ABSTRACT

GARDNER, GRANT EAN. Morphological and histological aspects of the spermatheca as they relate to sperm organization in the grasshopper species *Schistocerca americana* and *Dissosteira carolina* (Orthoptera: Acrididae). (Under the direction of Marianne Niedzlek-Feaver)

The spermatheca of the acridid Orthoptera *Schistocerca americana* and *Dissosteira carolina* both consist of a ductus seminalis and a receptaculum seminis that ends in two blind sacs called the apical and preapical diverticula. The diverticula of acridid grasshoppers show high morphological variation that might imply functional differences. A microscopic examination of the structure of the spermatheca surface of both species found the presence of numerous gland ductules, but a lack of acanthae typical of many acridids. A histological study of macromolecules in the spermatheca of mated females found large carbohydrate and protein secretions present in all chambers. The secretion was not present in virgin *S. americana* but was present in virgin *D. carolina*. These secretions are likely glycoproteins either secreted by female gland ductules or contributed from males. Lipids were limited to small droplets contained within epithelial cells lining the walls of the spermatheca of both mated and virgin females. Histological sections of the spermatheca were used to track the course of sperm bundles in the chambers at various intervals following copulation initiation. In *S. americana* sperm bundles are found primarily in the diverticula and appear to be degraded in the apical diverticulum. In *D. carolina* sperm bundles are seen in all chambers of the spermatheca except the ductus seminalis and maintain a constant distribution an hour into copulation. Implications of this study are discussed in relation to the function of acridid spermatheca and sperm organization.

MORPHOLOGICAL AND HISTOLOGICAL ASPECTS OF THE SPERMATHECA AS THEY RELATE TO SPERM ORGANIZATION IN THE GRASSHOPPER SPECIES *SCHISTOCERCA AMERICANA* AND *DISSOSTEIRA CAROLINA* (ORTHOPTERA: ACRIDIDAE)

by
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Chair of Advisory Committee

BIOGRAPHY

Grant Gardner was born on November 21, 1978 in Corpus Christi, Texas. After a nomadic existence, he graduated from Charlotte Latin High School in 1997. He attended Vanderbilt University where he earned a B.S. in Biology with a minor in Psychology on December of 2000. At Vanderbilt Grant conducted his undergraduate research in insect evolutionary ecology under Dr. Daniel Funk. After graduation, Grant spent time as a lab technician at the Vanderbilt University Medical School Department of Molecular Biology and Immunology working on capsid protein structure in HIV. Grant then returned to his North Carolina to pursue his graduate degree in Zoology under Dr. Marianne Niedzlek-Feaver at North Carolina State University.

ACKNOWLEDGMENTS

I express deep gratitude to my advisor Dr. Marianne Niedzlek-Feaver for her help and patience in molding a fledgling researcher into a passable scientist. I would also like to thank Dr. Betty Black, Dr. Lewis Deitz and Dr. Fred Gould for being so willing to serve on my committee. Thanks need to go to all those who helped along the way; to Sandra Horton at the North Carolina State University Veterinary School who initiated me in histology work and Greg Sword with the United States Department of Agriculture who helped with animal rearing and husbandry advice. Thanks go to Ozkan Ozden, for his advice and patience with helping on the cryostat. Also, big thanks go to all the undergraduate researchers who assisted in the "dirty" work and hopefully learned something along the way. Thanks are due to my family. Finally, I thank my wife, Heather, for putting up with many long weekends out in the field and many long nights sitting at the computer.

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INTRODUCTION

In insect mating systems, often an extended period lapses between the time females receive sperm from males and the time when eggs are fertilized. Depending on the species, the interval between insemination and fertilization can vary from a few hours to many years. Delayed fertilization in insects is possible in part to the longevity of male spermatozoa. In a couple of extreme examples, ants have laid fertile eggs 15 years following mating (Wheeler 1910) and honey bees (*Apis mellifera* L) have laid eggs up to seven years after copulation (Taber and Blum 1960). Although male sperm viability is one important factor in delayed fertilization, sperm longevity is also dependant on the female reproductive organ called the spermatheca. This organ receives sperm during copulation and can both store and retain the gametes until fertilization occurs (Clements and Potter 1967, Davey and Webster 1967).

The spermatheca is formed from an invagination on or between the eighth and ninth abdominal sternite of female insects and is embryonically derived from ectodermal tissue (Matsuda 1976). This basic tissue plan is one of the few points of similarity between the spermatheca of different insect taxa, as the gross morphology can differ greatly. There are numerous studies on the structure of this reproductive organ of various insect orders (for examples see, Tombes and Roppel 1972, Kocorek and Danielczok-Demska 2002, Martins and Sarrao 2002). The common short-horned grasshoppers (Orthoptera: Acrididae) present an appropriate model for studies in spermathecal gross morphology because of the high intra-family structural variability. This diversity is historically reinforced by the observations that acridid spermatheca vary so much in morphology that they have potential as a taxonomically defining character (Slifer 1940a, Dirsh 1957).

The basic structure of the spermatheca is similar for all acridid species, although the anatomical nomenclature varies in the literature. For consistency, this paper utilizes the definitions presented by Okelo (1975) to describe spermatheca gross morphology. The organ opens at the anterior gonopore and proceeds inward with a narrow chamber called the ductus seminalis. This long cuticular-lined tube narrows as it runs anteriorly, and eventually widens again creating the receptaculum seminis. Termination of the receptaculum seminis occurs at two blind sacs called the apical and preapical diverticula. The high variability in spermatheca structure typically involves the diverticula (Uvaraov 1966).

In acridid Orthoptera, males transfer spermatophores to the females via a reproductive organ that can penetrate far into the spermatheca. Spermatophores consist of a bulb that remains in the male and a tubular portion that is transferred to the female during copulation (Mann 1984). Depending on the species, acridid grasshoppers can contribute varying numbers of sperm bundles to the female during a single mating. Regardless of the number of sperm bundles transferred, spermatozoa from a single mating are usually sufficient for complete fertilization of female eggs (Parker 1970). In order for a female to increase effective fecundity, however, she must typically mate with multiple partners (Ridley 1988).

Due to the storage capacity of the spermatheca and the likelihood of multiple matings in acridid grasshoppers, males of polygamous species are not guaranteed paternity of the female's offspring (Parker 1984). This can exert evolutionary pressure on males to adopt behavioral mechanisms that increase the likelihood of fathering young. Adaptations to increase sperm precedence such as mate guarding and sperm removal do appear to be common in polygamous acridids. Males of the grasshopper *Sphenarium pupurascens* (Charp.) will guard a female for up to 18 days following copulation to prevent access to her

by other males (Ceuva Del Castillo 1999). In males of the grasshopper *Dicromorpha viridis* (Scudder) sperm from previous matings is likely being removed or replaced from the female spermatheca by secondary males (Johnson 1998).

Besides overt male behavioral responses, the spermatheca of polyandrous females offers an environment suitable for post-copulatory male sperm manipulation. Spermatozoa from multiple males can compete internally to fertilize female oocytes in a process known as sperm competition (Parker 1970, Parker and Smith 1975). Males can also attempt to gain post-copulatory sperm precedence by contributing additional molecular products to the spermatheca during mating. These additional products originate from the male accessory glands and can have a multitude of effects on female reproductive response. Some Orthoptera males contribute nutrients in the form of amino acids that can act as an internalized nuptial gift (Friedel and Gillott 1977, Butlin et al. 1987). Male transferred molecular products can also act as oviposition stimulants (Pickford et al. 1969, Lange and Loughton 1985, Paeman et al. 1991).

Males are not alone in exerting influence on the precedence of sperm in the spermatheca. R.A. Fisher (1958) noted that females make a greater pre-copulatory parental investment in their offspring by contributing a larger, more energetically expensive gamete. Therefore, females should be the limiting agent in the average mating system, with males competing for females and females being highly selective of their mates (Trivers 1972, Borgia 1981). As with sperm competition, the spermatheca offers an environment where females could potentially affect the ability of sperm to fertilize eggs. Post-copulatory behaviors or physiological specializations such as this, that allow females to shift the paternity distribution of mates is known cryptic female choice (Eberhard 1995).

The numerous glandular cells surrounding the epithelium of the spermatheca does offer some circumstantial evidence for cryptic female choice. The function of these cells is still relatively unknown, although some studies have attempted to determine their role (Kaulenas 1992). In non-Orthopteran systems spermathecal glands assist in the maintenance of the spermatheca epithelium (Dallai 1975, Happ and Happ 1975, Huebner 1980). In studies on the boll weevil (*Anthonomus grandis* Boheman) spermathecal glands contribute to sperm motility (Villavaso 1975, Grodner 1979) and exhibit a sperm-attracting effect (Grodner and Steffens 1978). Sperm bundles of the acridid grasshoppers *Gomphocerus rufus* (Lubber) (Hartmann 1978) and *Chorthippus curtipennis* (Harris) (Hartmann and Loher 1974) are shown to be partially degraded via proteolytic enzymes secreted from glands present within the spermatheca.

