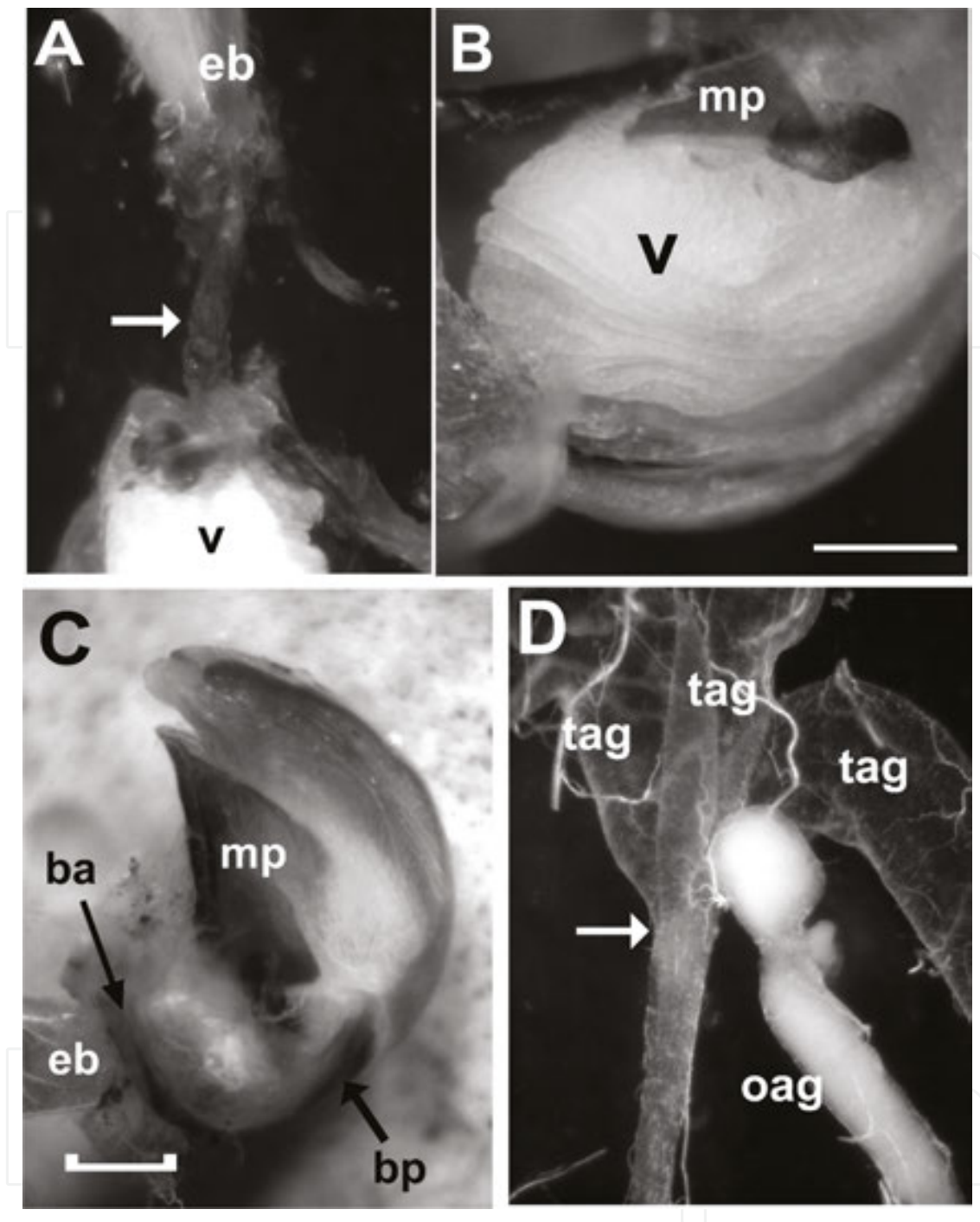
The posterior genital segment houses the aedeagus (see **Figure 8**). The aedeagus sits in a pocket lined with soft articulating cuticle and opened to the dorsal side of the posterior genital segment. During copulation, the aedeagus extends out of this pocket into the vagina. Viewed laterally, the aedeagus assumes a half-moon shape (**Figure 9C**). The curved portion of the aedeagus contains an elaborate bag-like structure formed from an invagination of soft cuticle with several overlying folds (**Figure 9B**). These folds allow the bag-like structure to be extended or compressed perpendicular to the flow of secretion from the male reproductive organs. In dissections where the vital dye, methylene blue, is added to the exposed abdomen, this dye is picked up by the reproductive glands and carried in their ducts to the aedeagus where it ends up in the space between the bag-like structure and the medial plate of the aedeagus. Methylene blue does not enter the bag-like structure suggesting that this structure is not designed to receive secretions from the male reproductive organs [37].

