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Spermatophore formation and transfer in the freshwater flatworm *Dugesia gonocephala* (Platyhelminthes, Tricladida, Paludicola)

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Abstract. Before copulation in *Dugesia gonocephala,* eosinophilic secretions of glands in the penis diaphragm and in the most proximal part of the ejaculatory duct accumulate in the funnel-shaped part of the ejaculatory duct and cohere to form a sclerotized, tubular structure, sealed at the distal end and covered internally with secretions of glands at the tip of the diaphragm conus. The elongating tube fills with a mixture of sperms, released in small clusters from both sperm ducts, and two types of seminal secretions produced by gland cells of the seminal vesicle. This causes the sealed end of the tube to inflate, forming a spherical, stalked bladder. As copulation begins, each mating partner inserts its penis with the protruding spermatophore into the vaginal area of the bursal canal of the partner. Penis insertion causes the penis papilla to elongate, the diaphragm conus to invert, and the seminal vesicle to expand. The latter is filled with a loosely packed substance. The increase in surface area is probably facilitated by the presence of epithelial cells with an expandable apical end. The spermatophore bladder expands to its full size during copulation as large amounts of sperms and seminal secretions are released into it. Filling of the spermatophore ends with the transfer of the spermatophore into the partner's bursa. No additional sperms or seminal secretions are transferred after spermatophore exchange is completed. Spermatophore transfer causes the lumen of the seminal vesicle to collapse and the diaphragm to regain its conical shape, suggesting that the seminal vesicle functions as a kind of mechanical pump.

Additional key words: Turbellaria, planarian, simultaneous hermaphrodite, penis

The rapid evolution of animal genitalia, particularly those of the male system, is a well-known phenomenon in the animal kingdom. Several hypotheses have been put forth to explain this tremendous variation in genital traits. The pleiotropy hypothesis states that a single gene may code for developmental processes in different parts of the body, affecting both genital and general morphology. Genital diversification may therefore just be a side-effect of natural selection favoring traits unrelated to reproduction (Ridley 1996; Arnqvist 1997). The evolution of animal genitalia may also be explained by the lock-and-key hypothesis, in that female genitalia are "locks" that allow access only to the appropriate male "keys," so preventing heterospecific matings (Shapiro & Porter 1989). Empirical evidence for this (popular) hypothesis is weak (Proctor et al. 1995; Arnqvist 1997, 1998). Recent studies explain the complexity of animal genitalia by focusing on processes of sexual selection, including phenomena such as cryptic female choice (females exerting post-intromission physiological control over paternity; see Eberhard 1996) and sperm competition (male–male competition on the level of ejaculates; see Parker 1970). Arnqvist (1998) found compelling evidence for the evolution of genitalia by sexual selection in insects. Profound morphological and functional studies are needed in order to further distinguish between these hypotheses.

Turbellarians, or free-living flatworms, are (with few exceptions) hermaphroditic, practicing cross-fertilization with reciprocal sperm transfer. Fertilization is internal. The genitalia are usually of great complexity, consisting of a protrusible penis or eversible cirrus, either of which may be armed with spines or thorns, and various accessory glands and vesicles for storing sperms (Hyman 1951). The function of these morphologically well-described organs is poorly understood: e.g., what is the function of the musculo-glandular, penis-like organs, usually called adenodactyles, commonly found in freshwater triclads? Hyman (1951) hypothesized that they are sexual stimulators, perhaps to induce the partner to donate sperms (Michiels 1998), but empirical evidence is lacking.

In fact, only a few studies deal with the functional morphology of the male genital organs of freshwater

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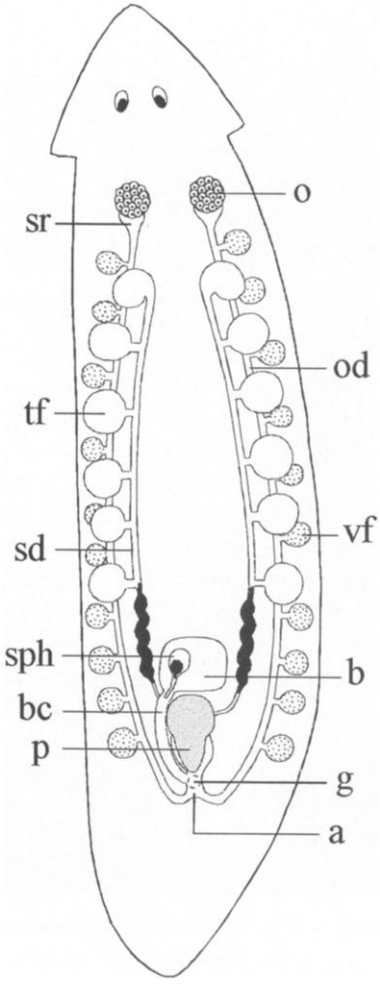


Fig. 1. Schematic dorsal view of the genital system of the hermaphroditic flatworm *Dugesia gonocephala.* Genital atrium (a); bursa copulatrix (b); bursal canal (bc); common ventral gonopore (g); ovary (o); oviduct (od); penis (p); sperm duct (sd); spermatophore (sph); seminal receptacle (sr); testis follicle (tf); vitellarian follicle (vf).

triclads, including the work of Fischlschweiger (1990, 1992) on the planarian *Girardia (= Dugesia) tigrina* (GIRARD 1850). In this species, sperms and secretory products accumulate in the lumen of the penis bulbus (= seminal vesicle) before copulation, and the resulting sperm mass is released into the bursa of the partner through the ejaculatory duct of the elongated penis papilla during copulation. This suggests that the seminal vesicle of planarians might function as a kind of sperm storage organ. The exchange of spermatophores, another common mode of sperm transfer in freshwater triclads (Sluys 1989), is scarcely documented. Information on this subject is usually limited to the fact that the spermatophore wall is formed by penial gland secretions (Weiss 1910; Sluys 1989, 1997; Sluys & Rohde 1991).

