Abstract

JOHNSON, JEFFERY ALLAN. A histological comparative study on sperm competition inside the spermathecae in the grasshopper species, *Dichromorpha viridis* and *Chortophaga viridifasciata* (Orthoptera: Acrididae). (Under the direction of Marianne Niedzlek-Feaver).

The mechanism of sperm transfer and sperm organization inside the spermatheca was investigated in two grasshoppers, *Dichromorpha viridis* (Scudder) and *Chortophaga viridifasciata* (DeGeer). The spermathecae were examined histologically from females whose copulations were interrupted at various prescribed intervals, either during their first or subsequent mating. Sperm organization inside the spermatheca from 24 to 120 hours after copulation had terminated was also investigated in *D. viridis*. Scanning and transmission electron microscopy were used to investigate the distribution and morphology of small hair-like structures inside the spermathecae.

In both species sperm were first observed inside the spermatheca approximately 30 minutes after the initiation of copulation. The majority of sperm transferred into the spermatheca were in the form of sperm bundles, or spermatodesmes. In *D. viridis* the rate at which sperm bundles were transferred appeared to decrease after 13 hours into copulation (average mating duration in the laboratory was 28 hours). In *C. viridifasciata* the rate of sperm transfer remained constant throughout copulation (average mating duration in the laboratory was 1.3 hours). The occurrence of both an abundance of individual sperm and sperm bundles was observed only in females of *D. viridis* who had mated previously and had a second copulation of less than 9 hours in duration and in females of *C. viridifasciata* that had a second copulation less than 45 minutes in duration. As mating continued, fewer and fewer individual sperm were observed, and by 15 hours into copulation in *D. viridis* and 1 hour in *C. viridifasciata* only sperm bundles were observed inside the

spermatheca. Therefore, it appears that the majority of the individual sperm in the above copulations are from prior matings, while most of the sperm bundles are from the last copulation. The interrupted matings of previously mated females indicate that at least some sperm bundles remain inside the spermatheca while individual sperm were removed by some mechanism, possibly sperm flushing by an excess of seminal fluid provided by the mating male.

In *D. viridis* some sperm bundles remained intact for at least 7 days after the termination of copulation. The sperm bundles must disassociate into individual sperm prior to fertilizing the female's eggs, and therefore it may be the female that provides the mechanism or chemical stimulus to initiate sperm bundle disassociation prior to oviposition or prior to a second mating or both.

The results of this investigation suggest sperm competition, perhaps mediated by female choice, as a primary reason for lengthy copulations in *D. viridis*. Male weight in *C.viridifasciata* has been documented to play a significant role in female choice and mating duration, whereas in *D. viridis*, other factors such as nutrient transfer may play a significant role in female choice and mating duration. In *D. viridis*, males may also act as mechanical plugs by remaining in copula for an extended time after a sufficient amount of sperm has been transferred, or males may be participating in the process of sperm removal by transferring an excess of seminal fluid to "flush-out" any sperm present from previous matings .

A histological comparative study on sperm competition inside the spermathecae in the grasshopper species, *Dichromorpha viridis* and *Chortophaga viridifasciata* (Orthoptera: Acrididae)

by

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Zoology
Raleigh, North Carolina
1998

Approved by:

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Biography

Jeffery Allan Johnson was born on December 30, 1972 in Columbia, Missouri. He graduated from high school at David H. Hickman High School in Columbia in 1991.

Jeff attended Drury College in Springfield, Missouri and graduated with an emphasis in General Biology in 1995. He then started his graduate education in the Zoology Department at North Carolina State University as a Master of Science candidate. Jeff did his research under Dr. Marianne Niedzlek-Feaver. He graduated in May 1998 with a major in Zoology and a minor in Entomology. At that time the future had endless possibilities.

Acknowledgments

I express my appreciation and gratitude to Dr. Marianne Niedzlek-Feaver, Chairperson of Advisory Committee, for her advice, guidance and support during this investigation. I also thank the other members of my committee, Drs. Lew Deitz and Betty Black for their suggestions and assistance.

I thank Sarah Liebling for her assistance in collecting individuals for the laboratory investigations, and Valerie Knowlton (NCSU Center for Electron Microscopy) deserves recognition for her assistance with the electron microscopy work.

A special thanks to my family and friends for believing in me and reassuring me of my flaws.

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Introduction

In many animals, sperm competition has been a major selective force in the evolution of mating strategies (Parker 1970, Smith 1984, Birkhead and Moller 1992). Sperm competition occurs when two or more males inseminate a single female during a reproductive cycle and compete to fertilize her eggs (Parker 1970). In the grasshoppers (Orthoptera: Acrididae), sperm precedence studies suggest sperm competition because the two males that mate with a female often do not contribute equally to paternity and sperm competition is the logical explanation (Bella *et al*. 1992, Hewitt *et al.* 1989, Hunter-Jones 1960, Parker and Smith 1975, Gwynne 1984, Walker 1980, Longo *et al*. 1993, Lopez-Leon *et al*. 1993). Competition among males to fertilize a female's eggs leads to antagonistic mechanisms where a given male either reduces the effectiveness of subsequent matings to protect his sperm or gains precedence in paternity over stored sperm (Parker 1984). The particular adaptations that develop depend on various factors such as the presence and form of sperm storage (i.e., spermathecae), sperm longevity, multiple mating opportunities, and energetic costs associated with reproduction in both sexes (Parker 1970, Smith 1984).

Beyond general descriptions and repertoires of mating behavior, not much work on mating strategies has been done on grasshoppers. The few detailed studies to date treat those species that are territorial or have complex pair formation behavior (Greenfield and Shelly 1985, Niedzlek-Feaver 1995, Otte 1972, Otte and Joern 1975, Shelly and Greenfield 1985, Shelly *et al*. 1987, Steinberg and Willey 1983, Wicker and Siebt 1985, Willey and Willey 1969). The present study treats two species, *Dichromorpha viridis* (Scudder) and *Chortophaga viridifasciata* (DeGeer), in which pair formation, according to Otte (1970), is simple and complex respectively.

Both *D. viridis* and *C. viridifasciata* belong to the family Acrididae (short-horned grasshoppers) of the order Orthoptera. *D. viridis* is currently placed in the subfamily

Gomphocerinae and *C. viridifasciata* is in the subfamily Oedipodinae (Otte 1981, 1984).

In the species *D. viridis*, no sign of any behavior that could functionally be categorized as "courtship" has been observed in the field (Gooding 1996). Although acoustical signals do not precede mating in the field of *D. viridis*, they are used by copulating males, presumably to thwart would be intruders. Males have not been observed to fight except in attempts by one male to dislodge a copulating male. The behavioral data indicate that it is the copulation itself that is the primary social concern in *D. viridis*. Given preliminary data that copulation duration is highly variable although generally lengthy (28.5 hours on average in the laboratory), this species becomes a choice candidate for studies directed at assessing the significance of "cryptic" female choice (choice exerted after pair formation) and male sperm competition in shaping the mating system.

In the species *C. viridifasciata*, pair formation behavior seems to be more complex than *D. viridis*. Females appear to "select" males and possibly judge a male's potential nutrient or gene contribution by a male's weight determined during the initial stages of copulation or use visual cues to compare size or physical conditions prior to the on set of copulation (Niedzlek-Feaver 1995). The species *C. viridifasciata* has been characterized as using pre-mating female choice rather than "cryptic" (post-mating) female choice. Although females of *C. viridifasciata* could exercise post-mating choice by remating with another male prior to oviposition and thus removing spermatozoa from the first male, the mating duration of *C. viridifasciata* (1.3 hours on average in the laboratory) suggests pre-mating female choice as a more likely scenario to judge a male's reproductive potential.

The objective of the present study was to investigate the mechanism of sperm transfer in *D. viridis* and *C. viridifasciata* and in doing so partially explain the

differences between the two species with regard to pair formation, and lengthiness and variability of copulation duration.

Materials and Methods

Collection and Rearing of Insects

Adults and nymphs of *Dichromorpha viridis* and *Chortophaga viridifasciata* were collected at North Carolina State University's Schenck Forest and Unity One Research Facility (Wake County, North Carolina).

Adults and nymphs were reared in either communal cages separated by sex or individual cages depending on the study being conducted. The light/dark cycle and temperature remained similar to field conditions at that particular time. Food consisted of a fine ground mixture of Purina cat chow, fish food, oatmeal, and rabbit food. Grass seedlings consisting of bermuda grass and a mixture of wheat and rye were also maintained in all the cages. Fluorescent and incandescent light was supplied for each cage, and Styrofoam cups filled with sand were provided for ovoposition sites in female cages.

To distinguish individuals, different color markings were applied to the pronotum as adults. Weight and measurements of total body, pronotum, femur, and wing lengths were also collected for adults.

Histological Survey

Single and Multiple Interrupted Matings

Either teneral or last instar females and males were collected in the field and maintained in the laboratory until controlled matings could be conducted. Females were housed separately from males, and individuals were marked for identification as soon as they molted into adults. Both single and multiple matings were conducted. Females were isolated from males for at least seven days prior to copulation. Between

6-10 females were placed in the male cage and allowed to form pairs. Once a pair had formed, they were removed and placed in a smaller cage to prevent takeover from another male. The females assigned to single matings were allowed to mate for a prescribed time interval. The females assigned to multiple matings were allowed to mate to completion on the first mating and then reintroduced to the male cage two to five days after the first mating for a second copulation. No oviposition occurred during the experimental period between first and second matings and therefore loss of ejaculates through use for fertilization can be excluded.

In both groups, copulations were interrupted at a prescribed time interval by placing the copulating individuals in a -70 degrees Celsius freezer. The spermathecae were dissected from the female, fixed in Carnoy's fixative for three hours, dehydrated with an ethanol series, and embedded in paraffin. Longitudinal serial sections were cut (7 micrometers) and stained with Feulgen's stain, which is specific for DNA (Boone and Drijver 1986). Feulgen's stain allowed easy recognition of sperm within the spermathecae. By comparing sequential sections so that individual sperm bundles could be identified, the locations and number of sperm bundles transferred to the female's spermatheca during interrupted matings could be assessed. These assessments represent minimum estimates of sperm bundles present because, despite efforts to the contrary, a few sections were probably lost as ribbons when cut or transferred to slides. Also, it was sometimes difficult to tell where one sperm bundle ended and another began. The side of conservatism was followed by tallying only two sperm bundles at the exact same location, if one appeared in that location two or more sections after the other had disappeared. Two sperm bundles that happened to lie on top of one another and were located closer to each other than two sections (14 micrometers) were counted as one bundle.

Sperm Organization x Hours After Copulation Finished (D. viridis only)

Individuals were allowed to mate to completion and then males were removed from the individual pair cages. The females were then placed in a -70 degrees Celsius freezer at the prescribed time period: 24, 48, 72, 96, or 120 hours after the termination of copulation (n=2 for each time period). No oviposition occurred within these prescribed time periods. Therefore loss of ejaculates through utilization for fertilization can be excluded. The spermathecae were prepared for histological examination as described above.

Sperm Organization with Regard to Oviposition (D. viridis only)

Adult females (n=8) were collected from the field and placed immediately in individual cages with oviposition sand cups for seven days. The females were then placed in a -70 degrees Celsius freezer and the presence of any egg pods in the sand cups noted. The spermathecae were prepared for histological examination as described above.