The male secretions reach the aedeagus through the ejaculatory duct which is the fused portion of the left and right ejaculatory bulbs. This duct is anchored to a ring of cuticle in the basiphallus which serves as the supporting base for the aedeagus. A pliable delicate duct extends from this ring into the aedeagus (**Figure 9A**), and carries secretions from the ejaculatory bulb into the aedeagus when the aedeagus is extended into the vagina. Since the secretions can be deposited in the space between the bag-like structure and the medial plate on the straight side of the aedeagus, the bag-like structure could serve as part of a pumping mechanism that forces secretions out of the aedeagus and into the vagina during the power stroke, but prevents back flow during the recovery phase of the pumping cycle.

#### 4.2. The male reproductive organs

The male reproductive system anterior to the ejaculatory duct is bilaterally symmetrical and each side consists of two reproductive organs—the testis and the seminal vesicle, and two types of accessory reproductive organs—the three lobes of the transparent accessory reproductive gland (tag) and the one lobe of the opaque accessory reproductive gland (oag)





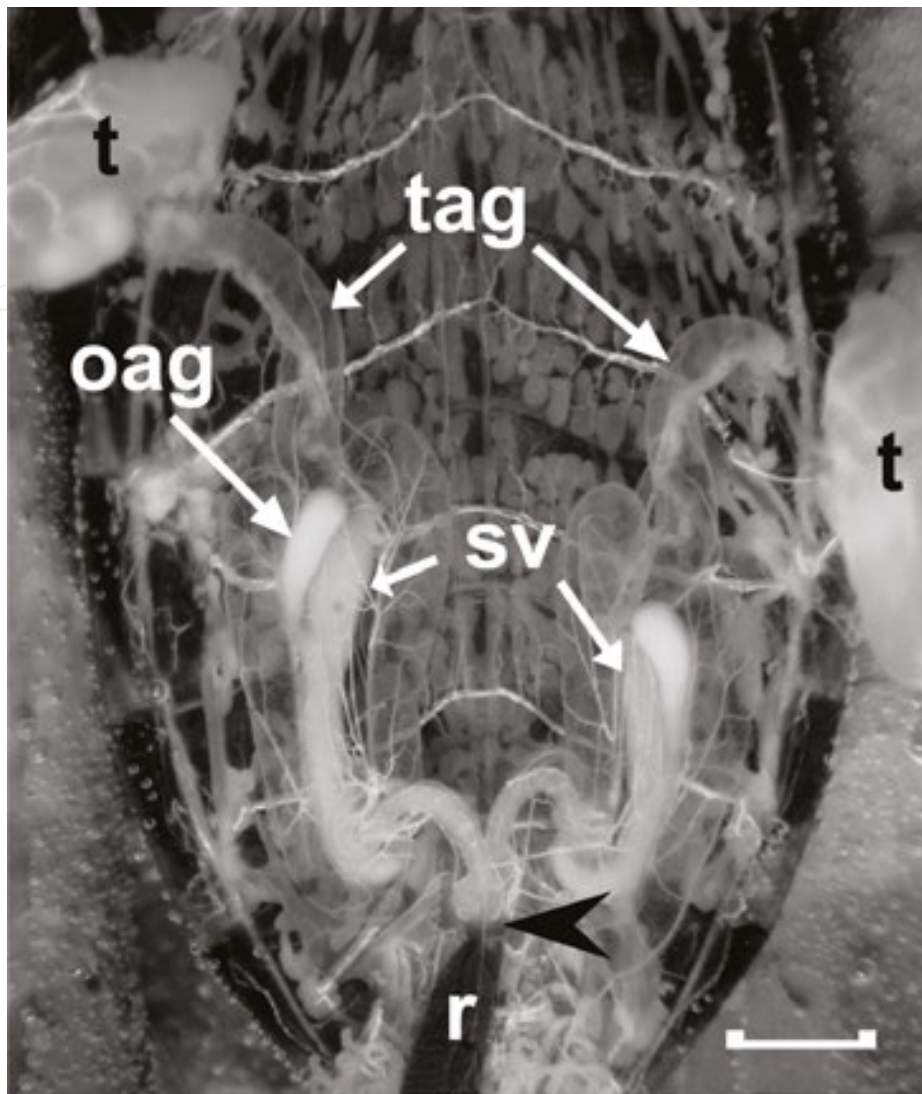
**Figure 9.** Structures in the male reproductive system of *Rhodnius prolixus* which provide a clue to the manner by which the male secretions are delivered to the vagina during copulation. (A) A small duct (arrow) from the base of the ejaculatory duct (eb) directs secretions into the aedeagus in the space above the valve (v). (B) Picture of the valve (v) showing that it consists of folds of soft articulating cuticle. Secretions enter the space between the medial plate (mp) and this valve. Scale bar: 0.6 mm. (C) A dissected aedeagus removed from the posterior genital segment. The whitish valve is just visible through the articulating cuticle between the medial plate and the curved back of the aedeagus. ba, anterior arm of the basiphallus; bp, posterior arm of the basiphallus. Scale bar: 0.5 mm. (D) The relationship between the ducts of the three transparent reproductive accessory glands (tag) and the opaque reproductive accessory gland (oag). They converge at the head of the common duct (arrow), travel down the duct separately merging into a single tube that empties into the ejaculatory bulb.