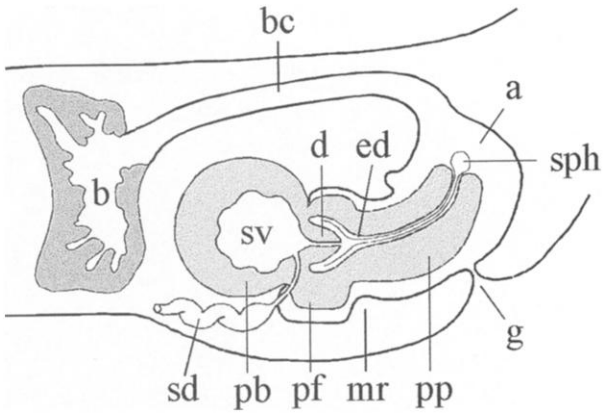


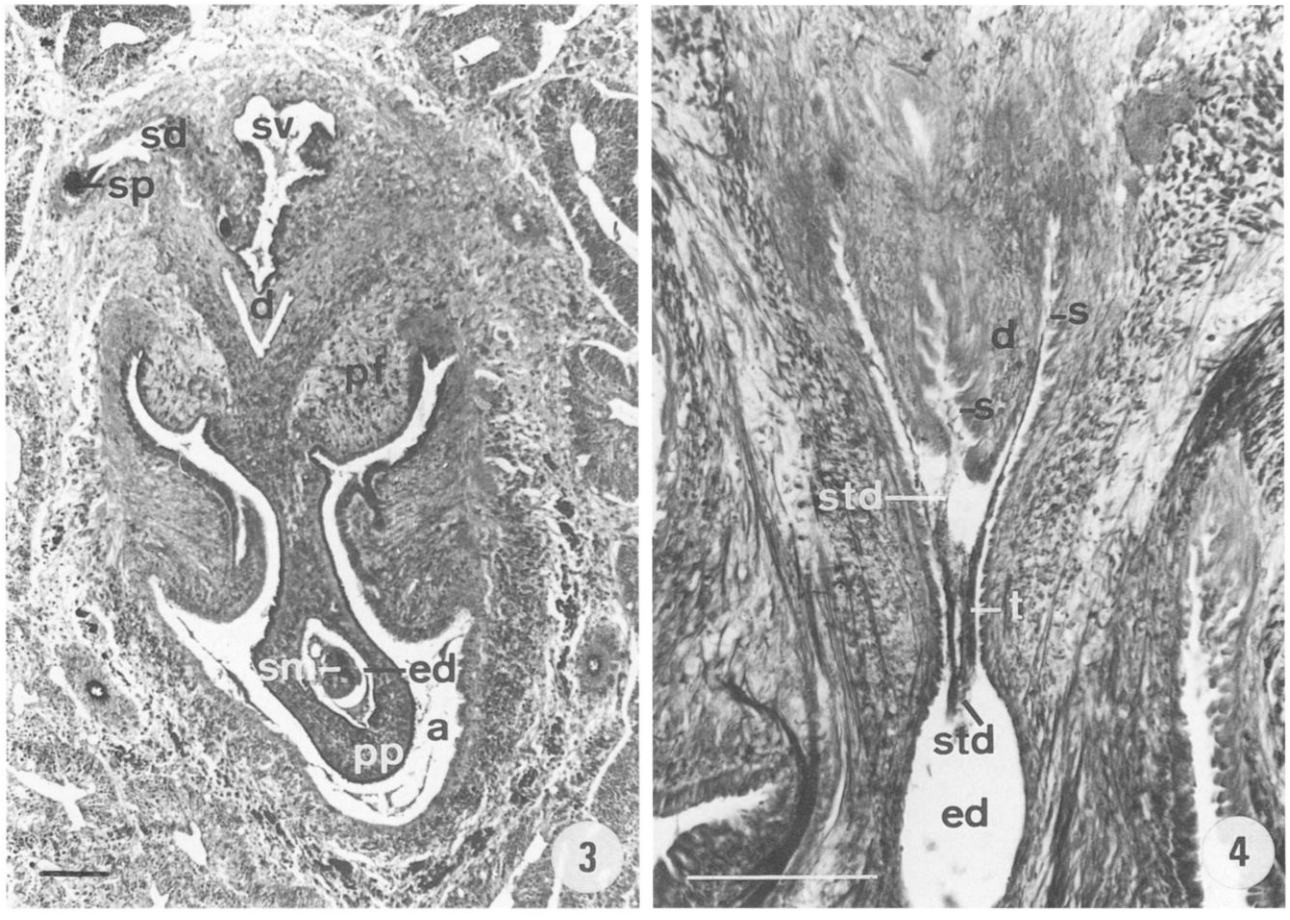
Fig. 2. Schematic sagittal view of the copulatory organs of the hermaphroditic flatworm *Dugesia gonocephala.* Atrium (a); bursa (b); bursal canal (bc); diaphragm conus (d); ejaculatory duct (ed); common ventral gonopore (g); muscular ridge (mr); penis bulbus (pb); circular penial fold (pf); penis papilla (pp); sperm duct (sd); spermatophore (sph); seminal vesicle (sv).

In this study we provide detailed information on the formation and transfer of the spermatophore of the planarian *Dugesia gonocephala* (DUGÈS 1830), using light microscopical and ultrastructural data. "Free" sperm transfer, as shown in the classical drawing of *D. gonocephala* by Burr (1928), is absent from our study population. In *D. gonocephala* every copulation is preceded by prolonged courtship behavior during which a spermatophore bladder inflates at the tip of the penis, expanding to its full size during copulation, as it fills with semen. Filling of the spermatophores ends 3 to 4.5 h after the start of copulation, when first one partner, then the other finishes ejaculation and completes spermatophore transfer (Vreys et al. 1997a). The transferred spermatophores contain equal amounts (by volume) of sperms (Vreys & Michiels 1998). When one or both partners have no spermatophore in the penis, as in 15% of the examined copulations, spermatophore transfer is unilateral or does not occur (Vreys et al. 1997a,b). During the days following copulation, sperms migrate from the spermatophore in the bursa to the seminal receptacles near the ovaries. The adaptive significance of the spermatophore may lie in its ability to protect migrating sperms from being digested by the bursal cells (Vreys et al. 1997a,c). Post-copula sperm digestion is a widespread phenomenon in free-living flatworms (Hyman 1951).

Methods

Study organism

Dugesia gonocephala is found under stones in small, shallow streams throughout continental Europe.



Figs. 3, 4. Penis of unmated worm. Frontal sections. LM. Scale bars, 100 μm. **Fig. 3.** Penis with unexpanded seminal vesicle, diaphragm conus extending into ejaculatory duct, and circular fold at the base, filling most of the atrial cavity. The posterior part of the sperm duct on the left contains clusters of sperms. A slightly inflated tube, filled with seminal material, is present in the posterior part of the ejaculatory duct. Atrium (a); diaphragm conus (d); ejaculatory duct (ed); penial fold (pf); penis papilla (pp); sperm duct (sd); seminal material (sm); sperms (sp); seminal vesicle (sv). **Fig. 4.** Secretions (s) of glands in the diaphragm conus (d) and in the most proximal part of the ejaculatory duct (ed) coalesce in the funnel-shaped part of the ejaculatory duct to form a sclerotized tube (t), sealed at the distal end. The inner side of the tube is covered with secretions (std) of glands at the tip of the diaphragm conus.

