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Sperm Storage in Females of the Smooth Newt (*Triturus v. vulgaris* L.): I. Ultrastructure of the Spermathecae During the Breeding Season

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ABSTRACT Sperm storage in cloacal spermathecae was studied in females of *Triturus v. vulgaris* collected early in the breeding season in southern England. Females collected in terrestrial situations, presumably unmated, were mated in the laboratory, and the ultrastructure of the transferred sperm and the spermathecae was observed at various intervals after mating. Sperm from a spermatophore cap lodged in a female's cloacal orifice can migrate into spermathecae within 1 hr after mating. Spherical structures on the axial fibers of some sperm in the cap could indicate immaturity. Disorderly clusters of sperm from the cap are still present in the cloacal chamber 12 hr after mating but are absent 24 hr after mating. During storage, sperm often are in tangled masses in the spermathecal tubules. The sperm are coated with spermathecal secretions, and some sperm nuclei were observed embedded in the spermathecal epithelium. Little evidence for spermiophagy early in the breeding season was found. During oviposition, mazes of sperm occur external to the spermathecal orifices, and sperm may be released in this condition onto eggs as they pass through the cloaca. The tangled clusters in which sperm are found from pick-up to oviposition are hypothesized as an adaptation to reduce the effectiveness of sperm competition from the ejaculates of rival males. Additional studies, using the same protocol and covering the entire cycle of sperm storage, are necessary to enable interspecific comparisons leading to phylogenetic hypotheses. *J. Exp. Zool. 283:51–70, 1999.* © 1999 Wiley-Liss, Inc.

Sperm storage glands, spermathecae, occur in the roof of the cloaca in females of the Salamandridae and the other six families of salamanders that comprise the suborder Salamandroidea (Sever, '91a, '92a). The histology of the spermathecae of the salamandrid *Triturus v. vulgaris* from southern England was described by Verrell and Sever ('88). They found that the spermathecae consist of numerous simple tubular exocrine glands that pass laterally from the dorsal half of the posterior cloacal tube and all but the most posterior part of the cloacal chamber (Fig. 1). The reproductive cycle and breeding habits of *T. v. vulgaris* have been studied extensively (e.g., Verrell and Halliday, '85b; Verrell et al., '86), and the nominate form is the most widespread and abundant species of urodele in Europe (Griffiths, '96).

The purpose of this study is to determine the pattern of sperm acquisition, storage, and use by females of *T. v. vulgaris* as part of a larger study on the reproductive biology of the species. Smooth newts have an extended breeding season during which the female takes several weeks to lay her full clutch of eggs. The processes that females use to fertilize their eggs have profound implications for both male and female behaviour. For example, females mate several times during the breeding season, creating the conditions for sperm competition, and females may exploit these conditions to mate successively with males of higher quality (Gabor and Halliday, '97). To understand such aspects of the mating dynamics of newts, we need detailed knowledge of what is happening to sperm within the female's reproductive system.

In this paper, we extend the observations of Verrell and Sever ('88) on sperm storage in the smooth newt by examining the ultrastructure of sperm storage during the breeding season. We provide the first data for *T. v. vulgaris* on the nature