Scanning Electron Microscopy

Virgin mature females were dissected in Hoyle's solution and the spermathecae were carefully cut open using a very sharp blade under a dissection microscope. The individual pieces were fixed in 3% glutaraldehyde (pH 7.4, phosphate-buffered saline) at 4 degrees Celsius for 24 hours. Secondary fixation in osmium tetroxide (2%) was followed by an ethanol dehydration series and critical point drying. Samples were mounted on stubs, sputter-coated with gold-palladium, and examined using a Joel T300 scanning electron microscope or a Philips 505T scanning electron microscope.

Transmission Electron Microscopy(D. viridis only)

Four *D. viridis* females who had mated multiple times and with a second mating interrupted at 2, 5, 6, or 9 hours into copulation were dissected in Hoyle's solution and fixed in 3% glutaraldehyde (pH 7.4, phosphate-buffered saline) at 4 degees Celsius for 24 hours. The samples were rinsed three times each in 20 minute changes of 0.1 M sodium phosphate buffer (pH7.4) and post-fixed in 1% osmium tetroxide for 3 hours at 4 degrees Celsius. The buffer wash was repeated and then the samples were dehydrated through an ethanol series. The samples were then placed into 1:1 Spurr's:ethanol for 1 hour. Infiltration continued with a 1 hour change of 3:1 Spurr's:ethanol, and then three 2 hour changes of 100% Spurr's with vacuum at room temperature. The samples were flat embedded in inverted BEEM capsules at 70 degress Celsius.

Thin sections were cut with a microtome using glass knives and collected on grids. Sections were stained with uranyl acetate followed by lead citrate, and examined with a Jeol 100S TEM.

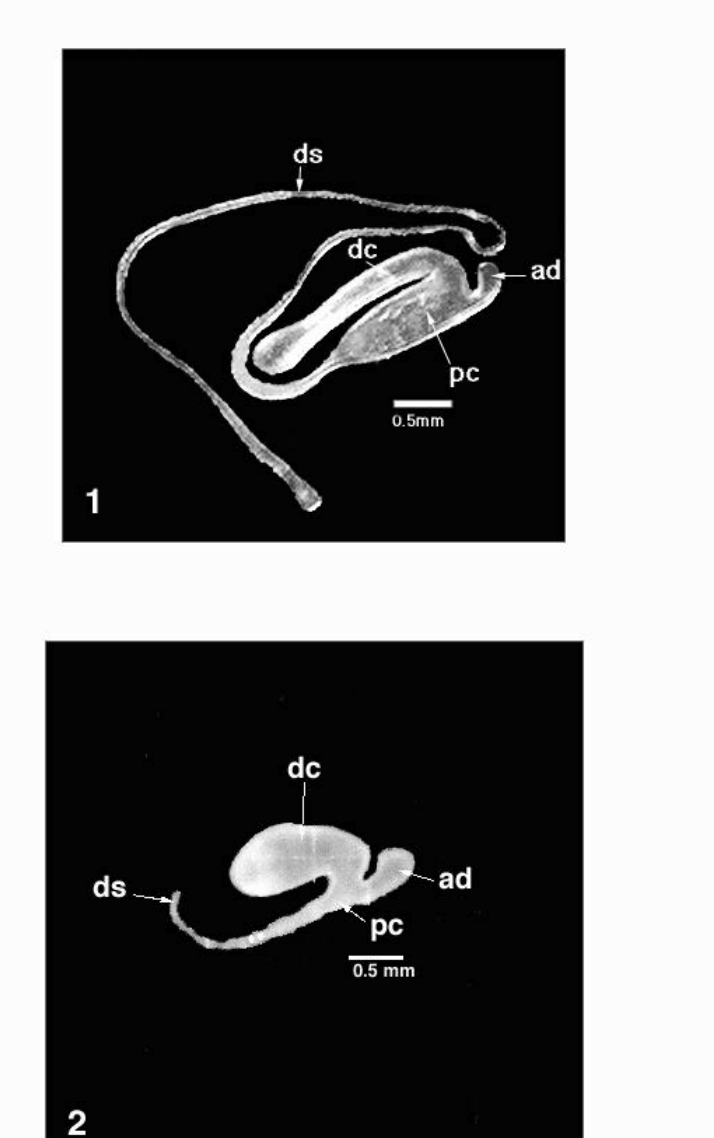
Results

Copulation Duration

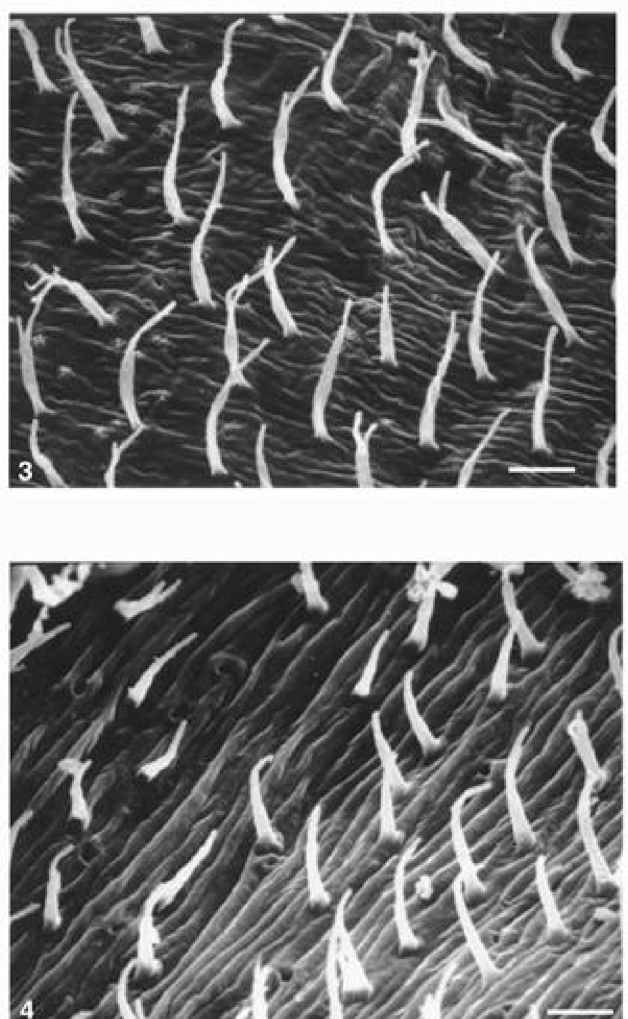
In the current study, mating was defined as taking place if the pair engaged genitalia. Because densities were high in the mating cages, however, "accidental pairings" were common. Feeding females bumped into males who then turned toward them and attempted to mount. Copulatory attempts lasting less than 15 minutes were not scored, to avoid an estimate of copulation duration that would be biased towards accidental encounters not common in nature. For *D. viridis* the mean copulation duration among pairs allowed to mate to completion was 28.5 hours (range: 17.0 to 55.0 hours). For *C. viridifasciata* the mean copulation duration among pairs allowed to mate to completion was 1.3 hours (range: 1.0 to 1.7 hours).

Histological Survey

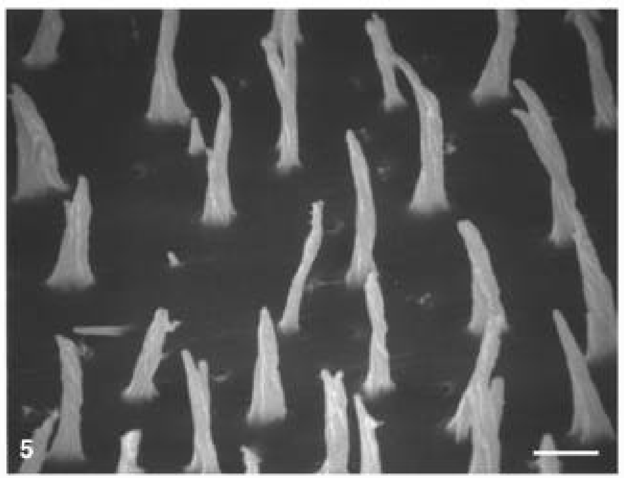
The sperm storage organ, or spermatheca, of *D. viridis* and *C. viridifasciata* consists of various chambers, the preapical diverticulum or distal chamber(dc) for example, and a long duct, the ductus seminalis (ds) (Figs. 1 & 2). The ductus seminalis connects the spermatheca to the genital chamber where fertilization occurs as the eggs pass from the oviducts into the genital chamber (Davey 1985). The external appearance and shape of the spermatheca of *D. viridis* and *C. viridifasciata* are very similar to those of *Locusta migratoria* Reiche and Fairmaire in comparison to other acridid spermathecae (Dirsh 1957, Gregory 1965). Gregory (1965) presented an excellent anatomical survey of the spermatheca, spermatophore formation, and sperm transfer in *L. migratoria*. Gregory described the ductus seminalis as forming a vestibule prior to its connection with the preapical diverticulum and apical diverticulum. *Dichromorpha viridis* and *C. viridifasciata* also have an apical diverticulum (ad) in the form of a small protuberance (Figs. 1 & 2). Herein the vestibule is called the proximal chamber (pc) due to the presence of small hair-like structures on the inside wall similar to the preapical diverticulum, or distal chamber of both species (Figs. 3-6). The spermathecae of *D. viridis* and *C. viridifasciata* differ in the size and shape of the proximal and distal chambers (Figs. 1 & 2) and in the appearance of the hair-like structures occurring within the spermathecae (Figs. 3-6). The distal chamber of *C. viridifasciata* is wider compared to that of *D. viridis*, whereas, the proximal chamber of *D. viridis* is wider than the same chamber of *C. viridifasciata*.

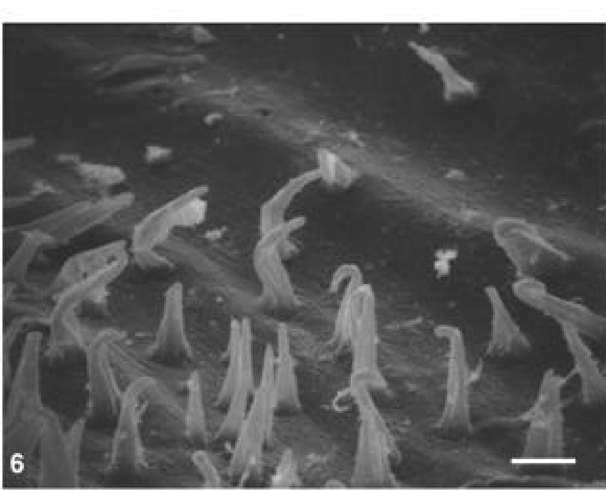


Figures 1 & 2. Light micrographs of spermathecae (*ad* = apical diverticulum, *dc* = distal chamber, *ds* = ductus seminalis, *pc* = proximal chamber). 1. *Dichromorpha viridis*. 2. *Chortophaga viridifasciata*. The ductus seminalis broke during dissection and is similar in length as that shown for *D. viridis*.



Figures 3 & 4. Scanning electron micrographs of the interior spermathecal surface in *D. viridis*. 3. Proximal chamber, 2500x (bar = 5 micrometers). 4. Distal chamber, 2500x (bar = 5 micrometers).