Individuals either multiply by transverse fission or reproduce sexually by outcrossing and copulation. Sexually mature individuals have a fully developed male and female reproductive system (Fig. 1). A pair of ovaries are situated just behind the brain, anterior to the vitellarian follicles. The oviducts open separately into the terminal part of the bursal canal (= vaginal area), a strongly muscular duct that connects the atrium (and its ventral gonopore) to the bursa copulatrix. The atrium is divided into a male and a common atrium by a muscular ridge of glandular nature (Fig. 2). From numerous testis follicles, sperms are discharged into the expanding sperm ducts, which are surrounded by circular muscles just before they enter the penis (Fig. 1). The penis consists of a strongly muscular bulbus embedded in the atrial wall, and a free intromittent papilla with a circular fold at the base (Figs. 2, 3). Within the bulbus is a cavity, the seminal vesicle, which is separated from the ejaculatory duct by a glandular, conical diaphragm, traversed by a narrow canal. The ejaculatory duct is funnel-shaped at the proximal end and runs medially through the penis papilla to open at its tip. For further details see De Vries & Ball (1980) and De Vries (1984).

Collection and microscopy

Specimens were collected in the Cottesserbeek near Cottessen (SE Netherlands, 50°9′N, 5°9′E) and cultured in the laboratory under constant conditions of 12

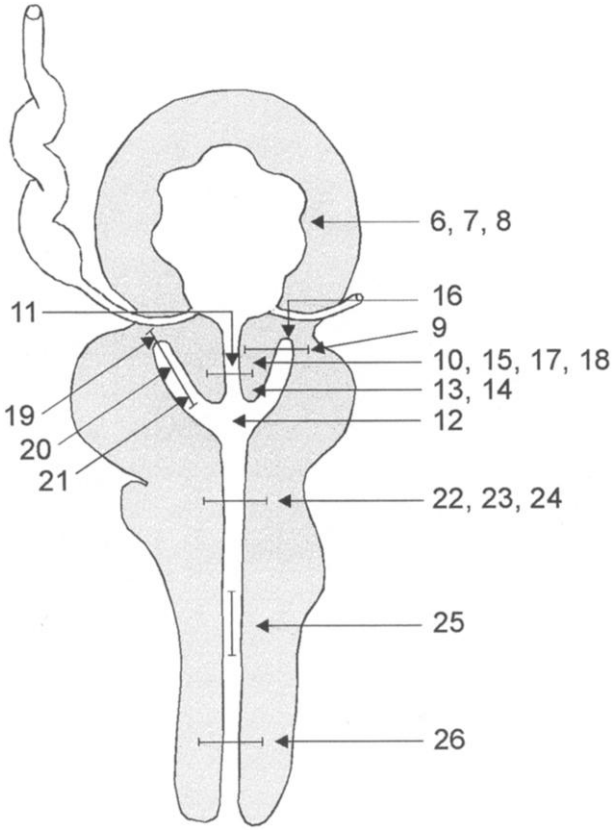


Fig. 5. Diagram of the penial parts where the TEM sections were made (see Figs. 6–26).

h light/12 h dark (day starting at 0700) and 14 ± 1°C. Animals were kept in constantly filtered and aerated stream-water and fed twice weekly with punctured fresh midge larvae (*Chironomus*).

Light microscopy: Unmated individuals isolated for 1–14 days (n = 12) and individuals taken at different times during (n = 14) and after (n = 8) copulation were fixed with Steinmann's fluid (1 part concentrated nitric acid, 1 part saturated solution of mercuric chloride in 5% sodium chloride, 1 part distilled water), stored in 70% ethanol, and embedded in paraffin. Serial 8-μm sagittal or frontal sections were stained with haematoxylin Erlich or Heidenhain, and counterstained with eosine (Romeis 1968).

Electron microscopy: Six individuals were kept in isolation for 7 days, then cut transversely just behind the pharynx. The posterior parts were fixed in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.2; 350 mOsm), postfixed in cacodylate-buffered 2% osmium tetroxide, dehydrated in an acetone series, and embedded in araldite. The animals are too large (up to 25 × 4 mm) to be fixed in pairs in copula for ultrastructural studies. Semi-thin and ultra-thin sections were made with a Reichert ultramicrotome. Semi-thin 0.3 μm sections were stained with thionine methylene blue. Ultra-thin 50–70 nm sections were stained with uranyl acetate and lead citrate for transmission electron microscopy (Phillips 400 TEM).

Table 1. Overview of the different secretions produced by the penis of *Dugesia gonocephala*.

Secretion	Source	Possible function
I, II	Seminal vesicle	Dilution and activation of spermatozoa after copulation
III	Tip of diaphragm conus	Lining granules: anti-agglutination of spermatophore wall
IV	Diaphragm conus & proximal part of ejaculatory duct	Cohering granules: formation of spermatophore wall
V	Middle & distal part of ejaculatory duct	Coating granules: protection of spermatophore wall
VI	Muscular ridge	Adhesion of gonopores during copulation

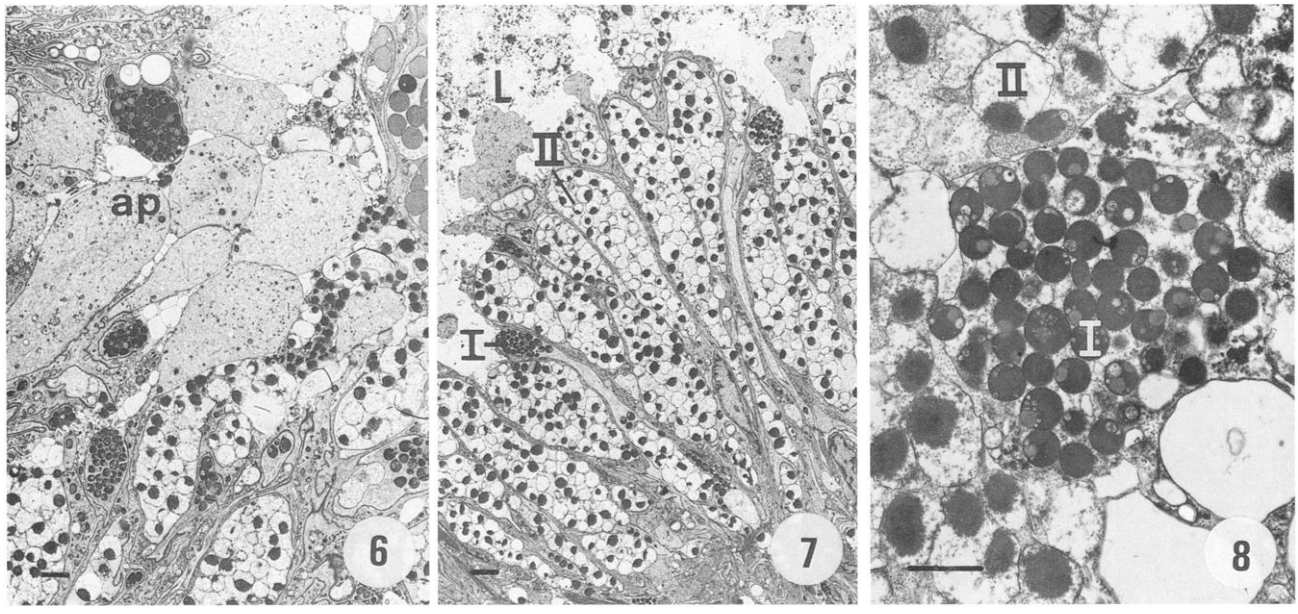
Results

Spermatophore formation: light-microscopy (Figs. 3, 4)

In unmated worms, eosinophilic secretions of glands in the penis diaphragm and in the most proximal part of the ejaculatory duct accumulate in the lumen of the funnel-shaped part of the ejaculatory duct and cohere to form a thin-walled, sclerotized tube, sealed at the distal end (Figs. 3, 4). The inner surface of the elongating tube is lined with secretions of glands at the tip of the diaphragm conus (Fig. 4). The outer surface bears a diffuse coating of highly eosinophilic secretions from glands in the posterior part of the ejaculatory duct; these diffuse secretions do not contribute to the tube wall.