(see **Figure 10**). The testes are located laterally near the mid-region of the abdomen. They are present in the penultimate larval stage (L5), and become connected to the seminal vesicle during metamorphosis. The testis consists of seven follicles folded onto each other and wrapped with a thin membrane. Two of the seven have a larger girth and a longer length than the other five, even in the L5 stage. As the testes increase in size during the adult stage, the growth is mainly attributed to the two larger follicles which increase considerably in length and girth [10], which has also been observed in other species of Reduviidae bugs [38].

Each testis is connected to the seminal vesicle by the vas deferens which extends a short distance from the testis, where the bases of the follicles are attached, to the tip of the anterior lobe of the tag. From this point, the vas deferens remains closely associated with the tag and courses along its side to the level of the lobe's base where the vas deferens connects to the seminal vesicle. Between the testis and the tip of the tag, the contents of the vas deferens tend to be transparent. From the tip of the lobe to the seminal vesicle, the contents are distinctly yellowish white and have a clump-like appearance. The seminal vesicle is a semi-rigid elongated sack which can increase considerably in girth as the adult matures. Its length is approximately the same length as the individual lobes of the tag (**Figure 10**), and its duct connects to the ejaculatory bulb posterior to the duct from the accessory reproductive glands.

Of the two types of accessory reproductive glands, the larger tag consists of three large tube-like lobes, and the smaller oag is a single elongated structure (**Figure 10**). The tag contains a clear proteinaceous material, and the oag contains a whitish milky substance, both of which are delivered to the female during copulation. Rather than sequestering from the haemolymph molecules made from another organ or tissue, the tag may make the secretions themselves [39], with their activity being under endocrine control [40, 41]. They also produce a polypeptide that is secreted into the haemolymph [42]. The tag possesses a relatively tough muscular lining that is supplied by motor axons which, when electrically stimulated, will cause each of the three lobes of the tag to constrict their girth and lengthen (personal observations). The tag secretions are viscous and pour slowly out of the lobe when it is cut. In contrast, the oag has a delicate lining, is easily damaged during dissection and its whitish secretions readily flow out of the lobe. It is widest at its anterior base, tapers towards its posterior end, and does not respond to electrical stimulation of the abdominal nerves. Early studies report that placing the contents of the oag onto an adult vagina can elicit strong twitch-like contractions of the vestibulum suggesting that this male secretion may aid delivery of the transferred spermatozoa to the spermathecae [20]. Because the response is described as capricious rather than consistent, this role is speculative.