This study focuses on two species of short-horned grasshoppers, *Schistocerca americana* (Drury) (subfamily Cyrtacanthacridinae) and *Dissosteira carolina* (Linnaeus) (subfamily Oedipodinae). We begin with a description of the gross morphology of the spermatheca of each species. This is followed by a histological survey of some of the potentially important macromolecules present in the spermatheca of both mated and virgin females. Finally, a histological analysis of the organization of sperm within the spermatheca following a single mating is conducted. The goal is to offer a comparative study of the general morphology and basic chemical nature of the spermatheca as it relates to sperm transfer and organization.

MATERIALS AND METHODS

Insect Collection and Rearing

Nymphs and adults of *Schistocerca americana* and *Dissosteira carolina* were collected at Research Farm Unit #1 (North Carolina State University) and Schenk Forest Research Facility (North Carolina State University). Both nymphs and adults of *S. americana* were generally associated with patches of tall field grasses. *D. carolina* nymphs and adults were often associated with bare patches along dirt roads or zones of sparse foliage. Adults of both species tend to fly long distances when startled and were collected using sweep nets. Voucher specimens of the two species were identified and deposited in the North Carolina State University Insect collection.

Nymphs of *S. americana* were found in high densities from June through late September and adults were found at variable densities throughout the year. Females of *S. americana* were collected as adults on April 8th and 9th, 2004 in an attempt to determine the presence or absence of sperm being stored over the winter months. *D. carolina* adults do not survive over winter, therefore, no corresponding data for this species was taken. *S. americana* were not seen to be actively flying until early April, nor were any matings observed in the field until mid May of 2004. The assumption was April would be early enough in the season to capture females that had not mated during the current spring season. Therefore, the presence of sperm in these individual adults should have been from copulations from the previous fall mating season. This assumption is reinforced by a study conducted at the same field site that observed matings in *S. americana* occurring only from early April to late June and then again from late July to early September (Kosal 1995).

Nymphs of both species caught in the wild were raised in wire mesh cages until adult eclosion. Following the final molt, adults were separated by sex to insure animals did not mate until they could be observed. Animals caught in the wild as adults were kept in communal cages in order to maintain lab cultures. Fluorescent and incandescent light was provided and light dark cycles ranged from 13:11 during the peak of summer and 7:17 during the winter. Attempts were made to keep the LD cycles consistent with the light cycles of the current season. Plastic spray bottles were used to douse the cages to provide a water source for the animals and also to increase the humidity within the enclosures. Plastic cups filled with sand were provided as oviposition sites in cages containing mated females.

Animals were fed with grasses grown in the Method Road greenhouses at North Carolina State University that consisted of wheat, rye and bermuda grass. Diets were supplemented with plantain and white clover collected seasonally from the field. Additional nutrients were provided in the form of a dry food mixture composed of ground Purina Cat Chow, Big Red rabbit food, wheat germ, and fish food. Cages were checked daily and food was supplemented as needed.

In order to identify animals for experiments requiring individual identification, animals were marked on the pronotum using Testor paint and labeled using a two-point/color system (Niedzlek-Feaver 2004). These animals were weighed to 0.001grams. Measurements of wing length, pronotum length, femur length and total body length were taken using a caliper.

Morphological Terminology

There is acceptance in the literature of the specific terminology of the various chambers of the acridid spermatheca, although a succinct definition is difficult to find. In an

attempt to clarify the spermatheca morphology for this study, the most recent study that attempts to technically define the chambers of acridid Orthoptera was used (Okelo 1975). Definitions of chambers are listed below with supplementary comments in parenthesis.

Ductus seminalis: The tubular canal through which the spermatozoa migrate to and from the receptaculum seminis. (This definition fails to indicate the termination point of the ductus seminalis. After the initial narrowing of the ductus seminis at the gonopore, the tube maintains a constant width for the majority of its length. For the purpose of this study the termination point is defined as the area where the anterior end of the ductus seminalis begins to widen once again).

Receptaculum seminis: The chamber or pouch at the anterior end of the ductus seminalis. (Okelo's definition includes the diverticula as part of the receptaculum seminis but in this study we exclude the diverticula as part of this specific chamber).

Preapical diverticulum: Bifurcation of the receptaculum seminis that curves posteriorly and touches the body of the receptaculum just posterior to the point of bifurcation.

Apical diverticulum: Bifurcation of the receptaculum semenis that ends anteriorly and is much narrower than the preapical diverticulum.

Spermatheca General Morphology

Spermatheca were dissected from preserved animals in fresh Hoyle's solution. The defined chamber was severed from the spermatheca using an ultra-fine scalpel and then cut along a single plane under a dissecting microscope. The surface of the tissue was observed by being laid completely flat on a slide with the inner surface of the chamber exposed toward the cover slip. Using a 0.5 x 0.5 square mm gridded glass cover slip, length measurements of the various chambers were made (n = 10). Measurements were made by laying the chambers

on a slide and allowing them to dry for one minute. We were then able to extend the chambers such that length measurements could be made. While under the microscope, counts were also made of the number of gland ductules visible at 400X magnification. These counts were then converted to a density figure (# of gland ductules per square millimeter). Counts of gland cell ductule density in each chamber of the spermatheca were made in order to note any significant differences between chambers that might provide evidence of varying female control within distinct regions of the spermatheca.

Macromolecule Histology

For each species, spermatheca were removed from three virgin and three animals which had been allowed to mate to completion. The spermatheca was divided into its respective chambers using an ultra-fine scalpel to remove each chamber from the organ body. *S. americana* spermatheca were divided into five separate chambers; ductus seminalis (ds), receptaculum seminis (rs), preapical diverticulum (pd), proximal apical diverticulum (adI), distal apical diverticulum (adII) (Figure 1). The apical diverticulum in *S. americana* was divided in two parts because of a distinct U-bend in the chamber that might imply functional differences in the regions. *D. carolina* spermatheca were divided into four separate chambers; ductus seminalis (ds), receptaculum seminis (rs), preapical diverticulum (pd), apical diverticulum (ad) (Figure 9). Each spermatheca chamber was combined with two other chambers from the same species and same mated or virgin status.

Chamber groups destined for rotary microtome sectioning were taken through an alcohol series and preserved in fresh Carnoy's solution for at least 24 hours. Spermatheca were dehydrated in an ethanol/HemoDe series and embedded in TissuePrep paraffin, following which serial cross-sections were cut at 6μm using a rotary microtome. Chamber

groups destined for cryostat sectioning were dissected from fresh specimens and embedded in cold optimum cutting temperature (OCT) embedding medium.

Muccopolysaccharide Activity

The presence of muccopolysaccharides in each chamber was determined using paraffin embedded sections and a Periodic-acid Schiff (PAS) staining procedure. Slides were sent to the North Carolina State College of Veterinary Medicine histology lab for staining. PAS is a general carbohydrate stain that picks up a variety of macromolecules including glycogen, mucin, mucoprotein, and glycoprotein. Basophilic polysaccharides stain a blue-maroon color and acidophilic polysaccharides stain a red-pink color.

Lipid Activity

The presence of lipids within the spermatheca was determined using cryostat sections and staining protocol from Chayen and Bitensky (1991). The slides were stained with Sudan Black B stain, which is a general lipid stain that gives a positive reaction for most classes of lipids. Lipids stain black to gray in color.

Protein Activity

To analyze the presence of proteins within the spermatheca both cryostat and paraffin sections were used. Cryostat sections were stained using a high pH, Fast Green staining protocol that gives a positive reaction for most protein molecules (Alfert and Geschwind 1953). Paraffin sections were stained using Fast Green as well and a protocol for general morphological counter-staining.

Single Interrupted Matings and Sperm Migration

Individually marked virgin females were placed in cages with marked males and allowed to copulate, with mating being defined as genital contact. Females were allowed to

mate for a predetermined time interval depending on the species with time intervals assigned to acquire fractions of total mating time for each species. The average total mating time for *S. americana* was approximately 5.5 hours and the interrupted mating intervals were designated at 1.5, 2, 3, 4, and 5 hrs. For *S. americana* there were natural matings and exploratory time matings taken at times smaller than 1.5 hours (0.25, 0.5, and 1 hour) to confirm the activity occurring at smaller copulation durations. The average total mating time for *D. carolina* was approximately 2.5 hours and the mating intervals were designated at 0.25, 0.5, 1, 1.5, and 2 hrs. Each of the assigned interrupted mate time intervals was completed with three replicate animals. At the end of the allotted time intervals animals were removed from the cage and were immediately sacrificed in a -20^{o}C freezer. Attempts were made not to dislodge mating pairs when removing them from the cage and in most cases pairs remained in copula.