Small clusters of sperms, which are released from the posterior, muscular regions of the sperm ducts, mingle with seminal vesicle gland secretions in the lumen of the unexpanded seminal vesicle (Fig. 3). The resulting sperm mass is transported through the narrow canal of the diaphragm conus into the lumen of the elongating tube, causing the sealed end to inflate, thus transforming the tube into a spherical, stalked bladder (Fig. 3). The inflated part usually protrudes from the penis tip. This pattern was observed in 7 of 12 unmated individuals. In the remaining 5 individuals, the inflated part contained only seminal secretions, but no visible sperms.

During copulation, the spermatophore is further



Figs. 6–8. Seminal vesicle of *D. gonocephala.* TEM. Scale bars, 1 μm. **Fig. 6.** Epithelial cells with a smooth, expanded apical end (ap). **Fig. 7.** Gland cells secreting (I) small, spherical, electron-dense granules and (II) large granules of light to medium electron density, with a small peripheral body of much higher density. Type II granules leave only narrow borders of cytoplasm in the cells. Both types of granules are secreted into the vesicle lumen (L). **Fig. 8.** Lumen of the seminal vesicle filled with dense groups of type I granules and "breaking down" type II granules.

filled with a mixture of sperms and seminal secretions, so expanding the bladder to its full volume (mean and SD = 0.26 ± 0.11 mm^3, range = 0.06–0.50 mm^3). Filling of the spermatophore ends with the transfer of the spermatophore into the partner's bursa. No sperms or seminal secretions are transferred after spermatophore exchange is completed.

Spermatophore formation: ultrastructure (Figs. 5–26)

Sections were made at the level of the seminal vesicle, the diaphragm conus, and the proximal, middle, and distal part of the ejaculatory duct (Fig. 5). Roman numbers refer to the secretions of the different penial parts (see also Table 1).

The inner epithelium of the seminal vesicle consists of non-secretory cells with a smooth, expanded apical end (Fig. 6) and two types of gland cells (Figs. 7, 8). The first type (I) secretes dense groups of small, spherical granules of high electron density. The second type (II) secretes large granules of light to medium electron density, with a small, peripheral body of much higher density. These large granules, which tend to break down in the lumen of the seminal vesicle, are so numerous, that they leave only narrow borders of cytoplasm in the cells (Fig. 7).

The non-secretory epithelial cells of the diaphragm conus are also characterized by a smooth, expanded apical end. The subepithelial gland cells are of two different types. The first type (III) produces large, electron-lucent granules (Figs. 9, 10), which are secreted in large numbers along the central diaphragm canal (Fig. 11) and at the tip of the diaphragm conus (Figs. 12–14). The second type (IV) produces small, electron-dense granules (Figs. 9, 10, 15), which are secreted into the funnel-shaped part of the ejaculatory