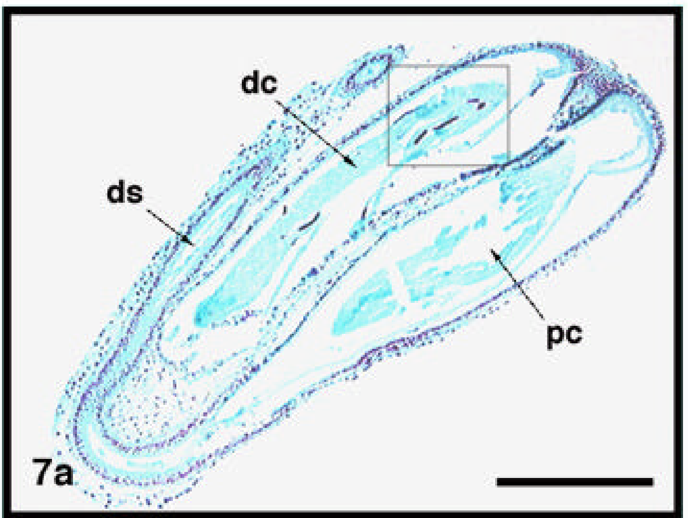
Figures 5 & 6. Scanning electron micrographs of the interior spermathecal surface in *C. viridifasciata.* 5. Proximal chamber, 2500x (bar = 5 micrometers). 6. Distal chamber, 2500x (bar = 5 micrometers).

Single Interrupted Matings with Virgins

Dichromorpha viridis

The first observation of sperm in the spermatheca occurred only after copulation had lasted approximately 30 minutes. During copulation the spermatophore of the male extends the length of the ductus seminalis and into the preapical diverticulum. Sperm is transferred from the male to the female inside a single spermatophore. The majority of the sperm transferred were in the form of sperm bundles, or spermatodesmes, with the spermatozoa being attached at their heads by a hyaline cap (Fig. 7). A few individual sperm were also transferred along with the sperm bundles (Fig. 8). The sperm were released into the spermatheca, and the number of sperm bundles inside the spermatheca gradually increased as copulation progressed (Fig. 9). At one hour into copulation, as many as five bundles were observed in the spermathecae and after three hours of copulation, as many as 14 bundles were observed in the spermathecae. Nevertheless, individual variation occurred. For example, in one mating of two hours in duration, no sperm were transferred, while in another of the same duration, five bundles were transferred.

The rate at which sperm bundles were transferred appeared to decrease after 13 hours (Fig. 9). For example, a mating of five hours yielded 26 bundles, a mating of ten hours 47 bundles, but a mating of 20 hours only 44 bundles. In all matings interrupted before 6 hours, most sperm bundles were found in the distal chamber (98% of all sperm bundles found). After that, equal numbers were found in the distal chamber and the proximal chamber. For example, in the mating interrupted at 13 hours into copulation, 47 bundles were found. Twenty-four bundles were located in the proximal chamber and 23 bundles were located in the distal chamber. Similarly, for a mating of 19 hours, 20 bundles were found in the proximal chamber and 25 in the distal chamber.



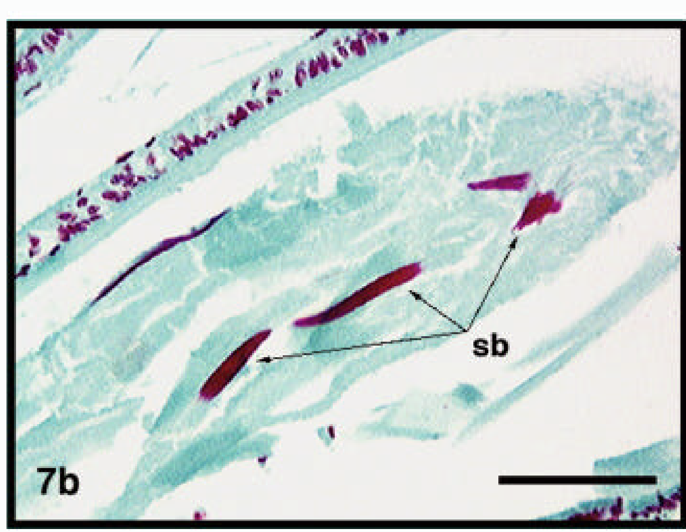


Figure 7. Light micrographs of spermatheca histological section in *D. viridis*; 1.5 hour single copulation (*dc* = distal chamber, *ds* = ductus seminalis, *pc* = proximal chamber, *sb* = sperm bundles). 7a. Sperm bundles in distal chamber (bar = 0.5 mm). 7b. Magnification (20x) of box in Fig. 7a showing sperm bundles in distal chamber (bar = 0.1 mm).

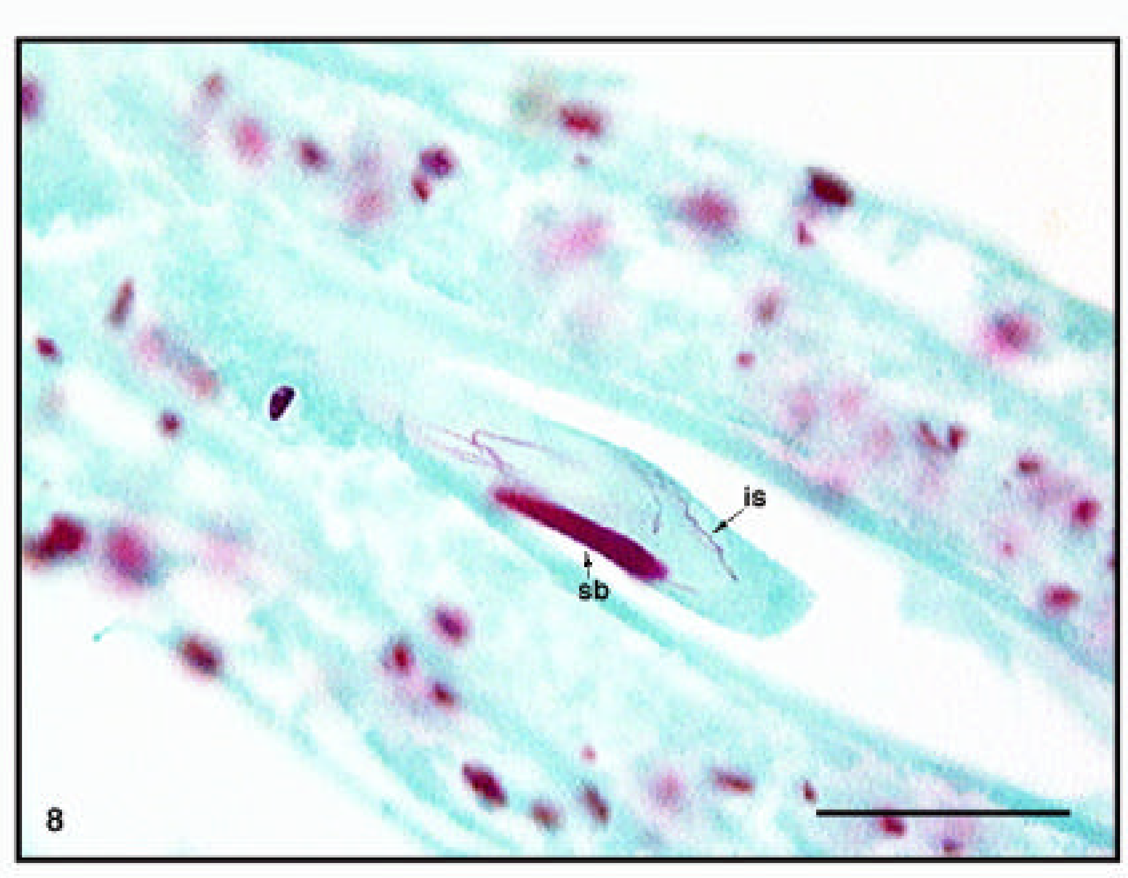


Figure 8. Light micrograph of ductus seminalis histological section in *D. viridis*: spermatophore with sperm bundle and individual sperm; 1-2 hour single copulation (mag bar = 0.05 mm) (*is* = individual sperm, *sb* = sperm bundle).

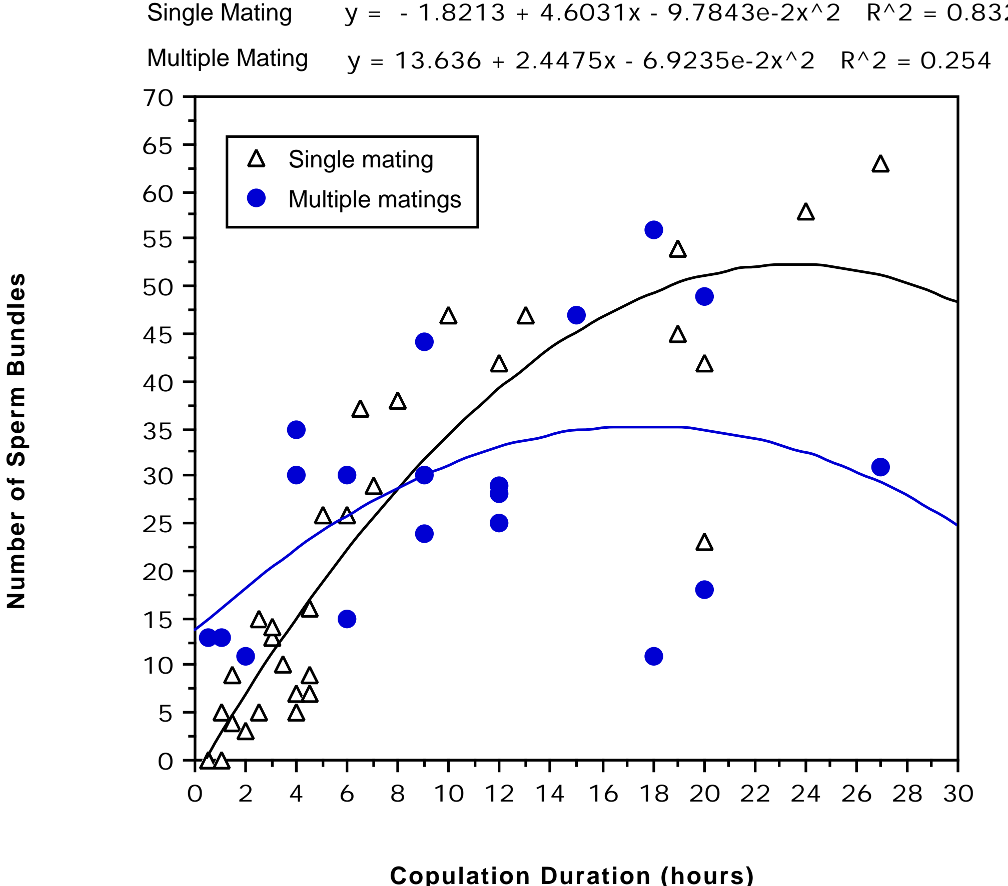
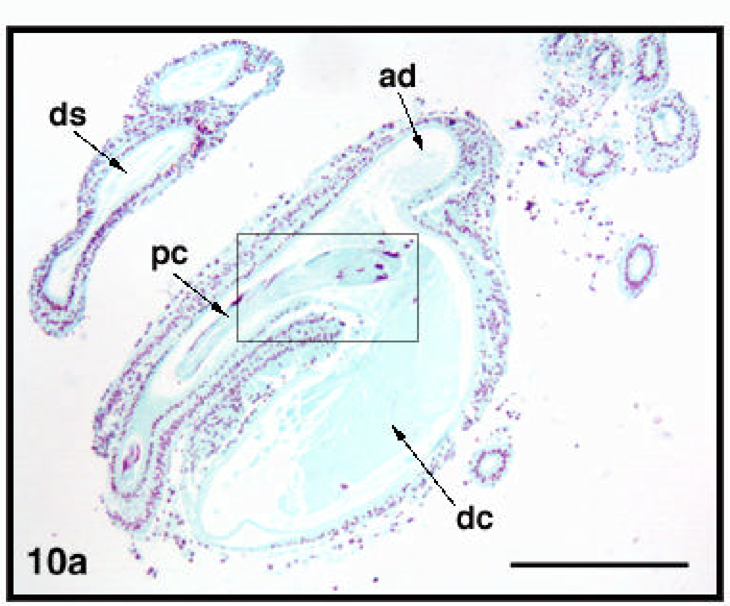


Figure 9. Relationship in *D. viridis* between copulation duration and number of sperm bundles inside the spermathecae of single and multiple mated females.