Spermatheca from interrupted matings were sectioned using paraffin sections and a rotary microtome. Slides were stained using Feulgen's staining protocol that yields a positive reaction for DNA and could be used to identify the distribution of sperm bundles within the tissue sections (Boone and Drijver 1986). Following mounting of the slides, sequential sections of the tissue were digitally photographed under a light microscope at a 100X magnification. From these photographs, an assessment of the number and chamber location of loose sperm and sperm bundles in the spermatheca were made. These estimates were conservative and probably represent a minimum number of bundles transferred.

RESULTS

Schistocerca americana

Spermatheca General Morphology

Figure 1 shows the general structure and chamber divisions of the spermatheca of *Schistocerca americana*. The ductus seminalis chamber began at its opening at the gonopore and terminated approximately 1 mm posteriorly from the branching of the preapical diverticulum. The receptaculum seminis began at the widening of the ductus seminalis. The kidney-shaped preapical diverticulum was easily defined from its branching point on the proximal side of the receptaculum seminis. The apical diverticulum was defined from the point where the receptaculum seminis the preapical diverticulum branched from the receptaculum seminis to its blind terminating point. The apical diverticulum typically consists of four perpendicular curves or bends, which give the chamber a zigzag look. For *S. americana* the division between the early branching of the apical diverticulum and the late branching of the apical diverticulum was between the second and third perpendicular turn. The lengths of all defined chambers can be seen in Table 1.

The outer topography of the spermatheca was dominated by small gland ductules (Figure 2, Figure 3). The ductules were similar in appearance in all areas of the spermatheca although there was a tendency for the gland ductules along the ductus semenalis to be slightly more elongate than those of the other chambers. A Friedman test concluded that there was no significant difference in the density of gland ductules between individual chambers. The average density calculation resulted in a value of 904 ductules per square millimeter. It is important to note that the number of ductules did appear to be highly variable even among chambers from different sample animals although no significant

differences were found. This could be due in part to manipulation of the spermatheca during dissection that could have dislodged gland ductules. The inner surface of the spermatheca was free of bristles or acanthae that have been noted in areas of the inner surface of some other acridid Orthoptera (Ahmed and Gillott 1982, Johnson 1998, Lay et al. 1999).

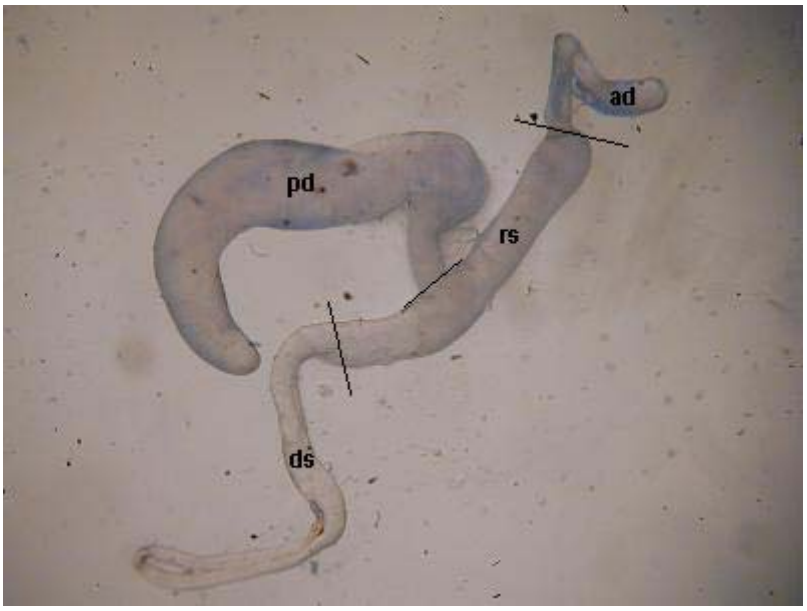


Figure 1: Light micrograph of the spermatheca of *S. americana* (40X) following removal of fatty tissue. Chamber divisions are shown (ds = ductus seminalis, rs = receptaculum seminis, pd = preapical diverticulum, ap = apical diverticulum). The narrow tube of the ductus seminalis runs to the gonopore.

Table 1: A comparison of average chamber lengths of the spermatheca of *S. americana* (n = 10).

	length (mm)	standard deviation
ds	10.4	0.0114
rs	0.9	0.0329
pd	1.5	0.0532
ad	2	0.0158

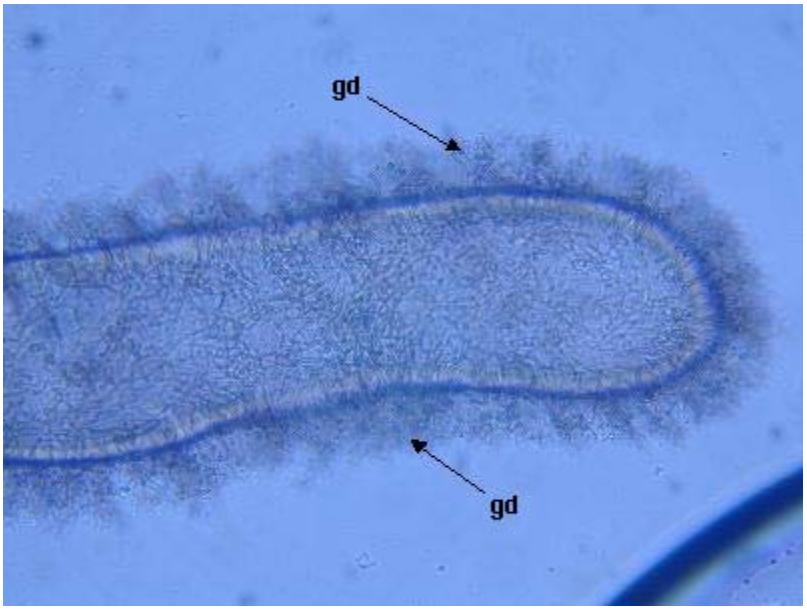


Figure 2: Light micrograph of the end of the apical diverticulum of *S. americana* (400X). Arrows indicate large concentrations of gland ductules (gd) that cover the surface of the chamber.

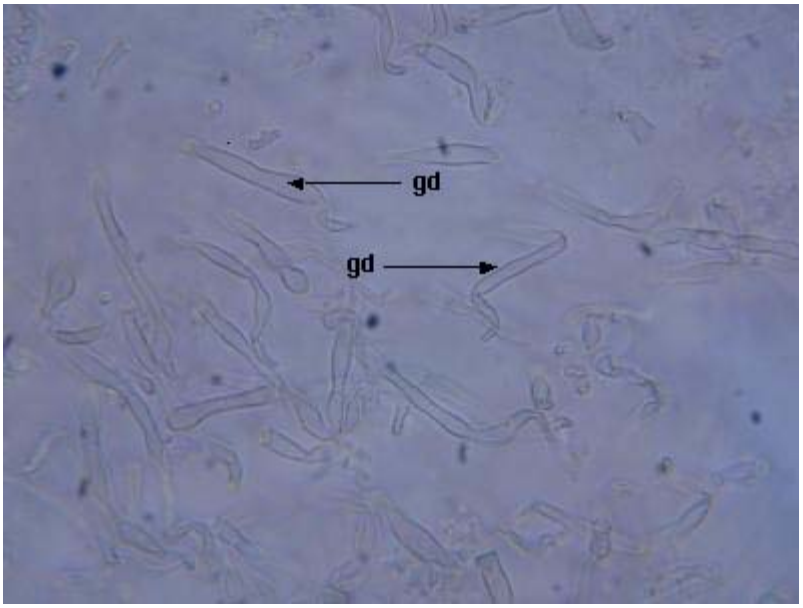


Figure 3: Light micrograph of the outer surface of the spermatheca of *S. americana* (1000X) zoomed to show the structure of the surface gland ductules (gd).

Macromolecule Histology

In virgin females the epithelial tissue including the gland ductules of the spermatheca were weakly to moderately PAS positive. The cuticular inner surface of virgin females stained a strong positive for acidophilic polysaccharides. The nuclei of the epithelial cell wall stained blue to purple for basophilic polysaccharides that is characteristic of tissue with high concentrations of DNA. In mated females, the epithelial cells and ductules also stained a weak to moderate positive using PAS. Sperm stained a very positive blue indicating the presence of a highly basic polysaccharide substance composing the sperm bundles. The location of sperm was in the receptaculum seminis, apical diverticulum-I, and the preapical diverticulum. A secretion in the inner lumen of the chamber that was not present in virgin females was moderate to highly PAS positive and often surrounded sperm bundles and loose sperm (Figure 4, Figure 5). Gland ductules along the edge of the epithelium were lightly visible with this stain.