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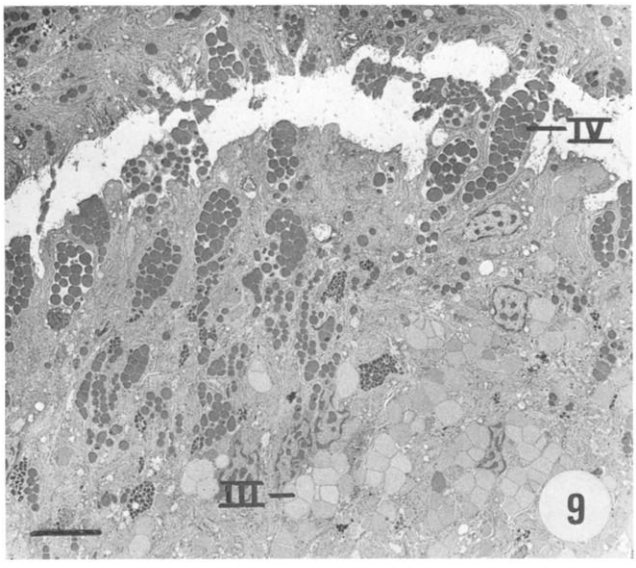
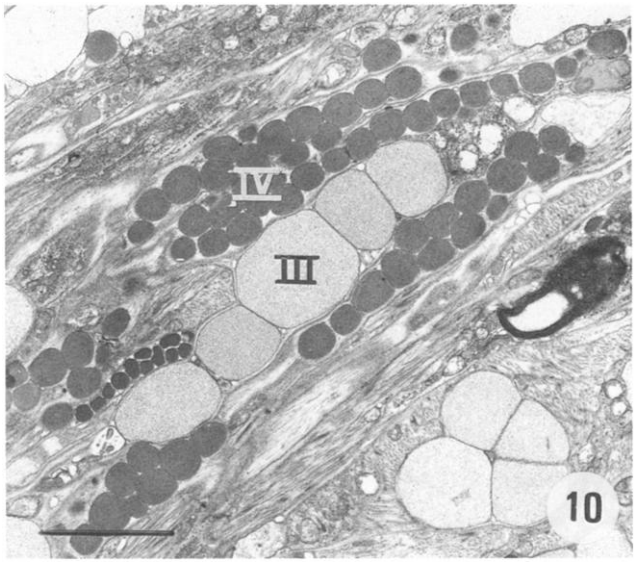
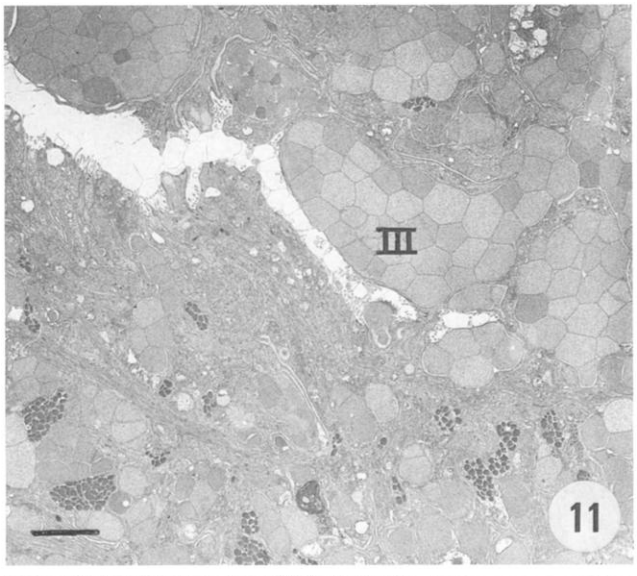
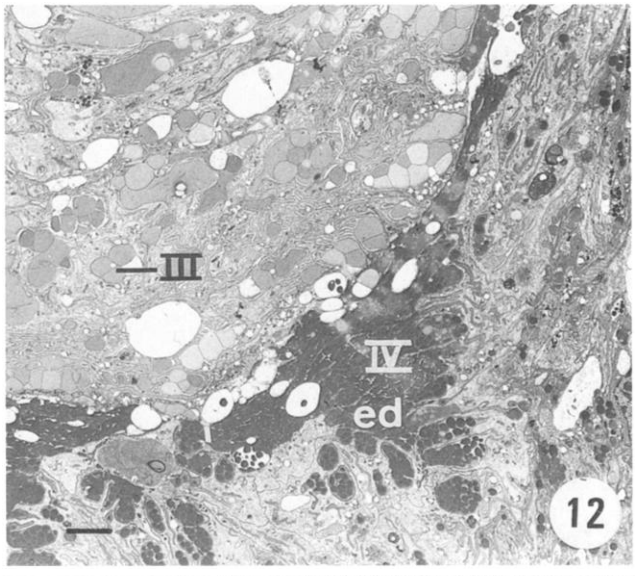
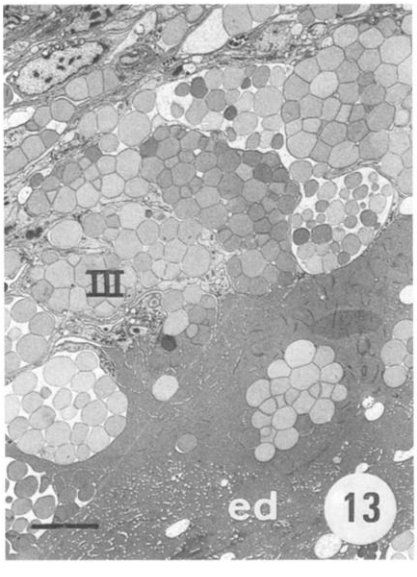
Figs. 9–15. Diaphragm conus of *D. gonocephala.* TEM. Scale bars, 3 μm. Figs. 9–11, 13–15, transverse sections; Fig. 12, longitudinal section. **Figs. 9, 10.** Two types of granules produced by subepithelial gland cells of the diaphragm conus: large, electron-lucent granules (III) and small, electron-dense granules (IV). **Figs. 11–14.** Secretion of type III granules along the central diaphragm canal (Fig. 11) and at the tip of the diaphragm conus (Figs. 12–14). Ejaculatory duct (ed). Note the presence of type IV granules in the lumen of the funnel-shaped, proximal part of the ejaculatory duct (Fig. 12). **Fig. 15.** Subepithelial gland cells producing type IV granules.