Each lobe of each accessory reproductive gland empties through its own duct, and these ducts enter a tube which makes up the proximal end of the common accessory reproductive gland duct (**Figure 9D**). As these ducts enter this tube, they do not merge into a single duct at this point, but extend down the tube to become a single lumen before emptying into the ejaculatory bulb. This tube has a muscular sheath, and displays spontaneous contractions that tend to shorten the tube pulling it posteriorly towards the ejaculatory bulb.



**Figure 10.** Live preparation of the abdomen of a male *Rhodnius prolixus* exposed to show the reproductive organs. t, right and left testes; tag, the anterior lobe of the transparent accessory reproductive gland; oag, left opaque accessory reproductive gland; sv, seminal vesicle; r, rectum. The ducts of the seminal vesicles and accessory reproductive glands, and the anus at the end of the rectum, come together to enter the anterior genital segment (arrowhead). Scale bar, 2 mm.

#### 4.3. Delivery of male secretions to the vagina

The manner by which the male secretions are delivered to the female reproductive system in insects varies between two extremes. At the one extreme, the female has two genital openings, one to the bursa copulatrix, and the other to the egg pore. The male produces a distinct spermatophore which is a proteinaceous package containing spermatozoa and this package is deposited into the bursa copulatrix. There, the spermatophore is broken open allowing the spermatozoa to migrate along the sperm duct to the spermatheca [43]. At the other extreme, the female has a single opening to her reproductive system. The male inserts a long intromittent organ through the vagina, into an insemination duct which leads to an elaborate spermatheca. At the end of the insemination duct, the male extends his intromittent organ

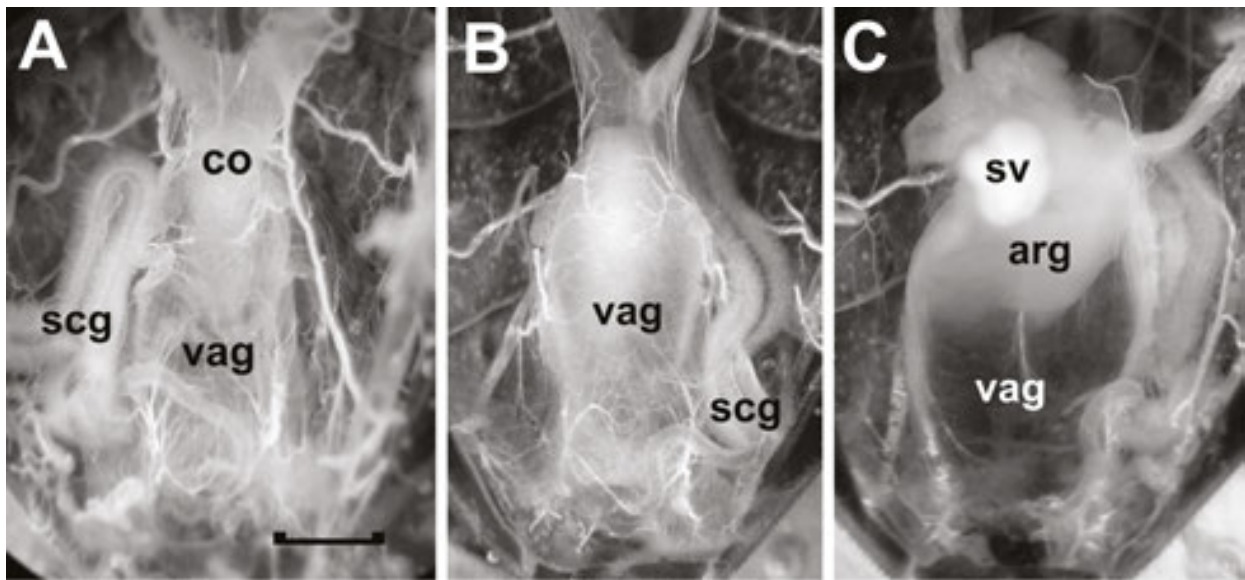
through a valve and pumps his secretions directly into the spermatheca. No spermatophore is needed [44].