Although positive staining for lipids using Sudan Black B was observed, no significant difference was seen in the spermatheca tissue of virgin versus mated females. There was positive staining in the outer epithelium area of the chambers (Figure 6). After further investigation, lipids were observed arranging into small lipid droplets.

Staining protocols using Fast Green as a stain for general proteins yielded a slight positive stain for most of the outer tissue layer of the spermatheca for both virgin and mated females. All spermatheca chambers in virgin females showed a distinct lack of secretions present in the lumen (Figure 7). In contrast, females that had mated showed a Fast Green positive secretion in the lumen of the majority of the spermatheca (Figure 8). Gland ductules were visible as well.

Figure 4: Longitudinal cross-section of the preapical diverticulum of a mated *S. americana* stained using Periodic Acid Schiff (100X). Note the presence of sperm bundles (sp) and PAS positive secretion (se) surrounding them that are contained within the lumen of the spermatheca cuticle wall (cu).

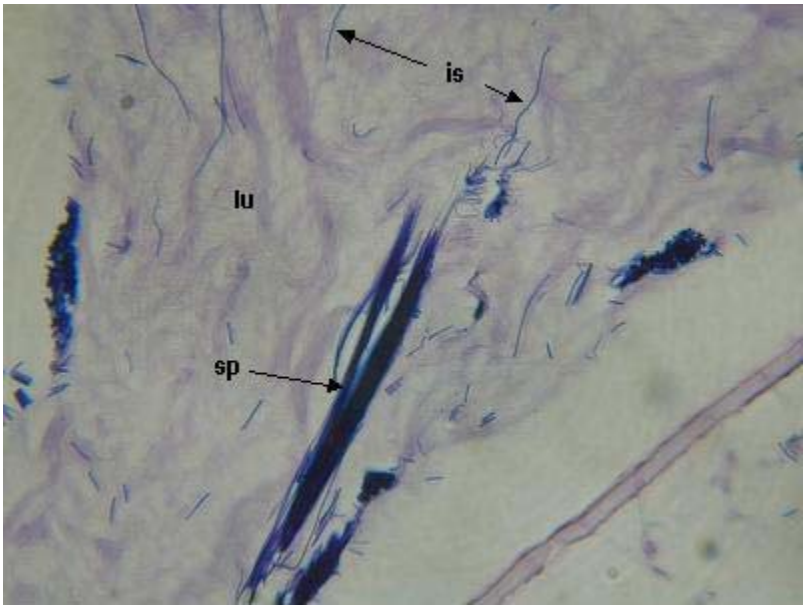


Figure 5: Longitudinal cross section of the receptaculum seminis of a mate *S. americana* stained using Periodic Acid Schiff (200X). The sperm bundles (sp) and broken sperm bundles (is) are clear visible in the lumen (lu).

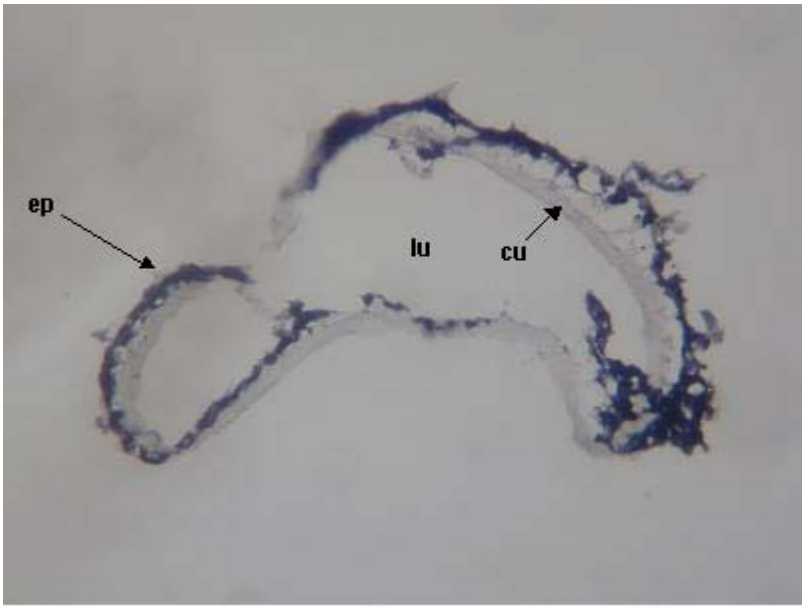


Figure 6: Transverse cross-section of the preapical diverticulum of a mated *S. americana* stained using Sudan Black B for lipids (100X) (ep = epithelial layer, cu = cuticle layer, lu = inner lumen of chamber).

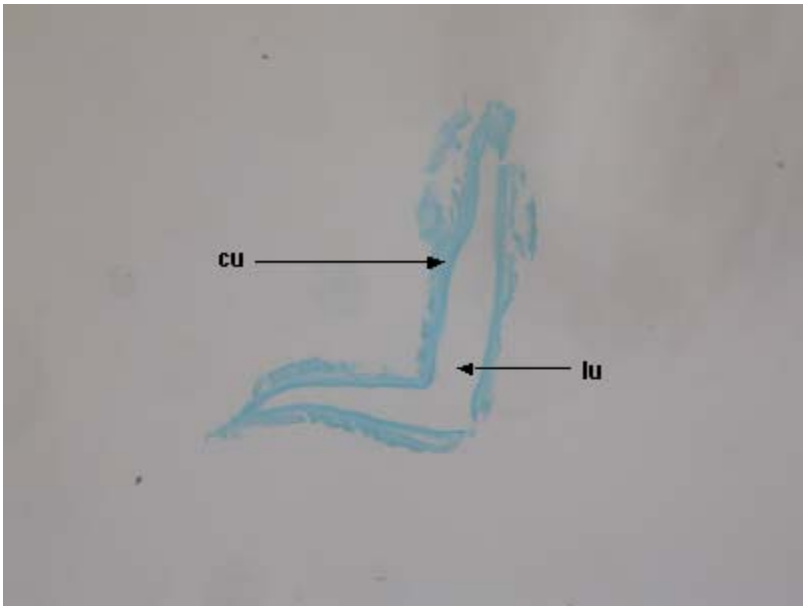


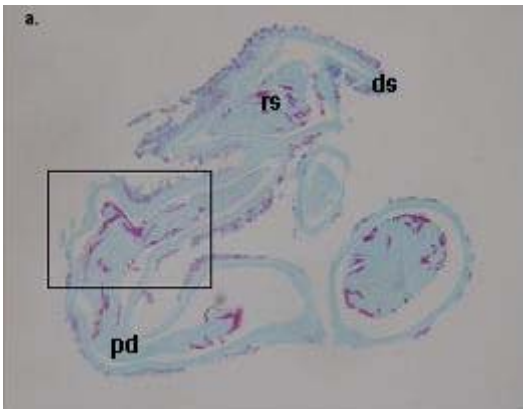
Figure 7: Longitudinal cross-section of the preapical diverticulum of a virgin *S. americana* stained using Fast Green for proteins (100X) (cu = cuticle layer, lu = inner lumen of chamber). Note the lack of any Fast Green positive secretions within the chamber lumen.

Sperm Migration

An example of a spermatheca used for sperm bundle counts stained using Feulgen's protocol is shown in figure 8. The average mating time for *S. americana* was five and a half hours, although animals were observed to mate for as long at seven hours. From initial mating trials there was not a large amount of sperm (three bundles) transferred until almost one hour into mating. After initial transfer, the number of bundles present in the spermatheca increases dramatically, with as many as 71 sperm bundles being present after an hour and a half. Sperm bundle transfer peaked at approximately three and half hours after which there was a definitive decrease in the number of bundles present (Figure 9). Along with distinct sperm bundles, there were chambers where large amount of loose sperm were observed. The presence of loose sperm did not appear to correlate with a specific time interval, but was consistently found in the receptaculum seminis.

By looking at individually defined chambers, there was a spike in the presence of sperm in the ductus seminalis at approximately an hour and a half following copulation (Figure 10a). The majority of the sperm bundles initially migrated to the receptaculum seminis after which most were concentrated until three hours (Figure 10b). As time progressed, the majority of sperm bundles moved out of the receptaculum seminis and migrated to the apical or preapical diverticula. Sperm bundle numbers in the preapical diverticulum remained remarkably consistent after three hours of mating and on to completion to (Figure 10c). The number of sperm bundles in the apical diverticulum shows a distinct arc-shape peaking at three hours indicating movement of the sperm in that chamber to other reaches following completion of mating (Figure 10d).

S. americana animals collected prior to the spring mating season did show significant amount of sperm being stored in the spermatheca. No sperm was found in the ductus seminalis and only one animal found to have sperm in the receptaculum semenis. The majority of sperm was found in the preapical diverticulum, with an average of 40 bundles being found in this chamber. The apical diverticulum also contained some sperm but it was in smaller concentrations with an average of 5 sperm bundles.