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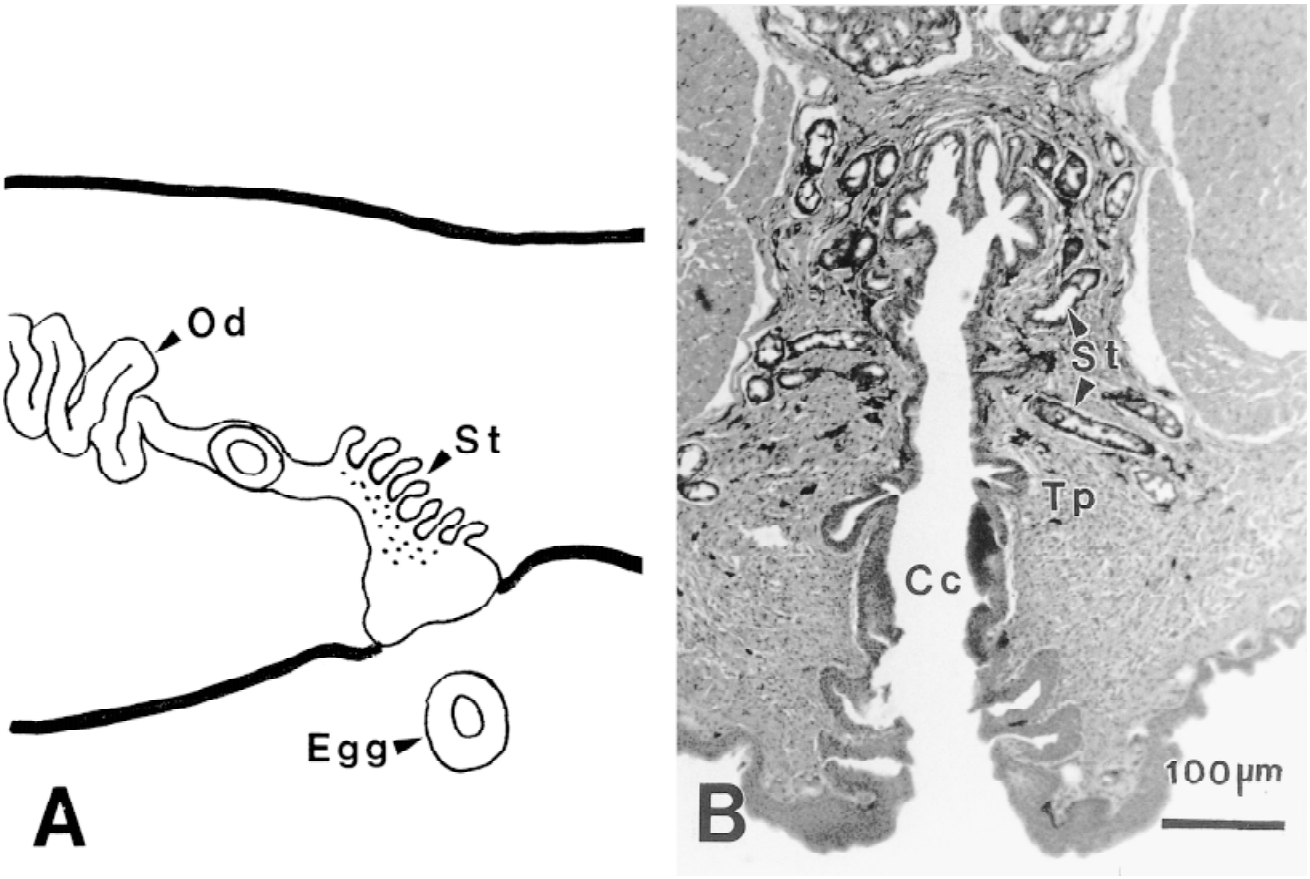


Fig. 1. Spermathecae of female *Triturus v. vulgaris*. (**A**) Midsaggital section through the cloaca and posterior oviducts. (**B**) Transverse section through the anterior cloacal chamber (Verrell and Sever, '88). Paraffin section stained with hematoxylin-eosin. Cc, cloacal chamber; Od, oviduct; St, spermathecae; Tp, tunica propria.

of the secretory process in the spermathecae, the length of time required for sperm to migrate into the spermathecae, the ultrastructure of stored sperm, and the occurrence of spermiophagy by the spermathecal epithelium. This paper is the first of a series that will detail the complete annual cycle of sperm storage in *T. v. vulgaris*, with comparisons to other subspecies and related taxa.

MATERIALS AND METHODS

All specimens were collected in the cities or vicinities of Oxford and Milton Keynes, U.K. Some individuals were collected at night in terrestrial situations and were assumed to be unmated females migrating to breeding ponds, whereas others were collected by dip-net from the ponds (Table 1). Certain females collected terrestrially were placed in aquaria filled with pond water in the laboratory, and, after at least 2 days of acclimation to an aquatic setting, matings were staged with males collected from breeding ponds at the same locales (Table 1). Because newts are not abundant in the U.K., and because we are much concerned with their conservation, we sought to keep the number of animals killed to the minimum necessary for this study.