The first description of sperm transfer in *R. prolixus* assumed that this species produced a distinct spermatophore. Khalifa [45] described the spermatophore in *R. prolixus* as a pear-shaped sac containing spermatozoa enclosed in a proteinaceous mass, and from his study of fixed tissue, Davey [36] proposed that before it is delivered to the female, this spermatophore is formed within the male spermatophore sac that everts from the end of the aedeagus into the female during copulation. While examining spermatophore formation in *Triatoma infestans* and *Rhodnius neglectus*, Pereira-Lourenço et al. [23] made observations which differ to that reported for *R. prolixus*. In these bugs, which are closely related to *R. prolixus*, there is no spermatophore sac and the spermatophores are transferred as a viscous or mucous substance directly to the vagina where they solidify. Chiang and Chiang [37] resolved this discrepancy by observing that the fixed tissue of *R. prolixus* observed by Davey [36] does not behave like a living tissue. As noted above in the description of the male reproductive system, the structure previously thought to be a spermatophore sac in fixed tissue of *R. prolixus* stays within the aedeagus, has no opening, and does not receive any secretions from the male reproductive system.

Considering these more recent findings, it is likely that all Reduviidae bugs lack spermatophore sacs, and the structure thought to be a sac is part of a pumping mechanism which enables the aedeagus to fill the vagina with the male secretions. Rather than resembling those arthropods which make encapsulated spermatophores that harden before they are inserted into the bursa copulatrix of the female [46], the Reduviidae are more closely related to insect species which lack spermatophores and deliver the semen by using a long intromittent organ that the male inserts through an insemination duct to the spermatheca [44]. In *R. prolixus*, the spermathecae are attached directly to the common oviduct so that no insemination tube is required since this location is very close to where the spermatozoa are delivered. Male Reduviidae bugs may still be described as spermatophore producers, but unlike the traditional description of a spermatophore, their spermatophores are naked, are formed directly in the female and harden after they have been delivered to the female.

As the male secretions are delivered to the female, they assume the pear-shape of the inside of the vagina with the narrower anterior end resulting from the male secretions being pushed up against the narrow base of the common oviduct (**Figure 11**). In a recently inseminated female, the secretions from the seminal vesicle appear as a clump of yellowish material at the base of the common oviduct whereas the rest of the vagina is filled with a slightly cloudy secretion from the accessory reproductive glands. Since the spermatozoa are positioned anteriorly, the seminal vesicle secretions are delivered first, followed by secretions of the accessory reproductive glands. With separate ducts to the ejaculatory bulb, differential motor activity from the central nervous system likely stimulates the seminal vesicles to deliver their secretions prior to transfer of the accessory reproductive gland secretions. In addition, the clump from the seminal vesicle is approximately the same size as the aedeagus suggesting that only one or two pulses from the pump in the aedeagus are needed to deliver the spermatozoa.

The remainder of the spermatophore consists of a large amount of secretion from the tag and oag. While in the body of the male, the secretions of the tag are transparent, but in the



**Figure 11.** A freshly dissected female of *Rhodnius prolixus* showing in (A) an empty vagina (vag), the collapsed common oviduct (co), and the secretory portion of the cement gland (scg), in (B) a vagina expanded to accommodate the male secretions shortly after the completion of copulation and in (C) the same vagina in B which has been cut open on its dorsal side and the contents pulled slightly out of the vagina. sv, secretions from the seminal vesicles; arg, secretions from the accessory reproductive glands. The clear area in the vagina is the region from which the male secretions were pulled away. Scale bar: 0.5 mm.

spermatophore in the female, they take on a slightly cloudy appearance (**Figure 11C**). This change likely results from a small amount of oag material mixing with a large amount of tag material which is possible due to the relationship between the ducts from the three lobes of the tag, and the single duct from the oag. All four ducts enter the distal end of the tube of the common accessory reproductive gland duct, and this tube is able to produce bursts of contractions that rhythmically constrict and shorten the tube. In addition, the lobes of the tag can contract due to motor stimulation thus forcing the material into their ducts, whereas material from the oag enters passively. This anatomical arrangement could allow the peristaltic-like contractions of the common duct to ‘milk’ the ducts of the four lobes of accessory reproductive glands at the same time resulting in a large amount of tag material being mixed with a small amount of oag material before they are delivered to the aedeagus and the vagina. This scenario, which is supported by the anatomy and physiology, suggests that the oag secretions are affecting the tag secretions rather than eliciting contractions of the vestibulum. Davey [36] postulated that the secretions from the cells lining the ejaculatory bulb mix with the tag secretions to lower the pH, causing the tag secretions to harden. However, the oag secretions may also serve in hardening the tag secretions. Determining the relationship between the secretions from the tag and oag promises to be a fruitful area of study.