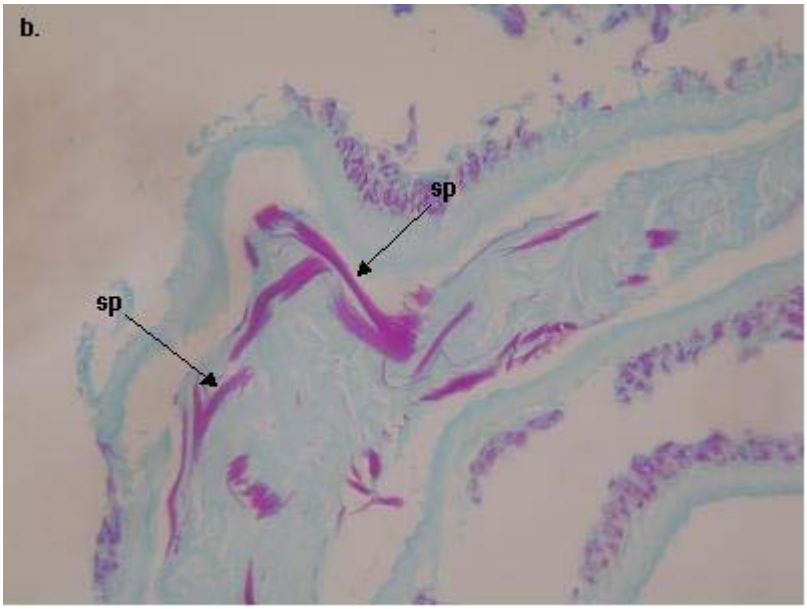


Figure 8: a. Longitudinal cross section of the spermatheca of *S. americana* having mated for four hours and stained using Feulgen's procedure (40X) (pd = preapical diverticulum, rs = receptaculum semenis, ds = ductus semenalis). b. Closer view of selected area of spermatheca in 3a with arrows indicating the sperm bundles (sp) (200X).

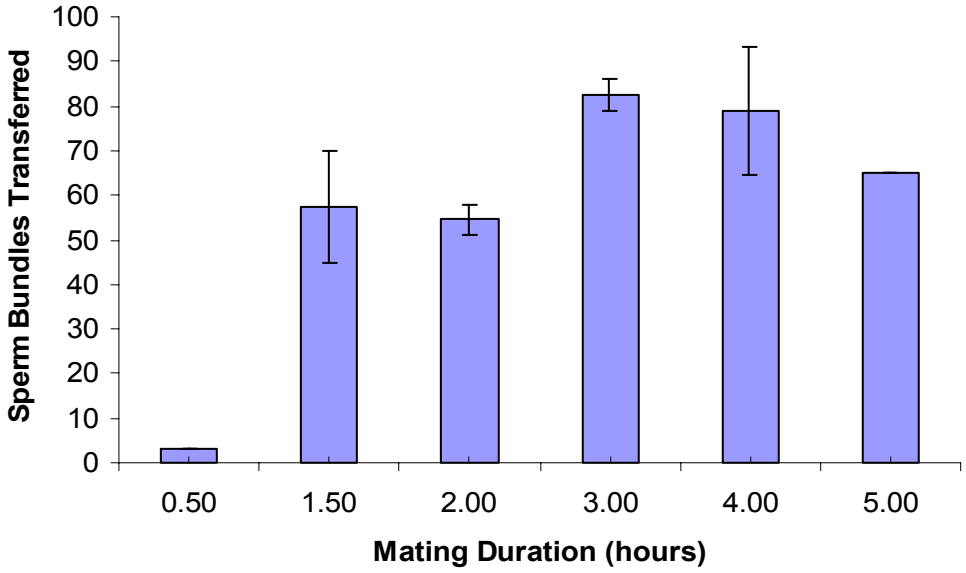


Figure 9: The total number of sperm bundles transferred to the spermatheca of female *S. americana* in a single mating. Each data point is the average of three samples with one unit of positive and negative standard deviation.

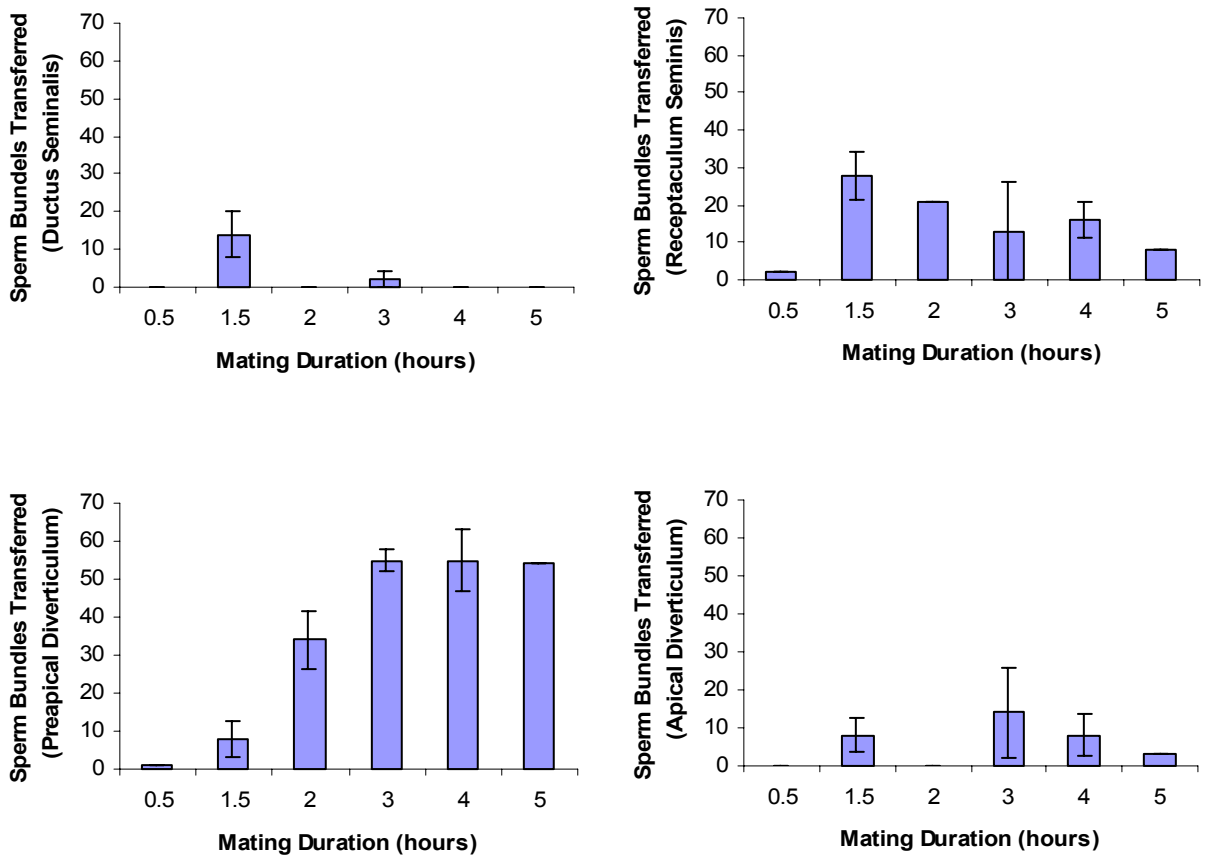


Figure 10 (a-d): Sperm bundles distribution with respect to time for individual chambers in the spermatheca of female *S. americana*. Note that for purposes of sperm distribution the apical diverticulum has not divided into two chambers.

Dissosteira carolina

Spermatheca General Morphology

The gross morphology of the spermatheca of *D. carolina* is structurally simple and follows the basic acridid plan. The ductus seminalis chamber for *D. carolina* was defined identically as that described for *S. americana* with the chamber ending 1 mm from the end of the receptaculum semenis. At the distal end of the receptaculum seminis, the spermatheca divides into apical and preapical diverticula at the proximal end of the receptaculum semenis. The divisions between the diverticula and the receptaculum are not as clear in *D. carolina* as they are in *S. americana*. Therefore the diverticula were divided at the point where they distinctly deviated from one another. The preapical and apical diverticula are both generally ovoid in shape. The apical diverticulum tends to be narrower and slightly more elongate (Figure 11) as per my definition. It is important to note that by looking at figure 11, it appears that the defined apical diverticulum appears to branch prior to the preapical diverticulum. Although this was not seen in every *D. carolina* spermatheca dissected, it was apparent in a few. Although other authors that describe the morphology of oedopodin grasshoppers do not indicate this, it could indicate that the diverticulum functions and definitions are inverted. Length measurements for all respective chambers are listed in table 2.