Specimens were killed by immersion in 5% benzocaine, and snout-vent length (SVL) was measured from the tip of the snout to the posterior end of the vent. The axial region from the posterior insertion of the hindlimbs to the caudal end of the vent was excised from freshly killed specimens and cut mid-sagittally. The right half was fixed for paraffin infiltration for light microscopy (LM). The left half was cut transversely at the anterior end of the vent. One of the resultant blocks

TABLE 1. Specimens utilized in this study[1,2]

Date collected	Date sacrificed	SVL	Condition[2]	Follicles			
				Ovary	Ovid	Dia[3]	SE
4 Mar	10 Mar	48	Terrestrial[4]	147	0	1.15	0.04
5 Mar	10 Mar	48	Terrestrial	196	7	1.21	0.04
9 Mar	10 Mar	48	Terrestrial[4]	174	0	1.20	0.03
9 Mar	10 Mar	42	In pond	120	13	1.20	0.04
9 Mar	10 Mar	42	In pond	111	12	1.14	0.04
9 Mar	11 Mar	48	Sacrificed 1 hr after mating	318	0	1.26	0.04
9 Mar	14 Mar	42	Sacrificed 12 hr after mating	186	0	1.12	0.04
9 Mar	14 Mar	45	Sacrificed 12 hr after double matings	162	0	1.18	0.05
9 Mar	14 Mar	44	Sacrificed 24 hr after mating	308	0	1.13	0.02
9 Mar	18 mar	43	Sacrificed 4 days after mating	206	0	1.14	0.04
9 Mar	20 Mar	54	Sacrificed 7 days after mating	492	0	1.19	0.04
9 Mar	20 Mar	44	Mated 13 Mar and 19 Mar; sacrificed 12 hr after last mating	263	0	1.17	0.04
9 Mar	21 Mar	42	Mated 17 Mar; laid 11 eggs and sacrificed 21 Mar	146	20	1.18	0.04

[1]Measurements are in mm. SVL, snout-vent length; Ovid, oviduct; Dia, diameter; SE, standard error.
[2]Specimens sacrificed after mating were collected terrestrially.
[3]Largest diameter of 11 vitellogenic follicles in the ovaries.
[4]Lacked sperm in their spermathecae.

of tissue was prepared for scanning electron microscopy (SEM), and the other was embedded in epoxy resin for thin (LM) and ultrathin sections for transmission electron microscopy (TEM). Carcasses of all specimens were preserved in 10% neutral buffered formalin (NBF) and are housed in the research collections at Saint Mary's College.

For the paraffin procedure, the tissues were initially fixed in NBF, rinsed in water, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections (10 μm) were cut with a rotary microtome and affixed to albuminized slides. Alternate slides from each specimen were stained with hematoxylin-eosin (general histology) or alcian blue 8GX at pH 2.5 (AB, for primarily carboxylated glycosaminoglycans) followed by the periodic acid–Schiff's method (PAS, neutral carbohydrates and sialic acids). Procedures followed Kiernan ('90).

For SEM and TEM procedures, the tissues were fixed in a 1:1 solution of 2.5% glutaraldehyde in Millonig's phosphate buffer at pH 7.4 and 3.7% formaldehyde buffered to pH 7.2 with monobasic and dibasic phosphate. For TEM, the tissues were trimmed into 1-mm blocks. After initial fixation, tissues were rinsed in Millonig's buffer, postfixed in 2% osmium tetroxide, and dehydrated through a graded series of ethanol.

For SEM, the tissue was then subjected to critical point drying, mounted on a metal stub with adhesive tape, and sputter-coated with gold. The specimens were examined with a JEOL JSM 820 (JEOL, Welwyn, Herts, UK). For TEM, tissues following dehydration were immersed in increasing concentrations of Epon epoxy resin (Agar Scientific, Stansted, Essex, UK) in absolute ethanol prior to polymerization in pure Epon for 12 hr at 60°C. Plastic sections were cut with an RMC MT7 ultramicrotome (Research and Manufacturing Co., Tucson, AZ) and DIATOME (Biel, Switzerland) diamond knives. Semi-thin sections (0.5–1 mm) for light microscopy were placed on microscope slides and stained with toluidine blue. Ultra-thin sections (70 nm) for TEM were collected on uncoated copper grids and stained with solutions of uranyl acetate and lead citrate. Ultrathin sections were viewed with a Hitachi H-300 transmission electron microscope (Nissei Sangyo America, Mountain View, CA). Terminology for sperm ultrastructure follows Picheral ('79).