Chortophaga viridifasciata

The first observation of sperm in the spermatheca occurred only after copulation had lasted approximately 15 to 30 minutes. Like *D. viridis*, the spermatophore of the male extends the length of the ductus seminalis and into the opening of the preapical diverticulum. Sperm is transferred from the male to the female inside a single spermatophore. The majority of the sperm transferred to the spermatheca were also in the form of sperm bundles, or spermatodesmes, and a few individual sperm were observed in the spermathecae of single mated females (Fig. 10). The sperm were released into the spermatheca, and the numbers of sperm bundles gradually increased as copulation progressed (Fig. 11). At 30 minutes into copulation, as many as 25 sperm bundles were observed in the spermathecae and at 45 minutes into copulation, as many as 51 sperm bundles were observed inside the spermathecae. At 1 hour into copulation, as many as 72 sperm bundles were observed inside the spermathecae.

Individual sperm were also observed to increase with copulation duration. Significantly more individual sperm were present 1 hour into copulation than at 30 minutes into copulation. The increase in individual sperm could be interpreted as early sperm bundle fragmentation. In all interrupted matings, most of the sperm bundles were observed in the proximal chamber (70% of sperm bundles observed) and appeared to still be in a spermatophore (Fig. 10). The majority of the individual sperm observed were also in the proximal chamber.



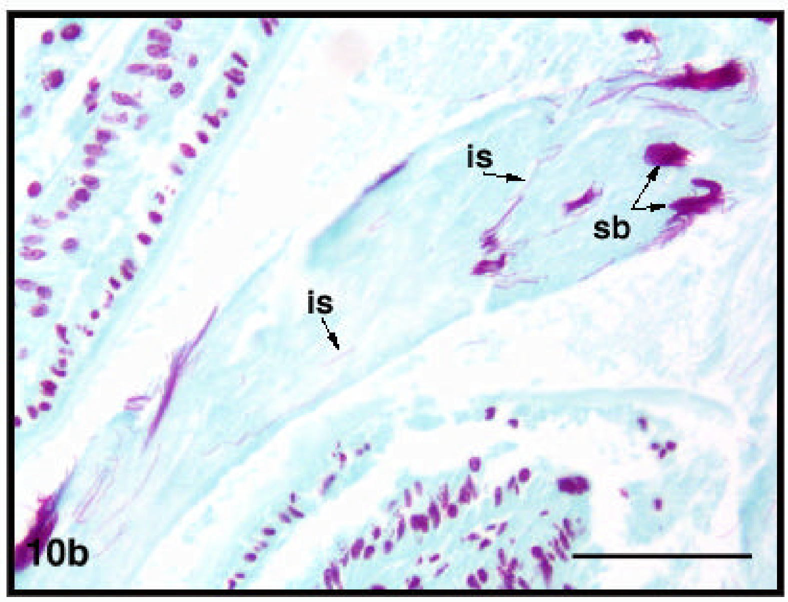


Figure 10. Light micrographs of spermatheca histological section in *C. viridifasciata*; 30 minute single copulation (*ad* = apical diverticulum, *dc* = distal chamber, *ds* = ductus seminalis, *is* = individual sperm, *pc* = proximal chamber, *sb* = sperm bundles). 10a. Sperm bundles and individual sperm in proximal chamber (bar = 0.5 mm). 10b. Magnification (20x) of box in Fig. 10a showing spermatophore with sperm bundles and individual sperm in the proximal chamber (bar = 0.1 mm).

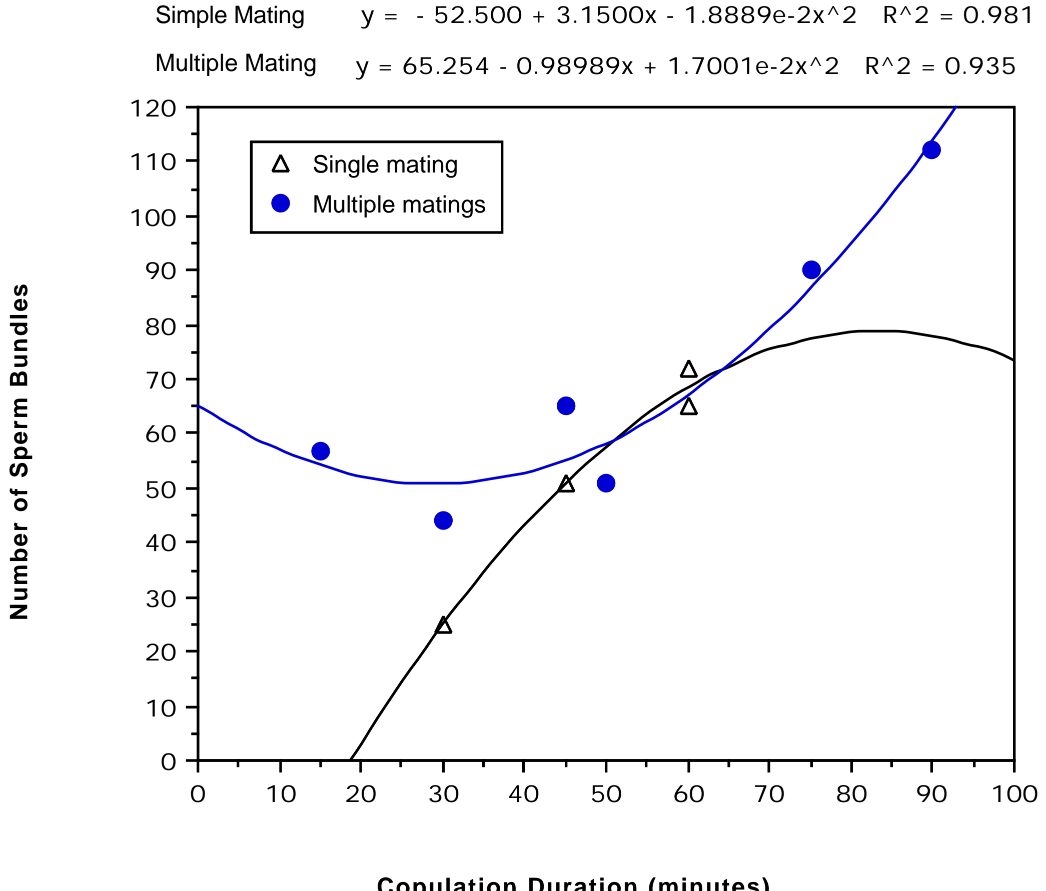


Figure 11. Relationship in *C. viridifasciata* between copulation duration and number of sperm bundles inside the spermathecae of single and multiple mated females.

Multiple Sequential Matings

Dichromorpha viridis

Both sperm bundles and individual sperm were found inside the spermatheca at early mating times in females who had mated twice (Fig. 12). The number of sperm bundles remained high throughout copulation. The numbers found for any given copulation period varied among individuals. For example, after one mating of 18 hours 56 sperm bundles were found in the spermathecae, in another of the same duration, only 11 sperm bundles were found (Fig. 9). Generally, the numbers of sperm bundles found after 4 or fewer hours of copulation in a female that had mated twice are higher than the numbers found in females that had mated only once.

The number of individual sperm present decreased as copulation progressed. After 1 to 4 hours, individual sperm were found throughout the spermatheca (Fig. 12a). Yet at 9 hours, only a few individual sperm were observed inside the spermatheca, and most were present in the proximal chamber of the spermatheca and inside the ductus seminalis. At 15 hours into copulation, only sperm bundles and no individual sperm were observed inside the spermatheca. A few individual sperm were observed inside the spermatheca at 18 and 20 hours into copulation, and their occurrence can be possibly noted as early fragments from the sperm bundles. In some histological sections where matings were terminated before six hours, individual sperm were observed in the "neck" between the distal and proximal chambers, apparently moving from one chamber to the other (Fig. 12). One interpretation consistent with these data is that sperm bundles loose their integrity, break down and the resulting individual sperm move or are moved inside the proximal chamber and eventually out of the spermatheca.

A larger number of sperm bundles were found earlier in the spermathecae of twice-mated females than once-mated females for the same copulation duration . For example, in copulations of 1 hour, 13 sperm bundles were found in the twice-mated

female, and only 5 bundles in the once-mated female (Fig. 9). After about 12 hours of copulation, there appear to be fewer sperm bundles in twice-mated females than in once-mated females.

The location of sperm bundles found inside the spermatheca varied among individuals. After 12 hours of copulation, 21 bundles were found in the distal chamber and 8 in the proximal chamber of one individual. Yet after 15 hours of copulation, 23 bundles were found in the proximal chamber and 7 in the distal chamber of another individual. Almost equal numbers were observed in both chambers in six individuals, more in the proximal chamber (at least 2x as many) in four individuals and more in the distal chamber of six other individuals.

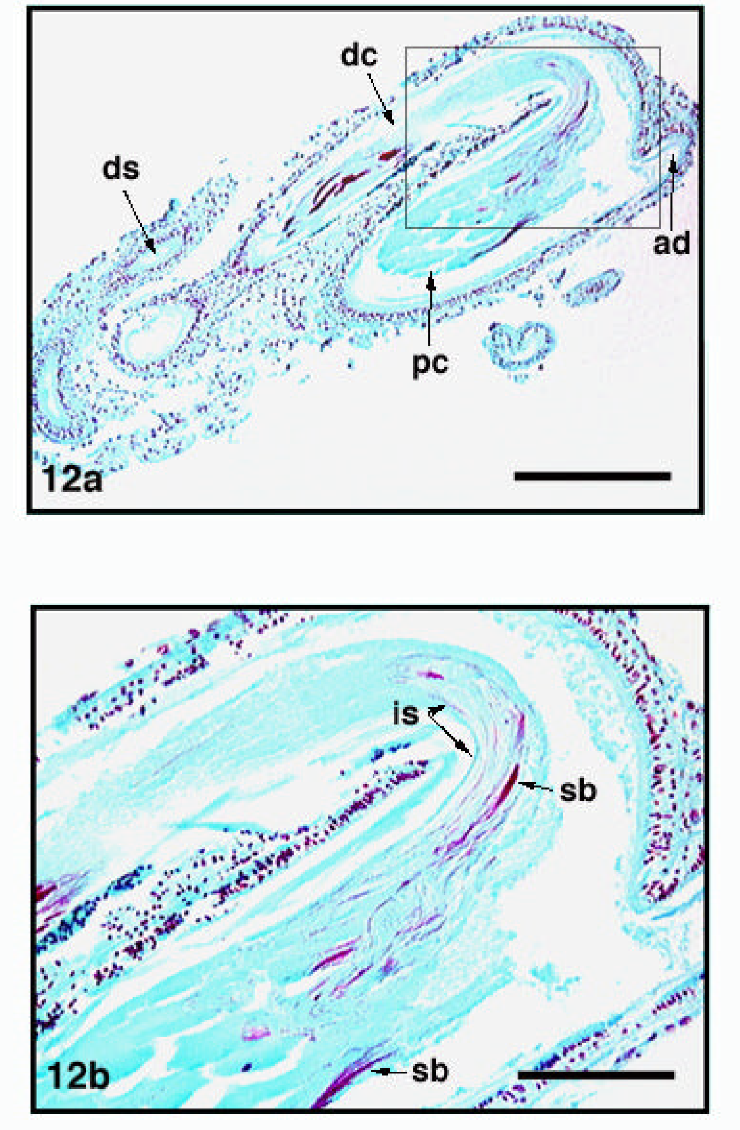


Figure 12. Light micrographs of spermatheca histological section in *D. viridis*; 4 hour multiple copulation (*ad* = apical diverticulum, *dc* = distal chamber, *ds* = ductus seminalis, *is* = individual sperm, *pc* = proximal chamber, *sb* = sperm bundles). 12a. Sperm bundles and individual sperm in distal and proximal chambers (bar = 0.5 mm). 12b. Magnification (10x) of box in Fig. 12a showing sperm bundles and individual sperm (bar = 0.2 mm).