The surface of the spermatheca was covered in glandular ductules similar in structure to *S. americana* (Figure 3). Unlike *S. americana*, all the ductules of *D. carolina* seem to be a consistent size along the entirety of the spermatheca surface. A Friedman test found no significant difference in the density of gland ductules between chambers. The

average density of was 1504 ductules per square millimeter. As with *S. americana*, no acanthae were noted on the inner surface of the spermatheca.

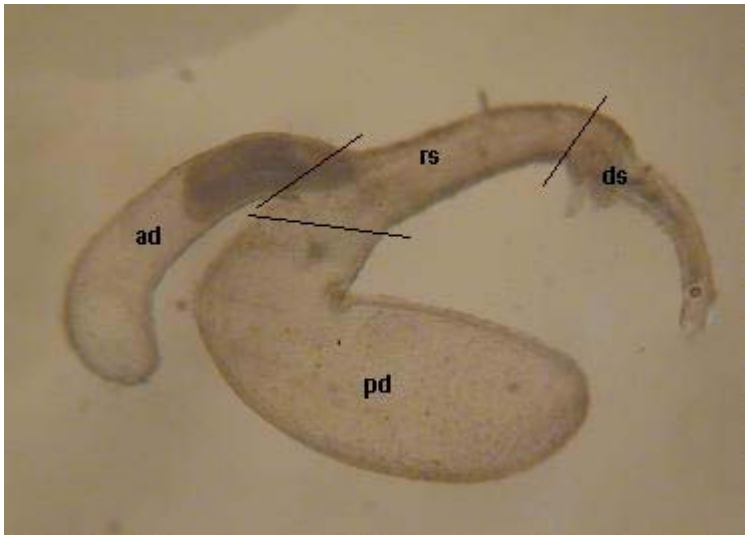


Figure 11: Light micrograph of the spermatheca in *D. carolina* (40X) following removal of fatty tissue. Chamber divisions are shown (ds = ductus seminalis, rs = receptaculum seminis, pd = preapical diverticulum, ap = apical diverticulum). The narrow tube of the ductus seminalis runs to the gonopore.

Table 2: A comparison of average chamber lengths of the spermatheca of *D. carolina* (n = 10).

	length (mm)	standard deviation
ds	6.6	0.0245
rs	0.7	0.0132
pd	1.3	0.0156
ad	0.8	0.0193

Macromolecule Histology

The spermatheca chambers of the virgin *D. carolina* were characterized by a weak to moderately PAS positive epithelium that consistently contained a heavily stained PAS positive inner-lumen secretion (Figure 12). In mated females sperm was noted in the apical diverticulum and stained highly PAS positive for basophilic polysaccharides. The chambers of mated females did not differ drastically from virgin females with the epithelium staining weakly to moderately PAS positive and showing highly PAS positive inner lumen secretions. Gland ductules were lightly visible with this stain.

The results for stains with Sudan Black B for *D. carolina* were analogous to those found in *S. americana* (Figure 6). There was no significant difference in the spermatheca tissue of virgin versus mated females even though there was a positive reaction for lipids. Again, there was a high concentration of lipids droplets located in the epithelial cells lining the spermatheca. The remainder of the tissue was only slightly positive for lipids and there was no inner lumen secretion positive for Sudan Black B stain.

Like *S. americana*, the outer tissue layers of *D. carolina* stained a consistent moderate positive using Fast Green. As found with PAS stain, the inner lumen of the spermatheca of both virgin and mated females contains protein positive secretions located in all the identified chambers (Figure 13). Gland ductules were lightly visible with this stain.

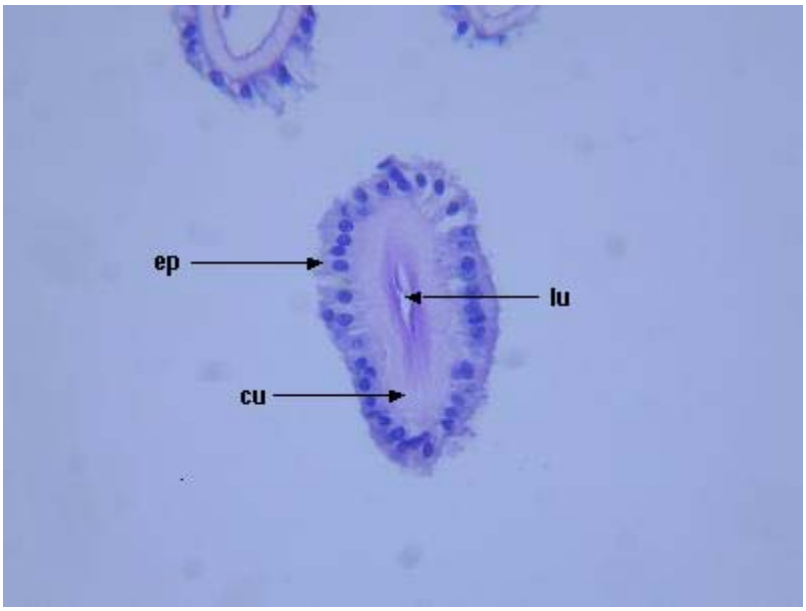


Figure 12: Transverse cross-section of the apical diverticulum of a mated *D. carolina* stained using Periodic Acid Schiff for carbohydrates (100X) (ep = epithelial layer, cu = cuticule layer, lu = inner lumen of chamber).

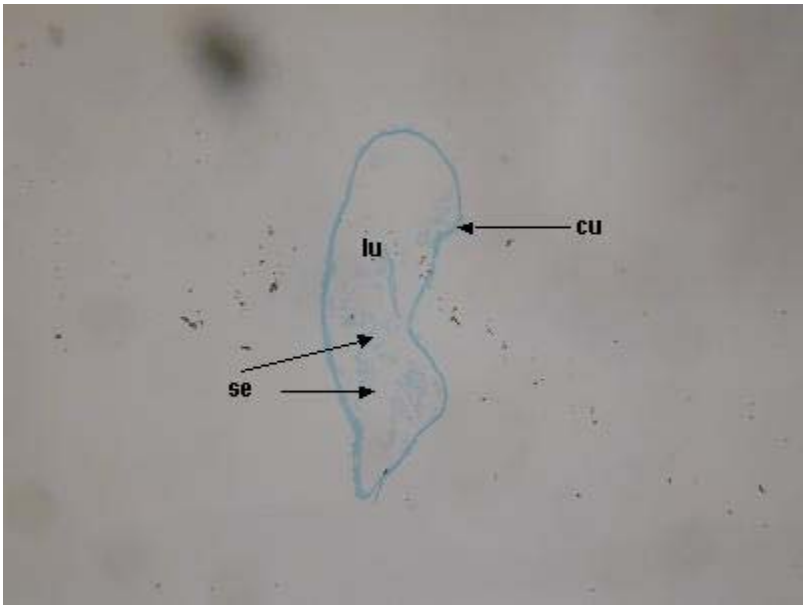


Figure 13: Longitudinal cross-section of the preapical divertculum of a virgin *D. carolina* stained using Fast Green for proteins (100X) (cu = cuticle layer, lu = inner lumen of the chamber, se = inner lumen secretions).

Sperm Migration

The total average mating time for *D. carolina* was two and a half hours with little variation. An example of a longitudinal cross section of the spermatheca stained using Feulgen's protocol can be seen in figure 14. Sperm bundles were first observed entering the spermatheca as early as 15 minutes following genital contact. There is a dramatic increase in the number of bundles from a half hour to an hour in which the number of sperm bundles double from the initial copulation. Sperm bundle transfer was at a maximum after one hour and then remained almost constant for the remainder of the pair's connection (Figure 15). Loose sperm was observed in the spermatheca, but did not appear to occur at any particular time interval or be limited to a specific chamber location.

In the beginning stages of copulation from initiation to approximately one half hour, sperm bundles appear relatively evenly distributed throughout the spermatheca (Figure 16 a-d). As copulation progressed past that time interval, the majority of the sperm bundles tend to migrate into the preapical diverticulum (Figure 16c). It also appears that after approximately one hour into copulation, the sperm bundles that have been transferred maintain their location through to the termination of the copulation.