Ovaries and oviducts were removed from the carcasses, and the number of dark, vitellogenic follicles of similar size were counted. A random selection of 11 follicles from the ovaries of each specimen was measured to the nearest 0.01 mm with an ocular micrometer in a dissecting microscope (Table 1).

RESULTS

Females collected in terrestrial situations and sacrificed prior to matings in the laboratory

Two of the three females collected on land were, as expected, unmated and lacked sperm in their spermathecae (Table 1, Figs. 2, 3). The spermathecae consist of cuboidal epithelium with oblong, eu-

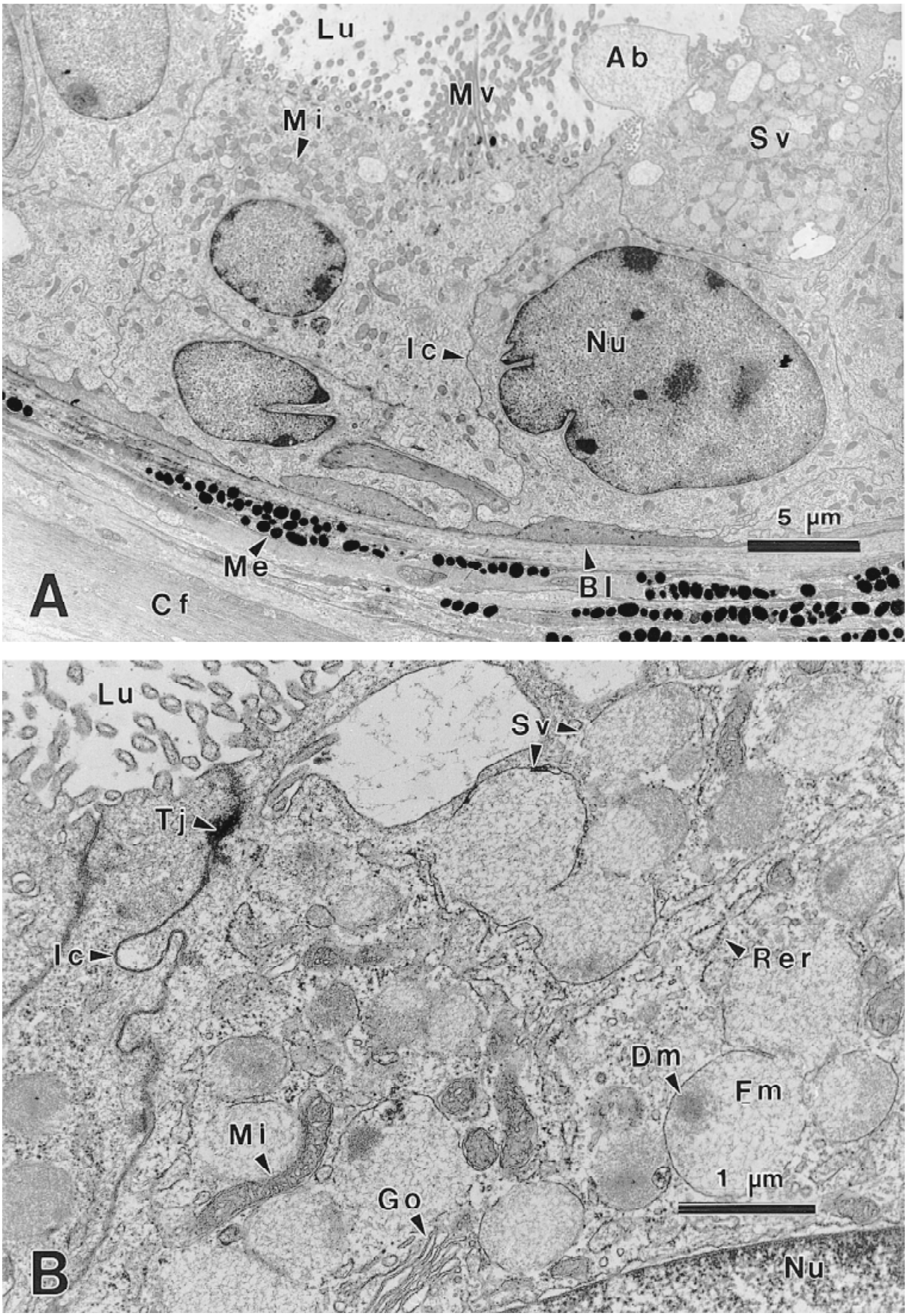


Figure 2.

chromatic nuclei and numerous apical microvilli (Fig. 2A). Melanophores are numerous superficial to the basal lamina (Fig. 2A). Intercellular canaliculi are narrow (Fig. 2A), and tight junctions occur at the luminal border (Fig. 2B). Active production and release of secretory vacuoles is apparent. Vacuoles filled with a flocculent material are numerous in the apical cytoplasm and associated with Golgi complexes, rough endoplasmic reticulum (RER), and mitochondria (Fig. 2A, B). Some vacuoles contain an electron-dense particle along their circumference (Fig. 2B). In paraffin sections, the apical cytoplasm (where secretory vacuoles are most dense) is PAS+ and AB–.

Secretory vacuoles apparently often coalesce into larger blebs (2–4 mm in diameter) that are released into the lumen by an apocrine mechanism, i.e., a portion of the apical cytoplasm containing secretory vacuoles is cleaved-off from the rest of the cell (Fig. 3A, B). The blebs consist of the flocculent material (Fig. 3A). Note that the bleb illustrated in Fig. 3A is associated with a region of cilia. Cilia are lacking in the spermathecae but line the cloacal walls (Sever, '92a), so this micrograph shows secretion at a spermathecal orifice. SEM revealed that the inner lining of the spermathecae is deeply furrowed (Fig. 3C). Occasionally, red blood cells are observed within the spermathecae, an artifact resulting from bleeding during dissection of the tissue (Fig. 3C).