Chortophaga viridifasciata

Both sperm bundles and individual sperm were found inside the spermatheca at early mating times in females who had mated twice (Fig. 13). As in *D. viridis*, the number of sperm bundles remained high throughout copulation (Fig. 11), and it was observed that the numbers of sperm bundles early in a second mating (i.e., 30 min) are higher than the numbers found in females that had only mated once at that same copulation duration.

The number of individual sperm present inside the spermatheca decreased as copulation progressed. After 15 to 30 minutes, individual sperm were found in both chambers, but significantly fewer were observed at 30 minutes than 15 minutes. At 45 minutes, even fewer individual sperm were observed inside the spermatheca, and most were present in the proximal chamber of the spermatheca and inside the ductus seminalis. A few individual sperm were observed only in the proximal chamber around 1 hour into copulation. At 1.5 hours into copulation an increase of individual sperm was observed and could be interpreted as early sperm bundle fragmentation.

A large number of sperm bundles were found earlier in the spermathecae of twice-mated females than once-mated females for the same copulation duration. For example, in copulations interrupted at 30 minutes, 44 sperm bundles were observed in a female who had mated previously and only 25 sperm bundles were found in a female who had not mated previously (Fig. 11). The number of sperm bundles observed inside the spermatheca remained higher in those who had mated previously compared to singly mated females of the same copulation duration.

The location of the sperm bundles inside the spermatheca varied among individuals. At 15 minutes into copulation, 9 sperm bundles were observed in the proximal chamber close to the preapical diverticulum and 48 sperm bundles were observed inside the distal chamber. Yet after 45 minutes into copulation, 32 bundles were observed in the proximal chamber and 33 bundles in the distal chamber. At 1.5

hours into copulation, 51 bundles were observed in the proximal chamber and 61 bundles were observed in the distal chamber.

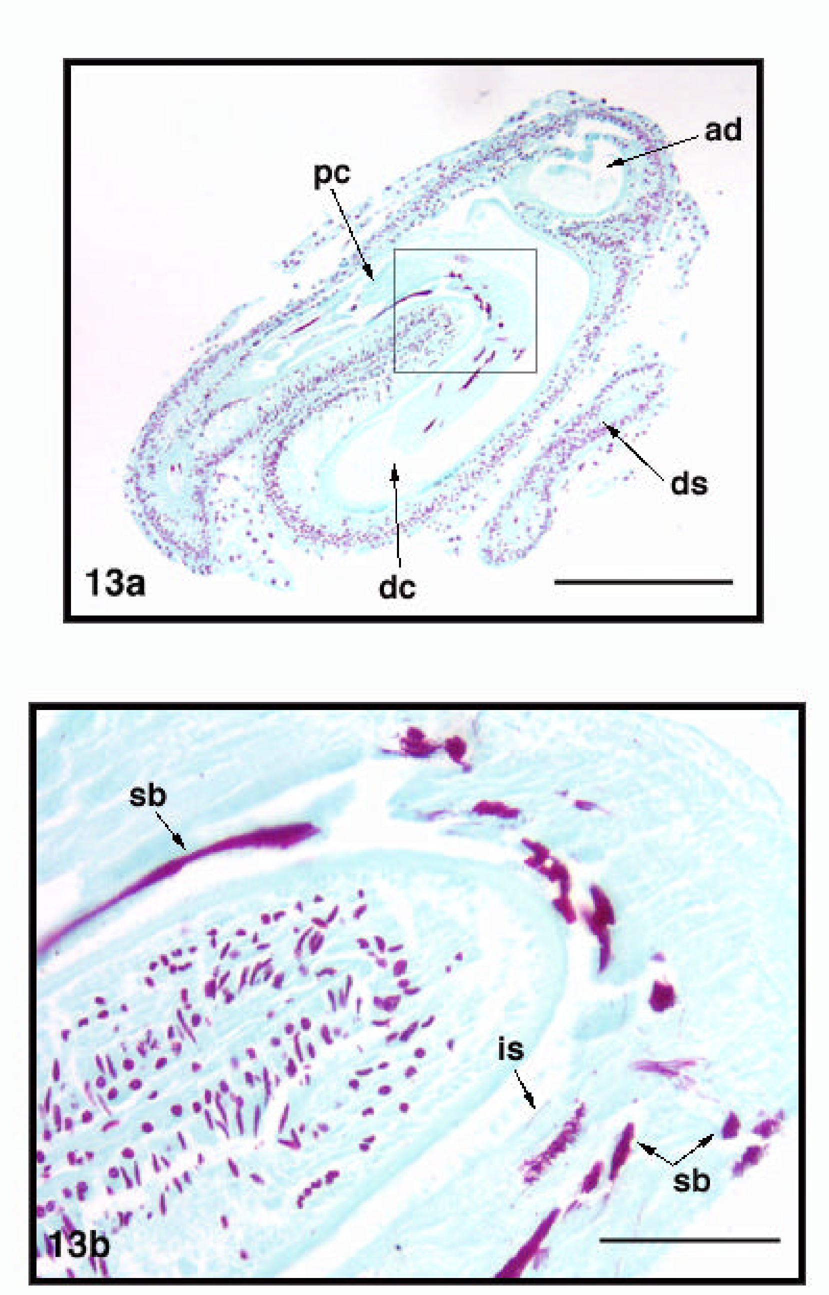


Figure 13. Light micrographs of spermatheca histological section in *C. viridifasciata*; 50 minute multiple copulation (*ad* = apical diverticulum, *dc* = distal chamber, *ds* = ductus seminalis, *is* = individual sperm, *pc* = proximal chamber, *sb* = sperm bundles). 13a. Sperm bundles and individual sperm in distal and proximal chambers (bar = 0.5 mm). 13b. Magnification (20x) of box in Fig. 13a showing sperm bundles and individual sperm (bar = 0.1 mm).

Sperm Organization x Hours After Copulation Finished (D. viridis only)

Histological cross sections of spermathecae were obtained from *D. viridis* females who had been isolated for 24, 48, 72, 96 and 120 hours (n = 2 for each) after the termination of copulation. Sperm bundles were only found within the distal chamber of the spermathecae, with one exception. Sperm bundles were observed in both distal and proximal chambers in those females who were isolated for 24 hours after copulation ended. Individual sperm were present in both the distal and proximal chambers of the spermathecae in all isolated females.

Sperm Organization with Regard to Oviposition (D. viridis only)

Adult *D. viridis* females were held in individual cages and allowed to oviposit in sand cups for up to seven days in the laboratory. In all females, individual sperm were found in both chambers of the spermathecae, and when found, sperm bundles were only observed in the distal chamber. The number of sperm bundles appeared to decrease with the number of egg pods laid. Three females did not oviposit. Thirty sperm bundles were found in two of these females, none in the third. Three females oviposited one egg pod, and two of the three had sperm bundles (n = 10 and 13 bundles). Two females oviposited two egg pods, only one of these females had three sperm bundles. Further data are needed to determine how many pods a female typically lays before remating, whether sperm bundles decrease with egg pods laid, and what occurs if females remate between ovipositions.

Scanning and Transmission Electron Microscopy

Dichromorpha viridis

The inside of the spermatheca consists of an array of small hair-like structures pointing into the lumen of the spermatheca (Figs. 3 & 4). The hair-like structures appear to fit the description of acanthae (type A) given by Richards and Richards (1979). Although their development had not been studied here, each appear formed from one cell and fused to the epicuticle without any associated nerve cells (Fig. 14). The hair-like structures are solid, and possibly, as described by Richards and Richards (1979), their cell processes have withdrawn and the space had become filled with acid mucopolysaccharide or underlaid with endocuticular material.

The acanthae occur throughout the inside of the spermatheca except on one side (the lower lateral side) of the distal chamber which is void of any observable structures (Fig. 15). Three kinds of acanthae occur inside the spermatheca: simple (Fig. 16), bifurcate (Fig. 17), and trifurcate (Fig. 18). The simple (unforked) acanthae occur in both chambers of the spermatheca, but the bi- and trifurcate acanthae are mostly found inside the proximal chamber with only a few bifurcate acanthae in the distal chamber. The acanthae are approximately 5 to 10 micrometers long and 1 to 2 micrometers wide at their base. The surface of the acanthae appear smooth to wrinkled, but lack observable pores (Figs. 16 - 18).

The inside wall, or epicuticle, of the spermatheca in both chambers were observed and described according to the terminology of Harris (1979). The epicuticle of the proximal chamber appear microrugose (wrinkled), and the acanthae appear directed into the lumen of the spermatheca (Fig. 3). The epicuticle of the distal chamber consist of three different conditions. The region close to the junction of the distal and proximal chambers appear microglabrous (smooth) to submicrorugose (slightly wrinkled), and the acanthae appear directed into the lumen of the spermatheca (Fig. 19). The medial epicuticle of the middle to distal region of the distal

chamber appear to have prominent microrugose (wrinkled) sculpturing (Fig. 4). The acanthae in the medial middle region are directed away from the proximal chamber, and those in the distal region are directed out and away from the epicuticle. The lateral epicuticle of the distal chamber had prominent microrugose (wrinkled) sculpturing, but no acanthae (Fig. 15). Likewise, the apical diverticulum had no acanthae and the epicuticle was microrugose.

Small pores were observed in the epicuticle throughout the spermatheca (Figs. 3 & 4), and their fine structure has previously been explored and given the term: secretory "units" (i.e., secretory cell and associated cell(s)) (Ahmed and Gillott 1982, Gupta and Smith 1969, Clemments and Potter 1967, Happ and Happ 1970, 1975). The secretory cells are specialized for the production of exportable proteins, or mucopolysacharides (Davey and Webster 1967, Clemments and Potter 1967, Ahmed and Gillott 1982, Davey 1985), and it is assumed that spermatozoa use this secretion for an energy source awaiting fertilization (Davey and Webster 1967, Villavaso 1975).