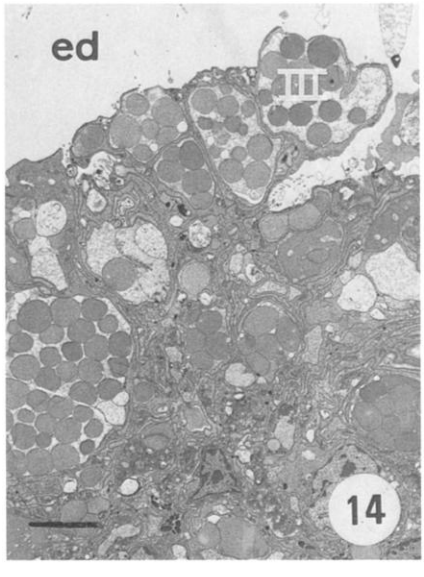


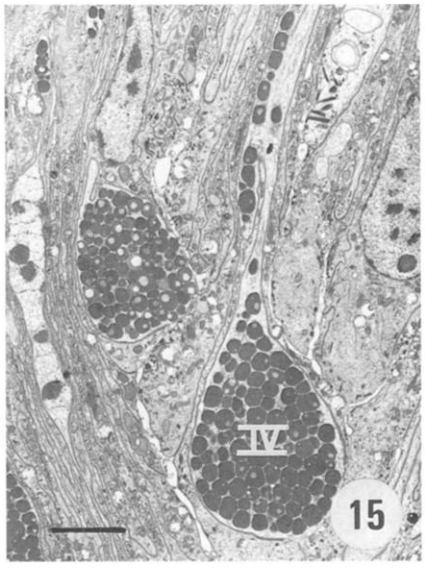


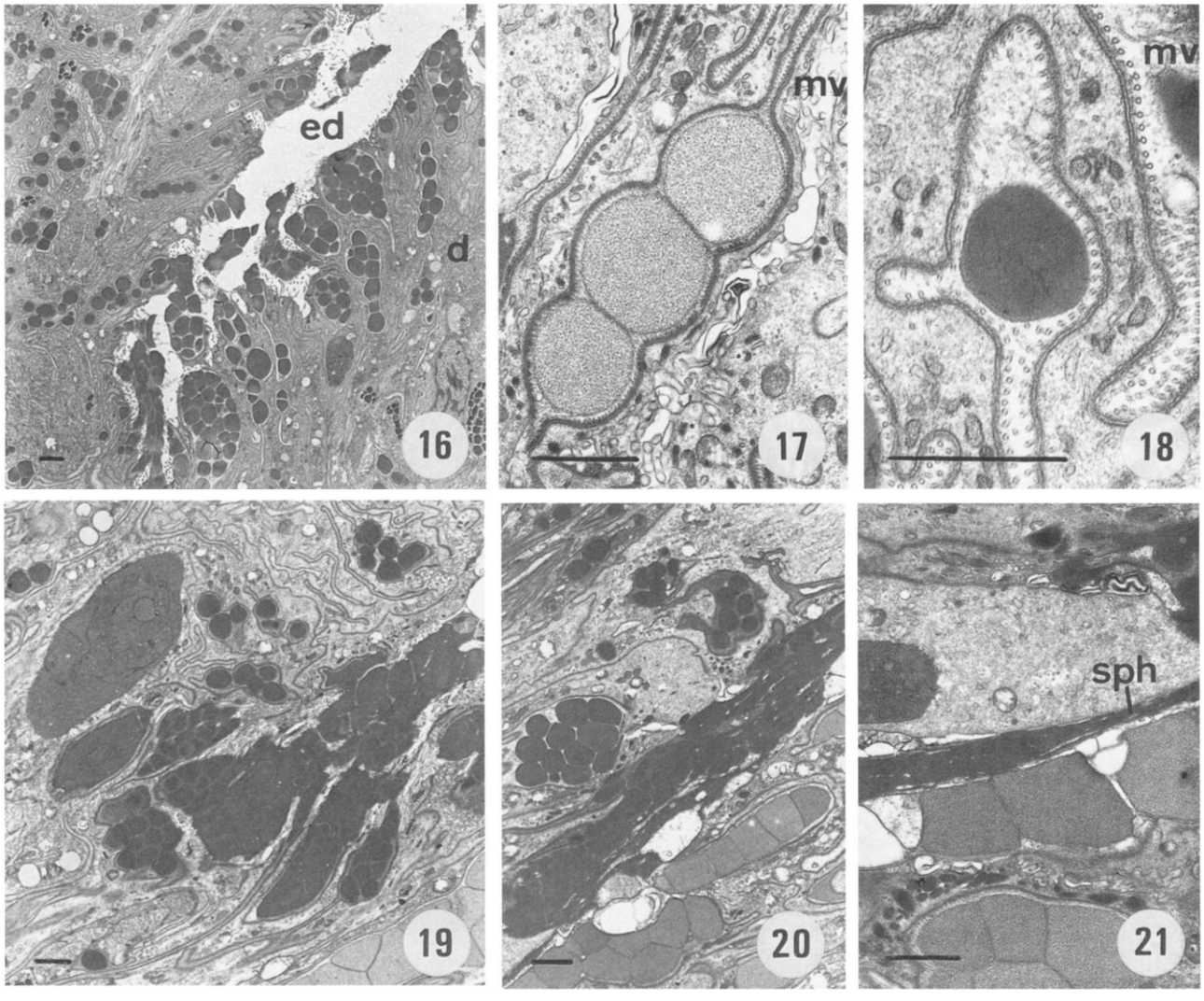












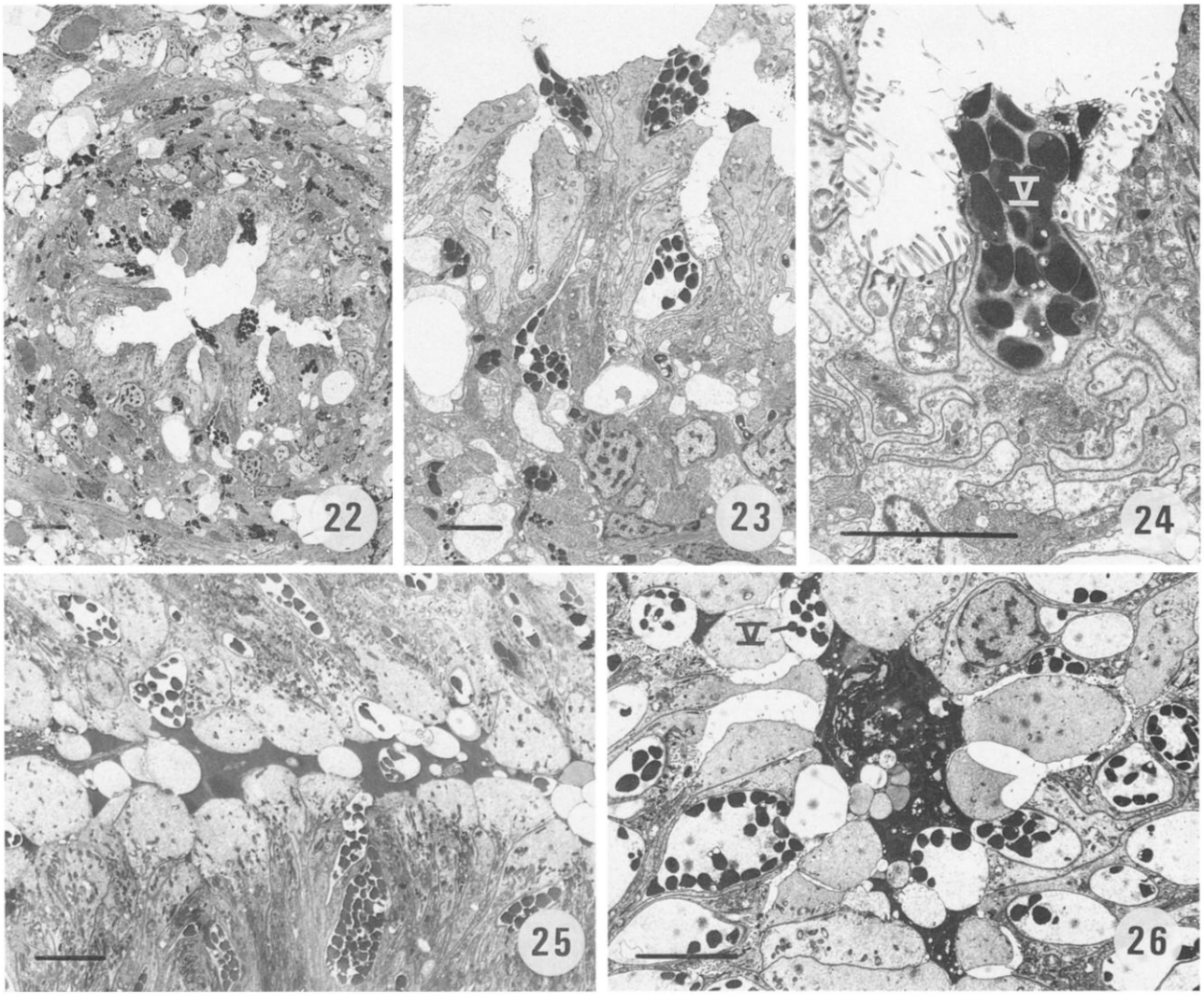
Figs. 16–21. Transverse sections of the diaphragm conus of *D. gonocephala.* TEM. Scale bars, 1 μm. **Fig. 16.** Subepithelial gland cells of the diaphragm conus (d) and of the most proximal part of the ejaculatory duct (ed) secreting type IV granules into the funnel-shaped, proximal part of the ejaculatory duct. **Figs. 17, 18.** Cell necks lined by microvilli (mv), of glands producing type III (Fig. 17) and type IV (Fig. 18) granules. **Figs. 19–21.** Type IV granules coalesce (Figs. 19, 20) to form a homogenous spermatophore wall (sph) (Fig. 21).

duct (Fig. 16). Type IV granules are also produced by subepithelial gland cells of the most proximal part of the ejaculatory duct (Fig. 16). The cell necks of both gland types are lined by microvilli (Figs. 17, 18). Once secreted, the small granules coalesce (Figs. 19, 20) to form a homogenous spermatophore wall (Fig. 21).