One female collected on land had mated and possessed oviducal eggs, indicating that either some oviposition had occurred or was imminent (Fig. 4, Table 1). In this female, sperm are numerous (Fig. 4A) and appear normal in cytology (Fig. 4B). Secretory activity is still intense, with numerous Golgi complexes, RER profiles, and secretory vacuoles in the cytoplasm (Fig. 4B–D). We also found occasional myelinic figures (My) in vacuoles in the apical cytoplasm (Fig. 4D).

Females collected in aquatic situations

Two females collected from a pond on 9 March were sacrificed the following day. Both had sperm in their spermathecae and oviducal eggs (Table 1). Observations of captive newts at the beginning of the breeding season indicate that females are highly responsive to males at this time (Hosie, '92; Halliday, '98).

Staged matings of females in the laboratory

Females mated in the laboratory were all collected in terrestrial situations and presumed unmated, although our experience with one individual (Table 1, Fig. 4) indicates that this is not conclusive. Still, we consider it likely that most females collected in terrestrial situations have not bred in the current season. The females we collected in terrestrial situations were allowed to acclimate in aquaria filled with pond water for at least 2 days before exposure to males that had been collected from breeding ponds.

One female was sacrificed 1 hr after mating (Figs. 5, 6). SEM revealed a tangled cluster of sperm just inside the cloacal orifice (Fig. 5A). The sperm are not in orderly arrays (Fig. 5B). A notable feature is the occurrence of multiple spherical structures along the tails of some sperm (Fig. 5C), perhaps indicating immaturity of the sperm (see Discussion). In a multiply mated female sacrificed 12 hr after last mating, we also found a mass of sperm just inside the cloacal orifice (Fig. 5D).