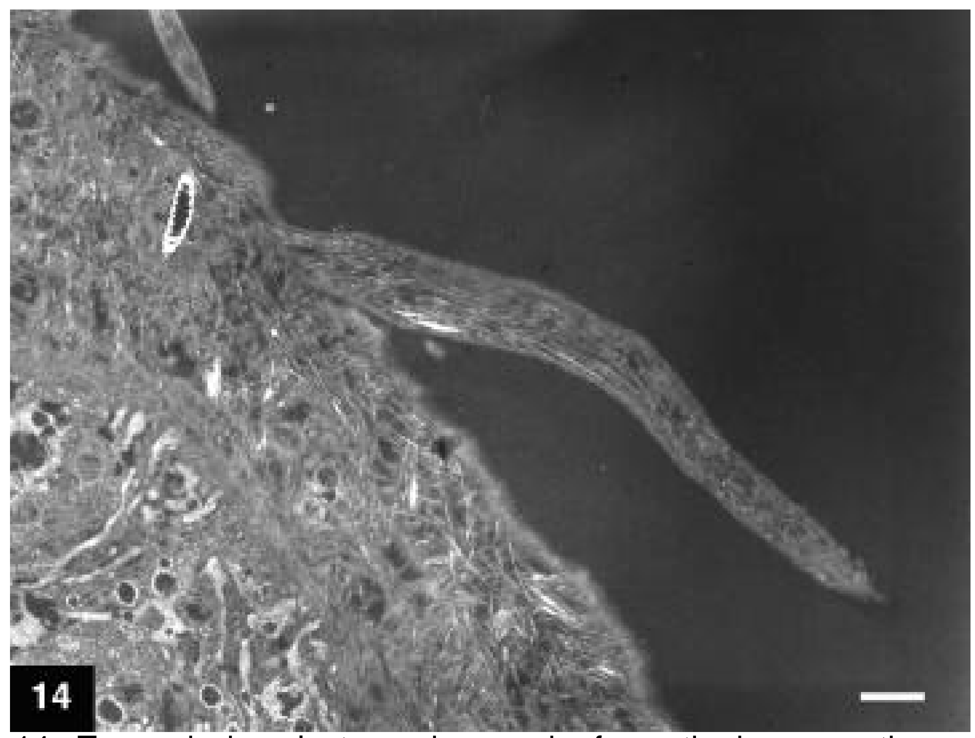


Figure 14. Transmission electron micrograph of acantha in spermatheca of *D. viridis*, 6000x (bar = 1 micrometer).

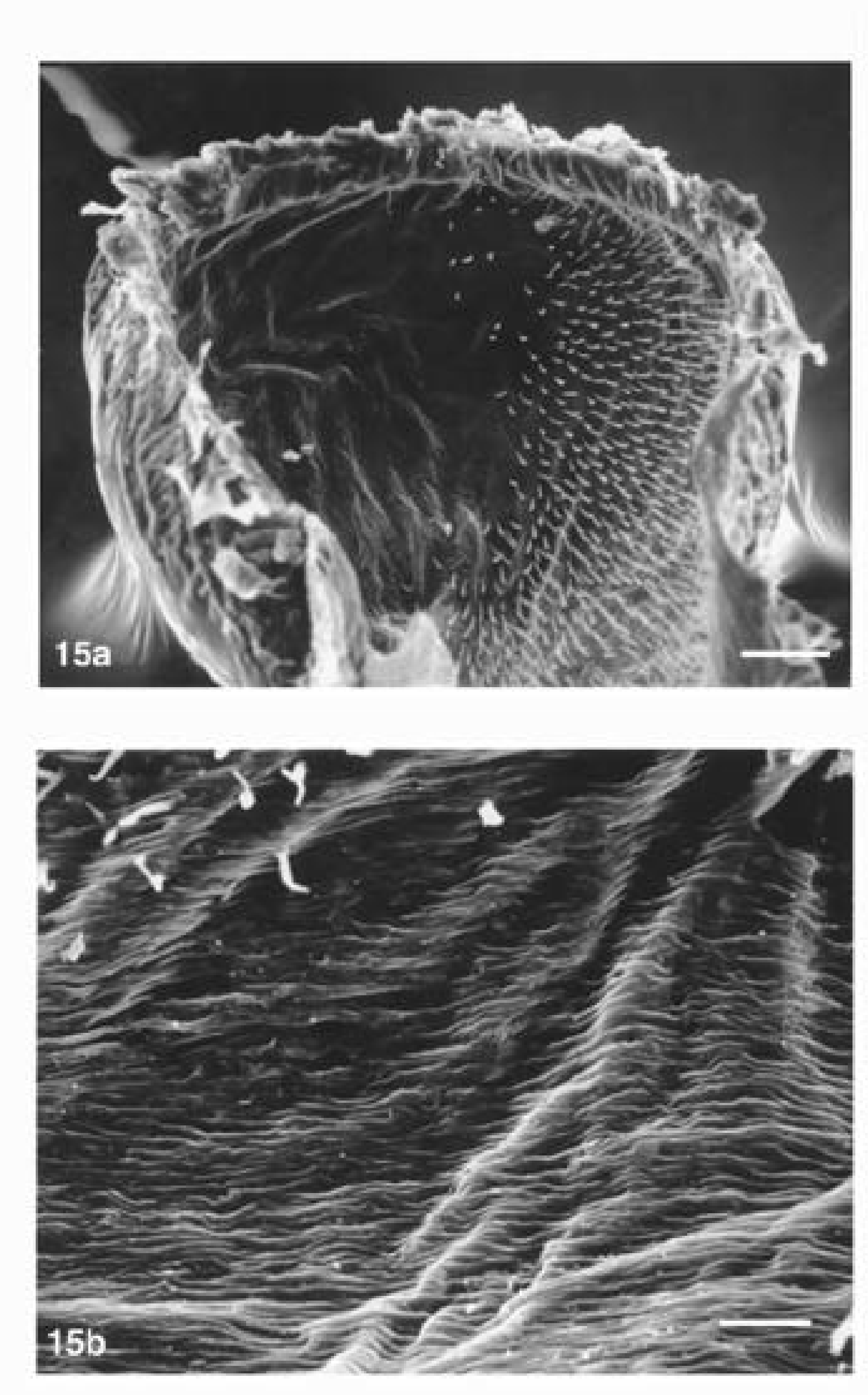
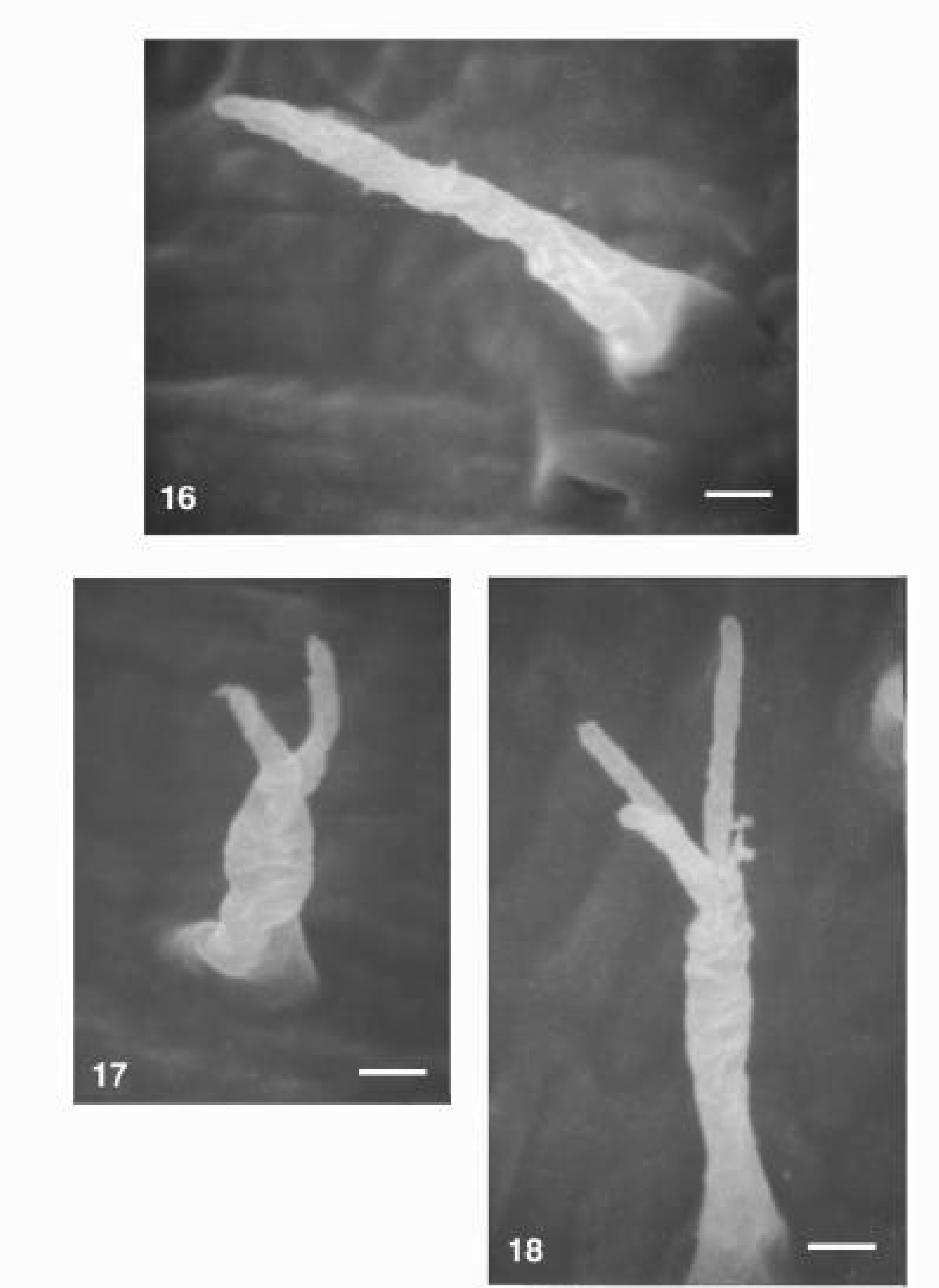


Figure 15. Scanning electron micrographs of the interior spermathecal surface in *D. viridis;* distal chamber. 15a. Distal aspect; medial side of chamber is on the right, 312x (bar = 40 micrometers). 15b. Lateral aspect; note absence of acanthae, 1250x (bar = 10 micrometers).



Figures 16 - 18. Scanning electron micrographs of acanthae from the proximal chamber of a spermatheca in *D. viridis*. 16. Simple acute acantha, 10000x (bar = 1.0 micrometer). 17. Bifurcate acantha, 10000x (bar = 1.0 micrometer). 18. Trifurcate acantha, 10000x (bar = 1.0 micrometer).