The epithelium of the middle part of the ejaculatory duct consists of non-secretory cells with an organelle-free apical region (Fig. 22), and subepithelial gland cells that produce big, irregularly shaped, electron-dense granules (V) (Figs. 23, 24). The secretory ducts are lined by microvilli (Fig. 24). The same is found in the distal part of the ejaculatory duct, but here the non-secretory cells have an expanded apical end (Figs. 25, 26).

Spermatophore transfer: light-microscopy (Figs. 27–31)

In unmated individuals, most of the atrial cavity is filled with the folded penis papilla. The seminal vesicle is unexpanded and the conical diaphragm extends into the lumen of the funnel-shaped part of the ejaculatory duct (Fig. 3). In copulating pairs, the tail ends of both mating partners are elevated and pressed together in the region of the widened gonopores, which cohere by highly eosinophilic secretions from glands in the muscular ridge (VI). Each mating partner inserts its penis papilla with the inflated spermatophore into the vaginal area of the bursal canal of the partner. The penis papilla elongates, the penial fold disappears, the dia-



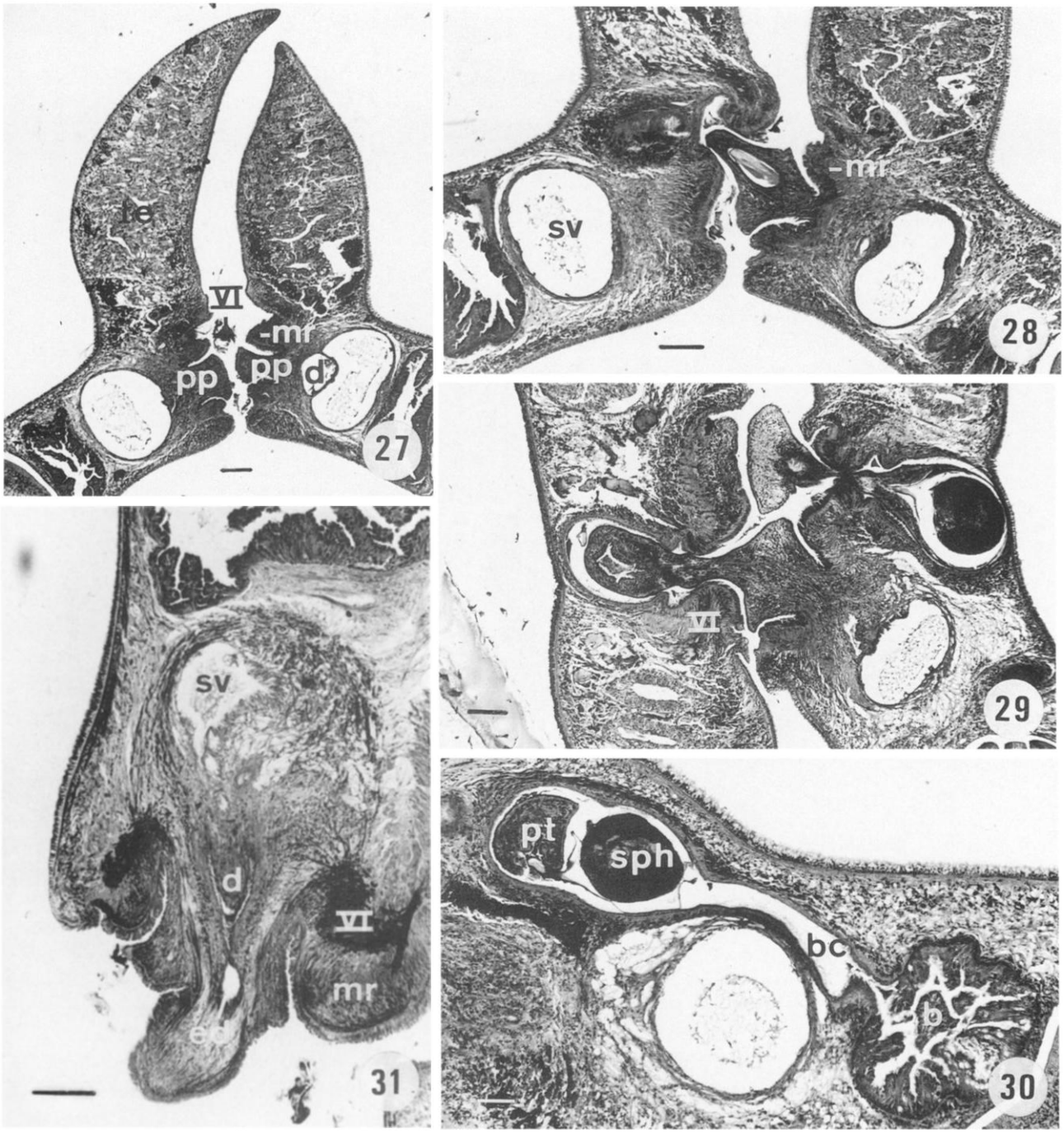
Figs. 22–26. Ejaculatory duct of *D. gonocephala.* TEM. Scale bars, 3 μm. Figs. 22–24, 26, transverse sections; Fig. 25, longitudinal section. **Figs. 22–24.** Middle part of the ejaculatory duct showing epithelial cells with an organelle-free apical region and subepithelial gland cells producing large, irregular-shaped, electron-dense granules (V). The secretory ducts are lined by microvilli (Fig. 24). **Figs. 25, 26.** Distal part of the ejaculatory duct showing epithelial cells with an expanded apical side and subepithelial gland cells producing large, irregular-shaped, electron-dense granules (V).

phragm conus inverts into the seminal vesicle, and the lumen of the seminal vesicle expands and is filled with a loosely packed (presumably fluid) substance (Figs. 27–30). The source of this substance remains unclear. With the transfer of the spermatophore into the partner's bursa, the seminal vesicle collapses and the diaphragm conus re-extends into the lumen of the ejaculatory duct (Fig. 31).

Discussion

Spermatophores play a crucial role in understanding planarian mating systems. In the freshwater flatworm *Dugesia gonocephala,* spermatophores are thought to have triggered the evolution of mate choice by size (Vreys & Michiels 1997), probably because "male" mating rate is limited owing to the time needed (~2 days) for spermatophore formation (Vreys et al. 1997a). Despite their relative importance from an evolutionary perspective, little is known about how planarian spermatophores are formed and transferred. This study revealed that spermatophores in *D. gonocephala* are formed in the ejaculatory duct of the penis by penial gland secretions. The same was described by Weiss (1910) for the planarian *Romankenkius hoernesi* (Weiss 1909), by Sluys & Rohde (1991) for *R. libidinosus* Sluys & Rohde 1991 and by Sluys (1997) for *R. boehmigi* (Weiss 1909).