Sperm are present in the spermathecae of the female sacrificed 1 hr after mating. The sperm are scattered throughout the lumina of the tubules and once again, are not in an orderly alignment (Fig. 6A–C). The luminal sperm appear normal in cytology (Fig. 6B). The presence of numerous secretory vacuoles and organelles involved in synthetic activity indicate that the epithelium is still actively producing secretions, and the intercellular canaliculi remain narrow (Fig. 6D). Portions of some sperm cells are adjacent to or embedded in the apical cytoplasm, but no obvious signs of degradation are apparent (Fig. 6C, D). However, unrecognizable debris is vacuolated in the spermathecal epithelium, and, as in the mated, terrestrially collected individual (Fig. 4D), myelinic structures also are present (Fig. 6D, inset).

We found sperm using SEM in the spermathecae of a multiply-mated female sacrificed 12 hr after mating (Fig. 7). The lining of the spermathecae contains much fibrous material together with occasional globules (Fig. 7A). The axes of the sperm are liberally speckled with debris, perhaps resulting from breakdown or interactions with the spermathecal secretory products (Fig. 7B). The

Fig. 2. Transmission electron micrographs of the spermathecae of a 48-mm SVL female *Triturus v. vulgaris* collected on land 4 March and sacrificed 10 March prior to mating. (**A**) Overview of spermathecal epithelium and adjacent luminal and stromal areas. (**B**) Detail of the apical cytoplasm. Ab, apocrine bleb; Bl, basal lamina; Cf, collagen fiber; Dm, electron-dense material; Fm, flocculent material; Go, Golgi complex; Ic, intercellular canaliculi; Lu, lumen; Me, melanin granules; Mi, mitochondria; Mv, microvilli; Nu, epithelial cell nucleus; Rer, rough endoplasmic reticulum; Sv, secretory vacuoles; Tj, tight junction.