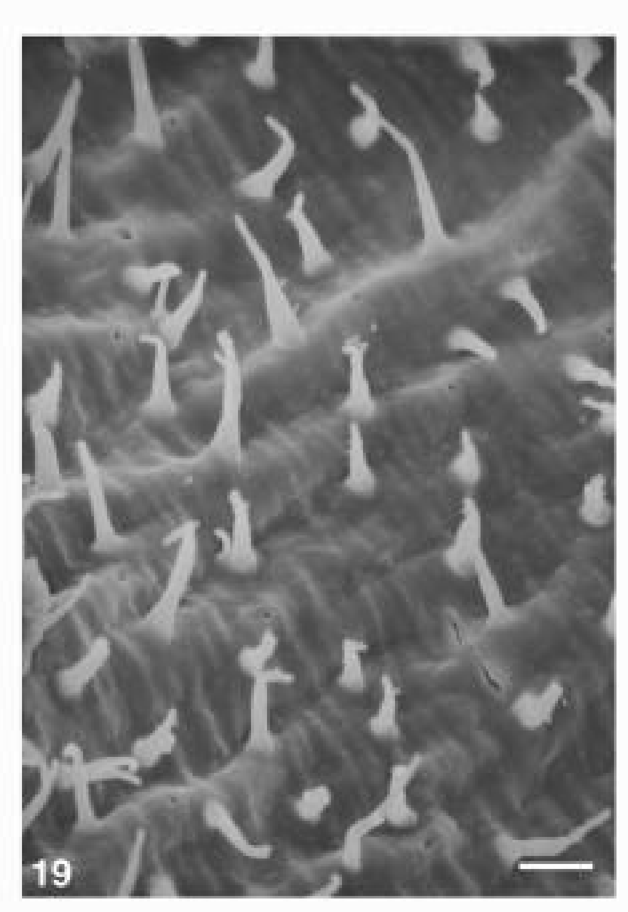
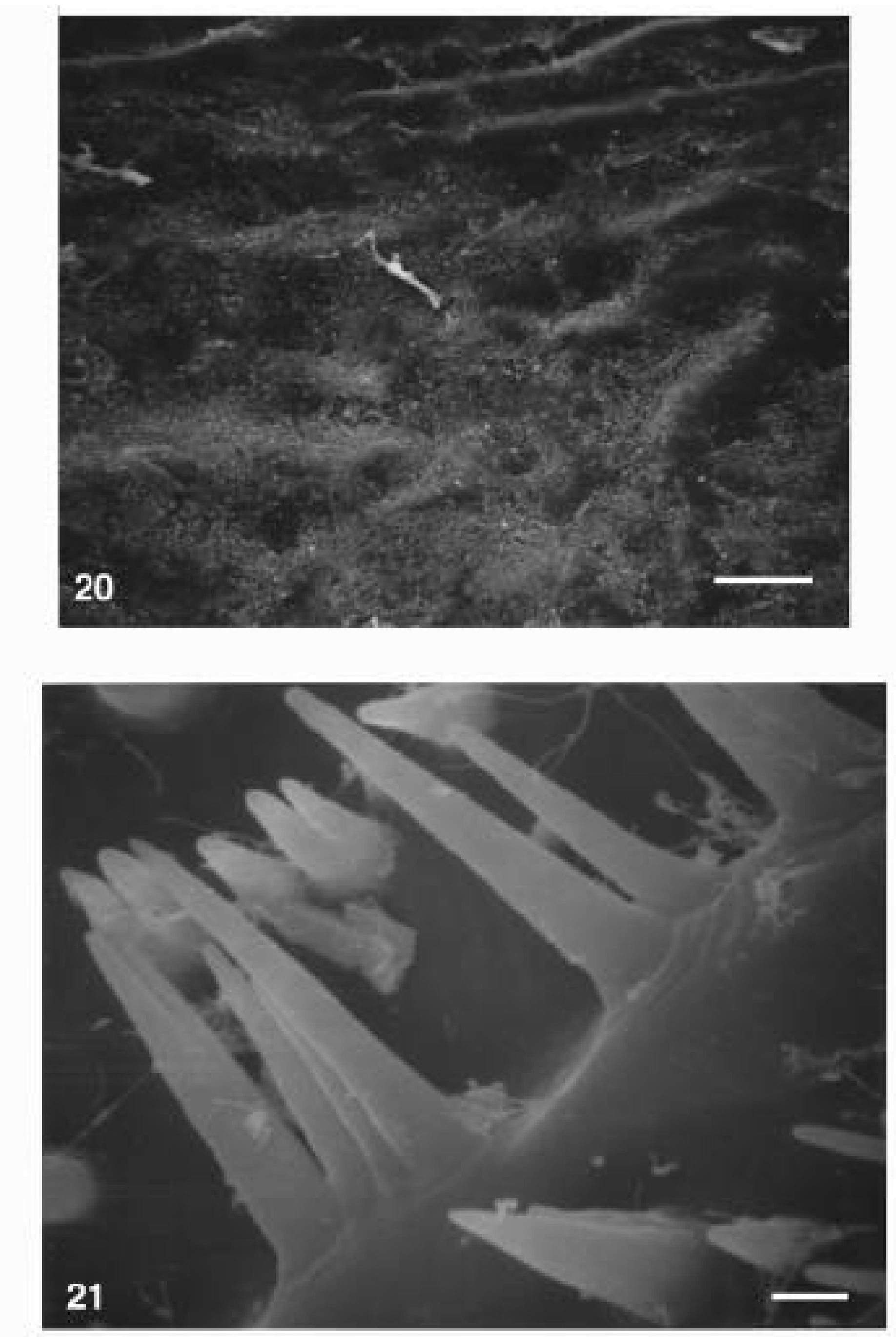


Figure 19. Scanning electron micrograph of the interior spermathecal surface in *D. viridis;* junction of proximal and distal chambers, 2500x (bar = 5 micrometers).

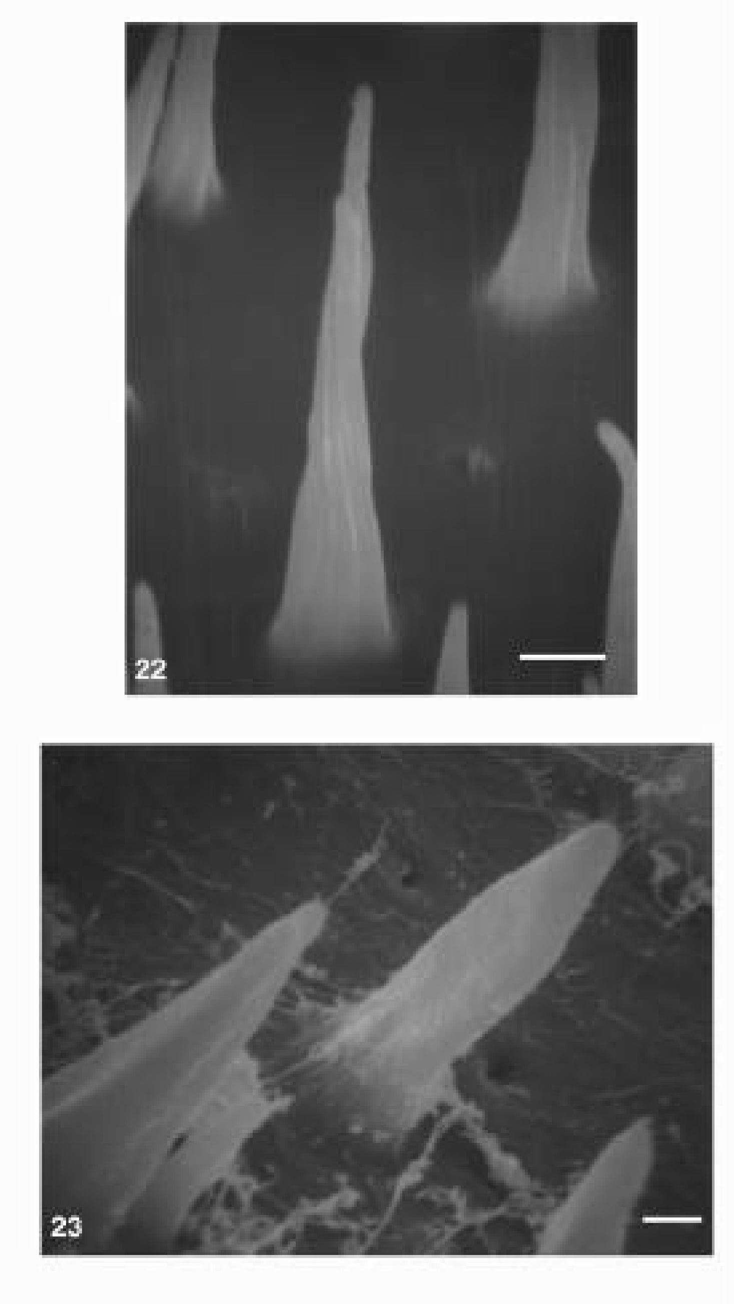
Chortophaga viridifasciata

Like *D. viridis*, the inside of the spermatheca consisted of small hair-like structures, or acanthae, pointing into the lumen of the spermatheca (Figs. 5 & 6). The entire proximal chamber had a large number of acanthae, while the distal chamber apparently lacked acanthae except for a small area in the middle of the lateral surface (Figs. 6 & 20). Some of the acanthae occurred in small clusters, mostly around the bend at the junction of the proximal and distal chambers (Fig. 21). The surface of the acanthae in the proximal chamber appeared smooth, without pores, but the acanthae themselves looked twisted from the base (Fig. 22). The few acanthae in the distal chamber appeared shorter and wider at the base than those in the proximal chamber (Fig. 23). The majority of the acanthae observed in both chambers were simple (unforked) and they where more acute than those of *D. viridis*.

The inside surface, or epicuticle, of the spermatheca appeared microglabrous (smooth) and was similar in both chambers. Small pores, or secretory “units” as described above, also occurred in the epicuticle throughout the spermatheca (Fig. 23). Unfortunately, the apical diverticulum was not prepared properly to see the inside of its chamber, so the occurrence of small hair-like structures could not be determined.



Figures 20 & 21. Scanning electron micrographs of the interior spermathecal surface in *C. viridifasciata*. 20. Distal chamber; note absence of acanthae, 1310x (bar = 10 micrometers). 21. At junction of proximal and distal chambers, 5200x (bar = 2 micrometers).



Figures 22 & 23. Scanning electron micrographs of acanthae from spermatheca in *C. viridifasciata*. 22. Proximal chamber, 7050x (bar = 2.0 micrometers). 23. Distal chamber, 10000x (bar = 1.0 micrometers).

Discussion

Insects, in particular, have evolved a variety of mating strategies. Unlike the sperm of many animals, insect sperm can remain viable for the duration of a female's life span (Page and Metcalf 1982, Starr 1984, Taber and Blum 1960). Most female insects have a sperm storage organ called a spermatheca; its form can take a variety of shapes and sizes (Dirsh 1957, Walker 1980, Smith 1984). In females that have mated multiple times, the spermatheca is the site where sperm from the last insemination compete with sperm from previous matings. The ability to store sperm prior to fertilization coupled with prolonged sperm viability within the spermatheca may be one reason why sperm competition is so common in the insects. Evidence for competition comes from studies indicating that when females mate with two males, the males do not contribute equally in paternity. Some insects show fertilization precedence for sperm from the initial mating (Gwynne 1988, Simmons *et al*. 1994, Bella *et al*. 1992) and others, as in the majority of studied cases, show sperm from the last insemination partially or completely displacing previously stored sperm to fertilize the female's eggs (Gwynne 1984, Hunter-Jones 1960, Walker 1980, Parker 1970, Birkhead and Hunter 1990, Longo *et al.* 1993, Lopez-Leon *et al.* 1993). Sperm storage and multiple mating by females may enable them to select stored sperm from "superior" partners for fertilizing their eggs.