The spermatophore of *D. gonocephala* consists of



Figs. 27–31. Sections of individuals fixed in copula. LM. Scale bars, 100 μm. **Figs. 27–30.** Before spermatophore transfer. Mating pairs with mutually inserted penises (pp), expanded seminal vesicles (sv) filled with a loosely packed substance, and inverted diaphragm coni (d). The tail ends (te) are elevated and pressed together. Note the highly eosinophilic secretions (VI) of glands in the muscular ridge (mr) between the two widened gonopores (Figs. 27, 28). In Fig. 30 the penis tip (pt) with inflated spermatophore (sph) is inserted in the vaginal area of the bursal canal (bc) of the partner. Note the distance between the penis tip (pt) and the bursa copulatrix (b). **Fig. 31.** After spermatophore transfer. A mating individual that separated from its partner during fixation. Note the collapsed seminal vesicle (sv), the diaphragm conus (d) extending into the lumen of the ejaculatory duct (ed) in the elongated penis papilla, and the strongly eosinophilic secretions (VI) in the muscular ridge (mr).

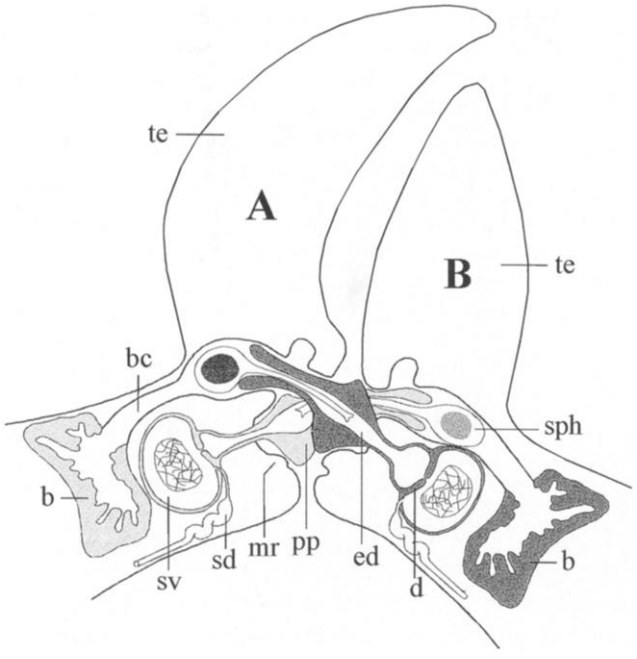


Fig. 32. Diagram of the genital organs of a copulating pair of *Dugesia gonocephala.* Reconstruction based on a series of sagittal sections of a pair fixed in copula after 3 h of copulation, before spermatophore transfer was completed. The two mating partners, A and B, are shaded in light and dark gray, respectively. Bursa (b); bursal canal (bc); diaphragm conus (d); ejaculatory duct (ed); muscular ridge (mr); penis papilla (pp); sperm duct (sd); spermatophore (sph); seminal vesicle (sv); tail end (te).

three layers, each with a different origin and function (Table 1). The middle layer, the actual spermatophore wall, is formed by small, cohering granules from glands in the diaphragm conus and the most proximal part of the ejaculatory duct. An inner layer of large granules, produced by glands in the tip of the diaphragm conus, probably prevents agglutination of the "sticky" wall, so keeping the lumen of the spermatophore open, in order to receive seminal material. Highly eosinophilic granules from glands in the middle and distal part of the ejaculatory duct form a diffuse outer coating, possibly to protect the thin-walled spermatophore from damage during transfer.

Before copulation in *D. gonocephala,* small clusters of sperms may already be discharged from the enlarged, middle sperm-duct regions (= spermiducal vesicles), into the lumen of the seminal vesicle, probably controlled, as in *Girardia tigrina* (Fischlschweiger 1990), by peristaltic movements of the posterior, muscular region of the sperm ducts. Released sperm cells mingle with two types of seminal secretions, very similar to those produced by *G. tigrina* (Fischlschweiger 1990). These secretions may dilute and activate the spermatozoa after the copulation, so aiding their migration from the partner's bursa to the seminal receptacles near the ovaries.

In contrast to *G. tigrina* (Fischlschweiger 1990), however, the mixture of sperms and seminal secretions (or seminal secretions only) is not stored in the seminal vesicle itself, but is transferred before copulation into the lumen of the tubular spermatophore, causing the sealed end to inflate and to protrude slightly from the penis tip. The seminal vesicle of *D. gonocephala,* therefore, merely functions as a prostatic organ, as is probably true for most freshwater triclads (see Hyman 1951), and not as a sperm storage organ, as in *G. tigrina* (Fischlschweiger 1990, 1992). In *G. tigrina,* leakage of sperms into the ejaculatory duct, and thus the likelihood of selfing, is probably reduced by the high viscosity of the seminal secretions (Fischlschweiger 1992). The risk of selfing is minimal in *D. gonocephala,* because the spermatophore in the penis receives all sperms released from the seminal vesicle.

The presence of a slightly inflated spermatophore in the penis induces mating in *D. gonocephala* (Vreys et al. 1997a). The copulation position (see Fig. 32) is similar to that of other planarians (for overview see Vreys et al. 1997b), in that the tail ends of both mating partners are elevated and pressed together in the region of the widened gonopores. Sticky secretions from glands in the muscular ridge, which probably correspond to the secretions from club-shaped cells on the rims of the genital pores (see Burr 1928), serve to hold the gonopores together (Table 1).

During copulation in *D. gonocephala,* the penis papilla extends substantially in length, probably aided by the disappearance of the circular fold at the papilla base. Elongation of the penis papilla must be accompanied by an increase in surface area of the lining epithelia. The smooth, expandable apical side of the non-secretory cells of the seminal vesicle, the diaphragm conus, and the distal part of the ejaculatory duct probably serves this function. We did not find epithelial cells with "insunk" basal parts that move to the surface during copulation, as Fischlschweiger (1992) described for *G. tigrina.* Microvilli in the cell necks of the different gland types, thought to aid transport of granules (see Rieger et al. 1991), might also help to reinforce the cell necks' walls, which are under severe tension during copulation.

In our sections, spermatophores were either attached to the penis, or deposited in the bursa of the partner. Never were they found free in the bursal canal. This suggests that a powerful mechanism is needed to transport the spermatophore over the distance between the penis and the bursa (see Fig. 30). We propose that the

fluid-filled seminal vesicle might work as a kind of mechanical pump. We reason that, because the transfer of a spermatophore results in collapse of the seminal vesicle lumen, and re-extension of the diaphragm conus into the ejaculatory duct, the content of the seminal vesicle is forced out, probably by strong contractions of the muscular penis bulbus, in this way propelling the spermatophore out of the ejaculatory duct and along the partner's bursal canal into the bursa. That ejaculation in *D. tigrina* also results in collapse of the seminal vesicle lumen (Fischlschweiger 1990, 1992) is noteworthy in this context.

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