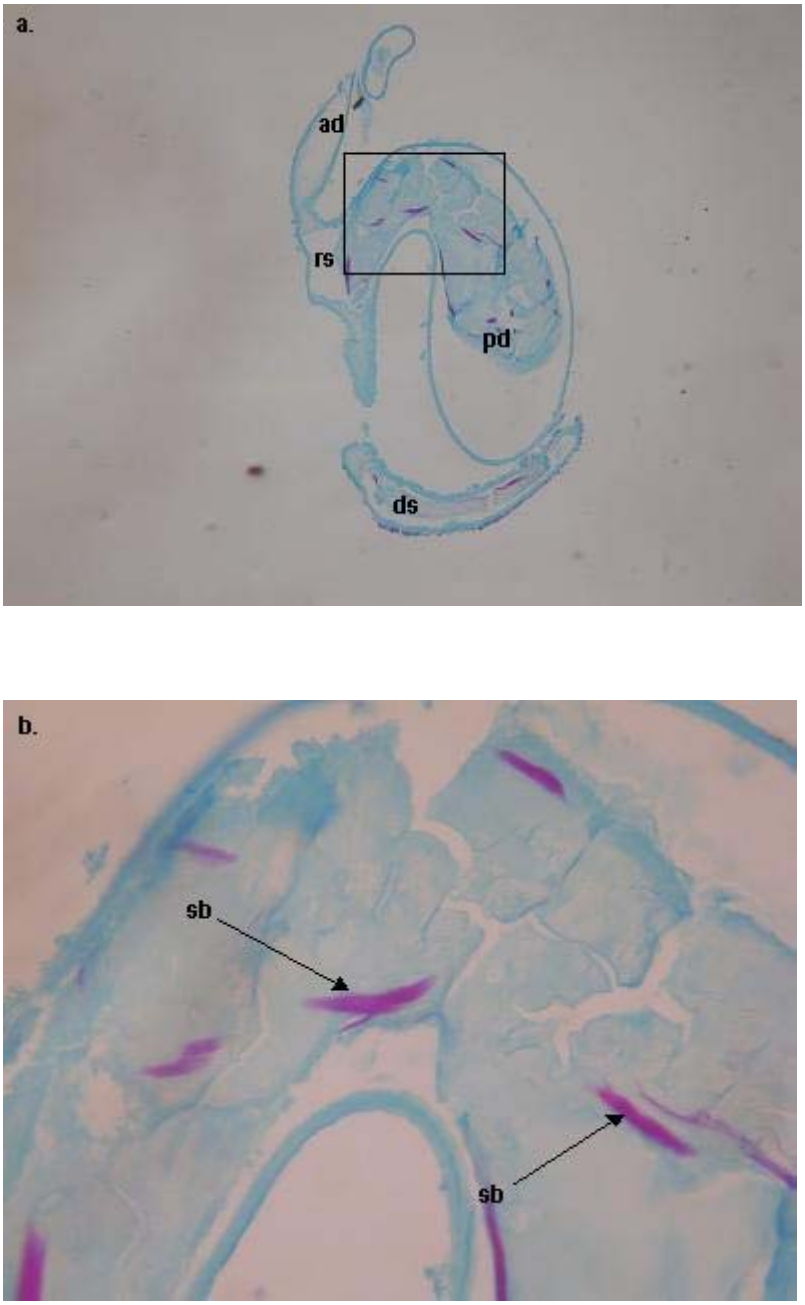


Figure 14: a. Longitudinal cross section of the spermatheca of *D. carolina* having mated for one hour and stained using Feulgen's procedure (40X) (pd = preapical diverticulum, rs = receptaculum semenis, ds = ductus semenalis, ad = apical diverticulum). b. Closer view of selected area of spermatheca in 3a with arrows indicating the sperm bundles (sp) (200X).

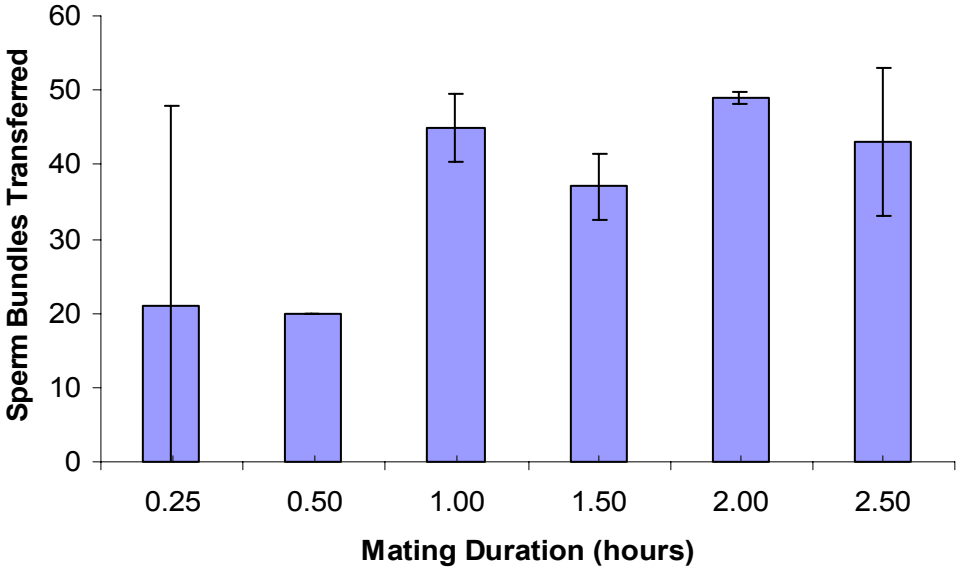


Figure 15: The total number of sperm bundles transferred to the spermatheca of female *D. carolina* in a single mating. Each data point is the average of three samples with one unit of positive and negative standard deviation.

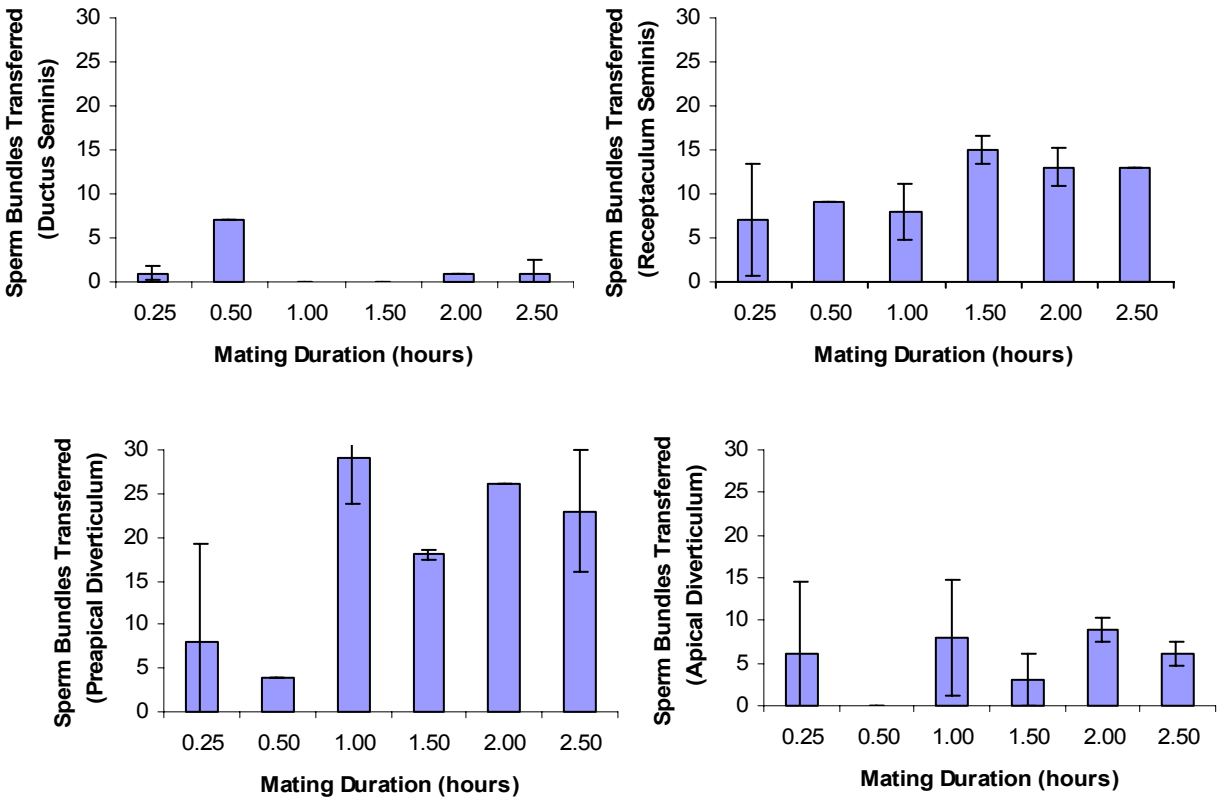


Figure 16 (a-d): Sperm bundles distribution with respect to time for individual chambers in the spermatheca of female *D. carolina*. Note that for purposes of sperm distribution the apical diverticulum has not divided into two chambers.

Discussion

General Structure

The gross structural diversity of the acridid spermatheca is well documented (Slifer 1939, 1940a, 1940b, 1940c, 1943, Katiyar 1956, Dirsh 1957, Kharibam et al 1982, Sathe and Joshi 1988), with the primary focus being on the use of the organ as a taxonomic character. Assis-Pujol and Lecoq's (2000) study addresses morphological variation of spermatheca with a comparative survey of Gomphocerinae (Orthoptera: Acrididae) that has reinforced the importance of analyzing the spermathecal structure by demonstrating its species-specific morphology. This structural diversity likely evolved from adaptations to the unique reproductive biology of specific acridid taxa. It remains unclear, however, as to what the functional significance of this variation might be. Recent studies on spermatheca diversity have focused on the ultrastructure of the organ in an attempt to determine the functional role of gland ductules and acanthae in sperm storage and organization (Lay and Hartmann 1998).