In general, copulation times vary among grasshoppers (Otte 1970, Reide 1987, Uvarov 1977). Many species like *Chortophaga viridifasciata* transfer enough sperm to fertilize an egg pod in less than one hour. Yet there are species such as *Dichromorpha viridis* that typically mate for a day in the laboratory. On average, five bundles of sperm are transferred per hour in *D. viridis*. Previous studies estimate at least 256 sperm per bundle (Longo *et al*. 1993, Pickford and Gillott 1976). An hour or two of copulation in *D. viridis* should result in enough sperm to fertilize an egg pod of approximately 26 eggs (although Pickford and Gillott (1976) showed a decrease in

hatchability with decreased copulation duration especially in the last pods a female lays). Obviously other factors besides adequate sperm deposition must play a role in determining a lengthy copulation duration in *D. viridis*.

Previous studies have proposed sperm competition among the reasons for lengthy copulations in grasshoppers. Gregory (1965) proposed that *Locusta migratoria* mated for an extended duration for a number of reasons other than the need to transfer most of the sperm into the spermatheca. He suggested that it took time to form a complex spermatophore that could act by blocking the deposition of other spermatophores. Parker and Smith (1975) suggested that one function of the lengthy copulation in this same species *L. migratoria*, was to achieve sperm precedence in fertilization. Others also have more recently proposed that a lengthy copulation duration is an adaptation to reduce sperm competition (Eberhard and Cordero 1995, Gwynne 1984, Thornhill 1984, Eady 1994, 1995). Proposed mechanisms include the male remaining in genital contact, or staying mounted on top of the female, to act like a mechanical plug. Or, the male may transfer substances within the seminal fluid that act to change a female's receptivity to other males or stimulate immediate oviposition or both.

The results of this study implicate sperm competition, perhaps mediated by female choice, as a primary reason for lengthy copulations in *D. viridis*. Histological evidence suggests that males may not only act as living plugs but replace at least in part previously deposited sperm in *D. viridis*, whereas in *C. viridifasciata* mating duration is significantly less and thereby dependent on mating behaviors other than mating duration to increase likelihood of paternity. In both species studied, even if it is the male rather than the female that directly controls sperm displacement, a female can exercise control over the paternity of her progeny simply by permitting a new partner to displace the sperm of a less suitable male with whom she had mated earlier.

Sperm competition appears to start as soon as the pair engages genitalia. Histological slides of spermathecae demonstrate large numbers of loose or individual sperm only during the initial stages of multiple matings. In both species under investigation, individual sperm appear in both distal and proximal chambers, and then all appear to move to the proximal chamber before disappearing from the spermatheca during a second mating. Yet in single matings of *D. viridis*, sperm bundles do not appear to loose their bundle integrity throughout copulation, and if any chamber is preferred for receiving sperm early in mating, it is the distal chamber. In multiple matings sperm bundles appear to disassociate so individual sperm can be removed by some mechanism, possibly sperm flushing by an excess of seminal fluid by the second mating male (Gwynne 1984, Parker 1970). Or the female may allow the male to transfer some chemical during mating that causes such disassociation.

Dragonflies are the noted insect example for mechanical removal of previous males' sperm by a copulating male, although males in some species of bushcrickets also remove a competitors' sperm before depositing their own (Ono *et al*. 1989, von Helversen and von Helversen 1991, Waage 1979). This kind of sperm competition is rarer than stratification (Birkhead and Hunter l990, Sakaluk 1985) in which the sperm of males who contribute earlier are placed by new contributions of sperm to the back of the spermatheca and so probably the back of the "line" leading to insemination.

The sperm bundles also must disassociate into individual sperm before they can fertilize the female’s eggs. Individual sperm were found in ovipositing females, and the number of sperm bundles appeared to decrease with the number of eggs pods laid in *D. viridis*. Sperm bundles in D. viridis were also shown to remain as bundles up to at least seven days after copulation had finished.

By what mechanism do sperm bundles loose their bundle integrity and disassociate into individual spermatozoa prior to fertilization? As prior to oviposition, during a second mating selective early disassociation of bundles may be initiated by

the female. A mechanism in which females choose cryptically (after copulation begins) would better explain the variation in the number and location of sperm bundles found among females who had previously mated than male replacement of sperm (Eberhard and Cordero 1995, Knowlton and Greenwell 1984). Females have been noted to influence sperm deposition, storage and use in insects (Lopez-Leon *et al*. 1993, Knowlton and Greenwell 1984, Ward 1993). Male characteristics also have been shown to influence the effectiveness of sperm removal (Simmons and Parker 1992). Which sex plays the more important role in *D. viridis* in controlling sperm displacement will be elucidated by studies as those planned in the future, and using molecular markers, to asses paternity in females of varying history and males with varying characteristics.

It seems likely that not all of the previous sperm is removed during the second mating. Some sperm from the first mating, if still in the form of sperm bundles, appeared to remain inside the spermatheca while the individual sperm disappeared. This probably explains why there were more sperm bundles inside the spermatheca in early multiple matings as compared to corresponding early single matings in both *D. viridis* and *C. viridifasciata* and why a higher variance was observed among multiple matings compared to single matings in this study. Also, it appeared that the distal chamber of the spermatheca in *D. viridis* and *C. viridifasciata* functioned as the primary storage facility for the sperm bundles. Early in the single matings of *D. viridis* more sperm bundles were found in the distal chamber. At 48 hours or more after copulation had terminated, sperm bundles were only found in the distal chamber of *D. viridis*.

Interestingly enough, the inside of the spermatheca of *D. viridis* and *C. viridifasciata* is scattered with small hair-like structures, or acanthae (type A), directed into the lumen of the proximal and distal chambers as described earlier in this paper. Acanthae have been observed in the reproductive tract of a variety of insects, but only a few references mention their presence in spermathecae (Ahmed and Gillott 1982,

Gregory 1965, Richards and Richards 1979) and their functions are a matter of speculation. Richards and Richards (1979) argued that the functions of acanthae can only be mechanical because they are without sockets and therefore not movable the way setae are. For the structures to be sensilla they must have a sense cell.

Acanthae have been observed and documented in a variety of insect species (see references in Richards and Richards 1979). Locations of acanthae vary on both internal and external surfaces; their presence may be constant and useful as taxonomic characters (Tuxen 1970, Dietrich 1989). An interesting and well documented location for acanthae in some insects is the proventriculus (see refs. in Boudreaux 1980). Hepburn (1969) believed that the acanthae probably serve as strainers in Mecoptera preventing the lose of food particles in the midintestine as they regurgitate digestive fluids into their prey and suck up partly digested liquid food. Unfortunately, few attempts have been made to ascertain the function of acanthae in spermathecae.

Gregory (1965) stated that the “fine cuticular spines that project inwards from the walls of the vestibule” in *Locusta migratoria* possibly function by rupturing the thin wall of the spermatophore to allow the sperm bundles to migrate within the spermatheca. Unfortunately, nothing more was written on the cuticular spines and no pictures of the hair-like structures, or possibly acanthae, were provided to compare with *D. viridis* and *C. viridifasciata*. It is possible that acanthae inside the spermatheca could function by rupturing the spermatophore, but why are acanthae also located throughout the distal chamber in *D. viridis* where the spermatophore does not reach?

The observed acanthae inside the spermathecae possibly function in preventing sperm bundle removal during copulation, but allow individual sperm to be displaced or flushed out by the excess seminal fluid provided by the second mating. Most of the acanthae in the middle of the distal chamber of *D. viridis* are directed away from the proximal chamber, thereby possibly helping to prevent sperm bundle removal

while smaller individual sperm are “flushed” out. Also, small amounts of circular muscle have been noted to occur outside the basal lamella of the spermatheca (Ahmed and Gillott 1982), and the spermatheca might be able to compress during copulation, or during sperm removal, thereby producing a “sieve-like” structure with the acanthae and prevent sperm bundle removal while smaller individual sperm are “flushed” out of the spermatheca. This would help explain why the removal of individual sperm in multiple matings was observed late in copulation without the removal of sperm bundles. Also, the surface of the acanthae appeared smooth, or without pores, thus indicating a potential mechanical function rather than chemoreception. The compression of the spermathecae around the spermatophore might also aid in preventing the spermatophore from being removed during copulation.

The absence of acanthae in the distal chamber except for a small area in *C. viridifasciata* coupled with their short copulation duration (1.3 hours on average in the laboratory) and premating female choice could possibly indicate a different form or mechanism of sperm removal or competition in this species compared to *D. viridis*. This would thereby place less emphasis on the function of acanthae in *C. viridifasciata* compared to *D. viridis*. The short copulation duration of *C. viridifasciata* along with the higher rate of sperm transfer than in *D. viridis* may put more importance on the amount of seminal fluid transferred during copulation to remove prior males' sperm. More research is needed to help determine a possible function of acanthae inside the spermatheca, and whether or not similarities exist among other insects concerning the internal morphology of spermathecae and their mating behaviors.

It has been observed that mating pairs of *D. viridis* can remain in genital contact for up to 72 hours in the field, and on average copulate around 13.2 hours (Gooding 1996). Results in this study show that the rate of sperm transfer appears to decrease after 13 hours into copulation in *D. viridis*. Many copulations longer than this duration

possibly function more to insure mate guarding against future matings than to transfer needed sperm, or as already proposed flush out (with or without female “help") a previous male's contribution.

In some grasshoppers males remain on a female’s back for some time after copulation (Wicker and Siebt 1985). Such contact has been hypothesized to be one way a male can guard against mating attempts and avoid sperm competition while a female is still receptive (Parker 1970). Males in other grasshopper species have been noted to leave sperm plugs in the females’ genital tract that act as mechanical barriers to subsequent spermatophore deposition by other males (Gregory 1965, Loher and Chandrashekaran 1970, Parker and Smith 1975). As suggested for *Eyprepocnemis plorans* Charpentier (Lopez-Leon *et al. 1993*), lengthy copulations in *D. viridis* may be more effective as the male himself acts as a sperm plug.

Guarding of sperm from displacement by females has been suggested as a explanation for lengthy copulation in other Orthoptera (see references in Vahed 1996). Guarding of sperm has also been suggested as an explanation for large protein investments observed in various orthopteran species (Sakaluk 1986, Simmons *et al.* 1993, Vahed and Gilbert 1996, Wedell 1991). A grasshopper was the first insect in which male accessory gland products were demonstrated to pass to the female during mating (Friedel and Gillott 1977). However, Vahed (1996) observed in katydids, that lengthy copulations are correlated with the loss of protein transfer or decrease in the amount of protein transferred. He suggested in katydids that lengthy copulation appears to take the place of "feeding" a female in guarding sperm, and it would be interesting to know if a similar situation occurs in grasshoppers.

Niedzlek-Feaver (1995) reported that females of *C. viridifasciata* appear to “select” males and possibly judge a male’s potential nutrient or gene contribution by a male’s weight determined during the initial stages of copulation or by using visual cues to compare size or physical conditions prior to the onset of copulation. Whereas

C. viridifasciata females may appear to use precopulatory assessment of male quality, *D. viridis* females appear to use postcopulatory ("cryptic") assessment of male quality by possibly choosing a male based on the amount of protein(s) transferred ascertained by stretch receptors in the walls of the spermatheca or some other sense mechanism inside the reproductive system. It will be interesting to see if *D. viridis* males, as reported for *Chorthippus brunneus* (Thunberg) (Butlin *et al* 1987), transfer protein(s) during mating and if they continue this transfer during lengthy copulations after the rate of sperm transfer decreases.

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