#### 4.4. Facilitating copulation

In *R. prolixus*, the male completes insemination in about 50 minutes ( $52 + 14$  minutes,  $n = 26$ , as reported in Ref. [10]), and has at least two physiological means to help maintain copulation for this length of time. One is sensory; the other is chemical.

If the sensory hairs on the ventral lateral region of the abdomen are gently stroked with a probe, the heartbeat is inhibited [47]. Such a reflex could be part of a general thigmotactic response in which the insect becomes less responsive to external stimuli when it wedges itself into a confined space (see p. 313 in Ref. [35]). This response could be elicited as these sensory hairs touch the surface of the enclosed area, and the stoppage of the heart beat may be part of the general calming of the whole body. The ventral region of the abdomen linked to this tactile inhibition of the heartbeat is the same region where the male places his abdomen during copulation, which, in turn, could generate a thigmotactic response to help calm the female.

This sensory thigmotactic response could be enhanced chemically by rhodtestolin, a cardio-inhibitor first discovered in testes extracts of *R. prolixus* [48]. When a test saline containing rhodtestolin is applied to the isolated heart, the heart becomes flaccid and all beating immediately ceases. Rhodtestolin is a small, heat stable protein, and its dramatic cardio-inhibitory effect is dose dependent and reversible [10]. It has yet to be determined how rhodtestolin concentrated in the testes makes its way to the female, but it is delivered during copulation since extracts of spermatophores removed from the female shortly after the completion of copulation show this cardio-inhibitory effect [10]. Being delivered to the female during copulation could enhance the sensory thigmotactic response, but if rhodtestolin has a general inhibitory effect on insect visceral muscle, its major role might be to relax the vagina muscles and to prevent the female from expelling the spermatophore prematurely. In preparations in which the tension generated by the vagina muscles is monitored by a force transducer, stretching these muscles generates strong spontaneous contractions that would expel any vagina contents, whether it is an egg or a spermatophore [22]. Therefore, rhodtestolin may be a visceral muscle relaxant which reduces the excitability of the vagina muscles so they do not contract in response to being stretched by the male secretions. This role still needs to be substantiated but it would increase the changes of the spermatozoa delivered to the vagina reaching the spermathecae before the male secretions are ejected from the vagina.

## 5. Summary

Our knowledge of the details of sexual reproduction in *R. prolixus* has lagged behind other aspects of its physiology since studies using this bug have tended to take advantage of the fact that a blood meal triggers the onset of physiological processes related to feeding. To help address this shortfall, the present chapter details the functional anatomy of male and female genitalia, and highlights a number of significant points which may apply to insect vectors of Chagas disease in general. First, the female genital chamber is best referred to as the vagina since the male of this species does not form a distinct spermatophore. Second, the male inserts his aedeagus directly into the vagina and delivers his secretions with the aid of a novel pumping mechanism. Third, the design of the accessory gland ducts provides a mechanism whereby the male secretions from the tag and oag can be mixed before they enter the vagina, suggesting that these secretions have a chemical relationship that warrants further investigation. Fourth, the male parameres and the third valvula of the female appear to be homologous sensory structures which may have a function in both sexes during copulation, or in the female

during egg-laying. Fifth, the sclerites of the first and second valvulae in the female, and their proximity to the excretory pore of the cement gland, suggests that they are involved in placing the cement gland secretions onto the ventral side of the egg. Finally, the sensory and chemical aids for copulation, which still need to be further explored, provide yet another intriguing aspect of insect reproduction first to be observed in *R. prolixus*.

This knowledge gained by detailing the mechanics of copulation and egg-laying in this well-studied insect sets the groundwork from which further investigation of this important physiological process in this bug can be carried out. Along with the completion of the *Rhodnius* genome project, which provides a resource to investigate the genes and proteins associated with reproduction, *R. prolixus* is well equipped to maintain its status as a popular insect model for teaching and research.

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