Both *Schistocerca americana* and *Dissosteira carolina* show significant numbers of gland ductules covering the entire surface of the spermatheca. Not all acridid species show ductules in such high concentrations and they may not be present in all grasshoppers. For example, electron microscopy studies of the spermatheca of *Chortophaga viridifasciata* (DeGeer) make no mention of these glandular structures (Johnson 1998). The ductules are likely similar to those described in *Locusta migratoria* (Linnaeus) (Lay et al.1999) and *Melanoplus sanguinipes* (Fabricius) (Ahmed and Gillott 1982) in that they serve as pathways from the spermathecal lumen to extracellular cavities located around epithelial gland cells. In acridids, it appears that duct systems function in both directions, with both secretory and resorptive properties (Lay et al. 2004).

Gland ductules as well as inner lumen secretions were visible with both PAS and Fast Green stains. As discussed earlier the ductules likely serve as the pathway for the lumen secretions in the spermatheca. The secretion is most likely made up of a protein / muccopolysaccharide mixture, possibly a glycoprotein as has been found in other insect species (Davey and Webster 1967, Happ and Happ 1975, Thukral 1976, Pal and Ghosh 1981, Dallai et al. 1993). The lumen secretions did not stain positive for lipids that seemed to be confined to small droplets compartmentalized in the epithelium. Lipid secretions have not been found in any other acridids, but have been found in the secretions of *Sitophilus granarius* (Linnaeus) (Bhatnagar and Musgrave 1971) and *Drosophila melanogaster* (Meig.) (Filosi and Perotti 1975).

Acanthae are found in the reproductive organs of several insects, although their function is still unconfirmed. Possible functions include rupturing of spermatophores to release sperm and prevention of sperm bundle removal in second matings but additional studies are needed to confirm this functional morphology. There are numerous examples of acridid species with spermathecal acanthae including *L. migratoria* (Gregory 1965), *M. sanguinipes* (Ahmed and Gillott 1982), *Dichromorpha viridis*, and *C. viridifasciata* (Johnson 1998). In this study, no acanthae were found in any of the chambers of the spermatheca of *S. americana* or *D. carolina*. There have been preliminary electron microscopy analyses of the spermatheca of *S. americana* that also seems to indicate that acanthae are lacking in this species (Niedzlek-Feaver, personal communication). This apparent lack of acanthae was unexpected and further electron micrographs will have to be made to confirm the presence or absence of acanthae in both *S. americana* and *D. carolina*.

Sperm Transfer

The possibility of differential organization of sperm in Oedipodinae and Cyrtacanthacridinae begins with insemination. Certain species in the subfamily Oedipodinae, such as *L. migratoria* (Gregory 1965), *Chimarocephala pacifica* (Thomas) (Loher and Chandrashekaran 1970), *Chortoicetes terminifera* (Walker), *Locustana pardalina* (Walker) (Pickford and Padgham 1973), and *Camnula pellucida* (Scudder) (Ewen and Pickford 1975) produce a single spermatophore that extends the full length of the ductus semenalis and deposits sperm directly into the receptaculum seminis. With few studies on the mating behavior and physiology of *D. carolina* it is difficult to say whether males transfer sperm along the same line as other oedipodins. It does appear that sperm is being deposited directly in the receptaculum seminis of *D. carolina* due to the lack of many sperm bundles appearing in this chamber over the course of copulation.

Several members of the subfamily Cyrtacanthacridinae produce multiple small spermatophores that penetrate only a short distance into the ductus semenalis. This kind of sperm transfer has been shown in other Cyrtacanthacridinae such as *Anacridium aegyptium* (Linnaeus) (Federov 1927) and *S. gregaria* (Forskal)*, S. pallens* (Thunberg)*, S. americana* and *Nomadacris septemfasciata* (Serville) (Pickford and Padgham 1973). As shown in this study sperm bundles eventually settle in diverticula of *S. americana*, therefore sperm must move along the ductus seminalis and into spermathecal chambers. It is possible that a chemical stimulus present in the glandular secretions cause sperm movement through the ductus semenalis as shown in the boll weevil *Anthonomus grandis* (Grodner and Steffens 1978). The need for movement of sperm along the ductus seminalis could explain why *S. americana* has secretions in the body of the spermatheca prior to mating as has been

suggested in another Acrididae, *M. sanguinipes* (Ahmed and Gillott 1982). More studies tracking the presence of glandular secretions and sperm following mating would be needed to confirm this in *S. americana*.

The fate of the majority of the sperm bundles in both *S. americana* and *D. carolina* seems to be the preapical diverticulum. In *D. carolina* over half of the sperm remains in the preapical diverticulum following completion of mating and in *S. americana*, approximately 80% of sperm bundles settle in this chamber. Post-copulatory concentrations of sperm in the distal chamber have been demonstrated in *D. viridis, C. viridifasciata* (Johnson 1998), and *M. bivattatus* (Say) (Hinn 1999). In *D. viridis* in the apical diverticulum directs sperm bundles into distal chamber and in *C. viridifasciata* it appears to go to the distal chamber remaining in the proximal chamber (Johnson 1998). The distal chamber in these studies conforms to the definition of the preapical diverticulum used here and the proximal chamber is what this study has defined as the receptaculum seminis. It is still unclear as to why such a complex spermatheca would concentrate such large percentages of transferred sperm to this particular chamber when this study and others have found little structural difference (aside from general shape) in the diverticula.

In *S. americana* sperm bundles are being maintained at equal numbers in the preapical diverticulum after approximately three hours of copulation. In the apical diverticulum and receptaculum seminis the number of sperm bundles is reduced after approximately three hours of copulation. It is possible that the decrease in sperm bundle numbers in the receptaculum semenis is due to their movement into the bifurcate chambers. The reduction in numbers in the apical diverticulum can only be explained by their degradation because the disappearance of sperm bundles in this chamber is not accompanied

by an increase in other chambers indicative of sperm migration. Sperm bundle degradation in the apical diverticulum has been exhibited in other acridid species as well (Longo et al. 1993). Contrarily, in *M. bivattatus*, the distal chamber acts as a site where the sperm bundles are digested for either nutritive properties or fertilization and the apical diverticulum is the location where sperm is stored (Hinn 1999).

The digestion of additional sperm is partially upheld by the findings that spermatheca of females having over wintered as adults show a distinct localization of sperm in the preapical diverticulum. This could mean that females are storing sperm over the winter months in the preapical diverticulum and digesting sperm in the apical diverticulum possible for the acquisition of additional nutrients. This is a tentative conclusion, as it is unknown whether females collected in the early spring had only mated once and the fact that small amounts of sperm bundles were still found in the apical diverticulum. Additional post-copulatory time intervals are needed to determine if the apical diverticulum is a source of sperm degradation in *S. americana*.

In *D. carolina* there appears to be no reduction in sperm numbers over the course of a single copulation as seen in *S. americana*. By tracking the course of sperm through the spermatheca it appears that a male contributes all its sperm bundles within the first hour of mating as sperm number peaks at around this interval. There is also limited movement of bundles within the spermatheca following the hour interval demonstrating that there is not a differential migration of sperm between these chambers. During copulation it appears that the apical and preapical diverticula serve only as storage chambers, however, as with *S. americana*, additional post-copulatory spermathecal sections need to be examined to confirm the sperm distribution in various chambers.

Copulation durations can be highly variable among species of grasshoppers (Uvarov 1977, Reide 1987). Some species, such *D. carolina* can successfully mate after only a two hour interval whereas other species such as *D. viridis* have been observed to mate for upwards of 28 hours in the lab (Johnson 1998). Several factors have been proposed as to the reason for lengthy copulation in grasshoppers including the need to form complex spermatophores, to achieve sperm precedence, to act as a mechanical plug, or to transfer additional substances with spermatophores. Because both *S. americana* and *D. carolina* remain in copula after all sperm bundles have been transferred, it is likely that mate guarding by acting as a mechanical plug is occurring.

In order to better understand the functional importance of the spermatheca of *S. americana* and *D. carolina* it will be important to look at other aspects of the reproductive behavior and physiology. Discussions on the mating biology of *D. carolina* is sparse in the literature and would benefit from further study. There is extensive information pertaining to the mating behavior and natural history of *S. americana* (Kosal 1995, Kosal and Niedzlek-Feaver 1997). This study focuses primarily on sperm organization after a single mating and with the likelihood of females mating with several partners in these species an analyses of sperm organization following multiple matings would be important.

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