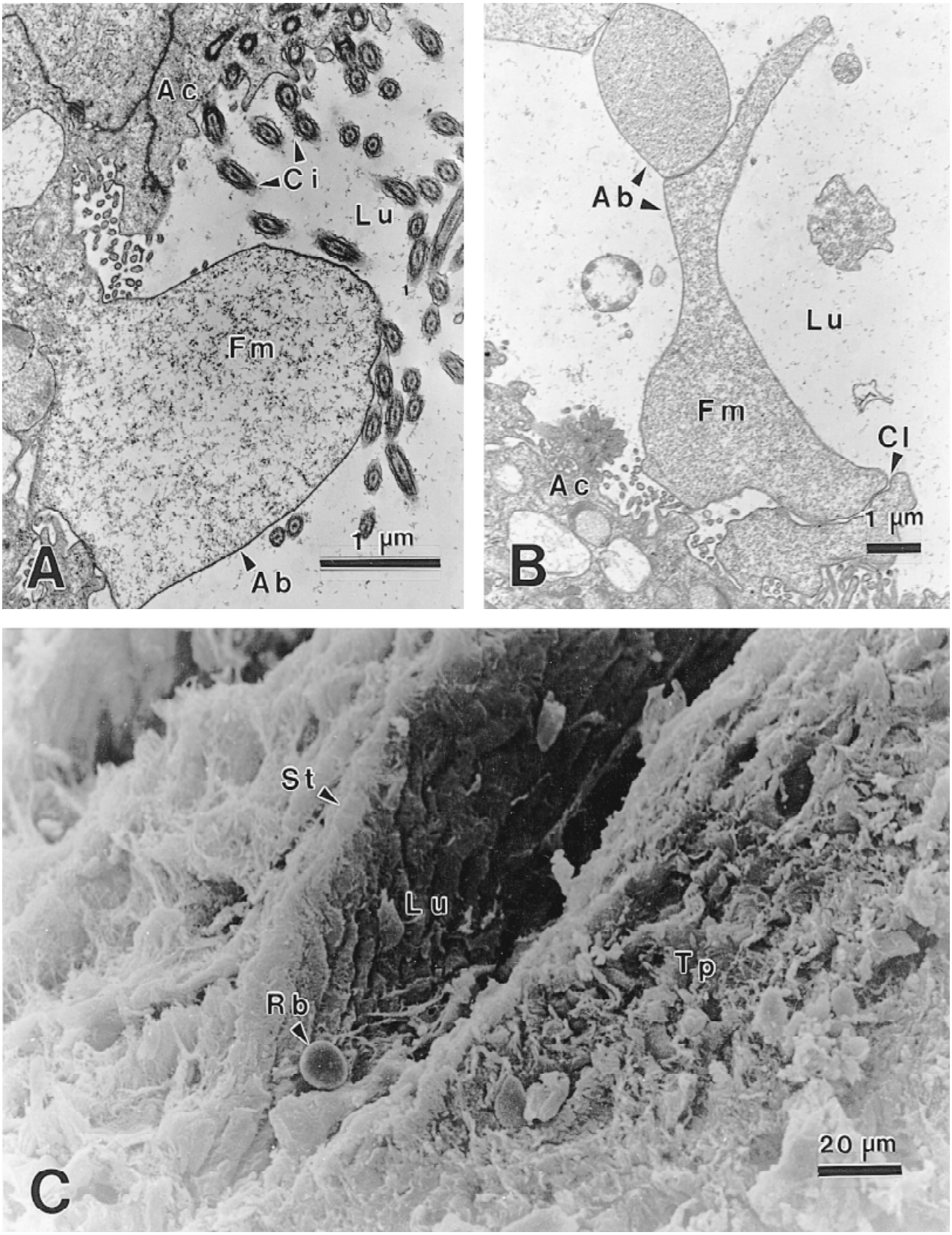


Figure 3.

heads of some sperm cells are embedded in the spermathecal lining (Fig. 7C).

In a female sacrificed 4 days after a staged mating in the laboratory, sperm appear less numerous than in females sacrificed within a day of mating (compare Figs. 6A and 8A). Also, less secretory activity is evident in the spermathecal epithelium. The euchromatic nuclei nearly fill the cells, and secretory vacuoles are few (Fig. 8B–D). Intercellular canaliculi are widened (Fig. 8B, D), and myelinic figures are numerous (Fig. 8D).

SEM of the inner lining of the spermathecae of a female sacrificed 7 days after mating reveals scattered small clusters of sperm (Fig. 9A), although some sperm occur singly (Fig. 9B). Sperm in clusters often are intertwined and spotted with debris, as in the female sacrificed 12 hr after mating (compare Figs. 7 and 9).

Finally, we examined a female who, 4 days after mating, began laying eggs, and this individual was sacrificed after ovipositing 11 eggs, all of which subsequently began development. The female also contained 20 oviducal eggs, indicating that more egg-laying was imminent. As in the other females, sperm are scattered in the spermathecal lumina (Fig. 10A) and appear normal in cytology (Fig. 10B). Again, compared to unmated females and those sacrificed within a day of mating, fewer secretory vacuoles are present and intercellular canaliculi are wider (Fig. 10B, C). Some degraded portions of sperm cells and other debris occur in the spermathecal epithelium (Fig. 10C). Tangled masses of sperm are found external to spermathecal orifices (Fig. 10D).

DISCUSSION

In southern England, smooth newts leave their terrestrial hibernacula in February and March and migrate to their breeding ponds (Verrell and Halliday, ’85b). Breeding activity commences upon arrival of newts at the ponds, but field studies suggest that the peak of mating activity, based upon frequency of courtship, intermale competition, and oviposition, is most intense in May and June (Verrell et al., ’86; Verrell and McCabe, ’88). Laboratory studies, however, suggest that females are most receptive early in the breeding season and that most females are inseminated, often by several males, within 6 days of arriving at a pond (Hosie, ’92).

In this study, females used in laboratory-staged courtships were collected during migration to ponds at the start of the mating season to ensure the collection of females that were unmated but in breeding condition (showing migratory behaviour, possessing eggs of ovulatory size, etc.). Subsequent papers will describe sperm storage in female newts at later stages of the annual reproductive cycle. Female smooth newts leave the breeding ponds in the summer months to assume a terrestrial existence until the next breeding season (Verrell and Halliday, ’85b).

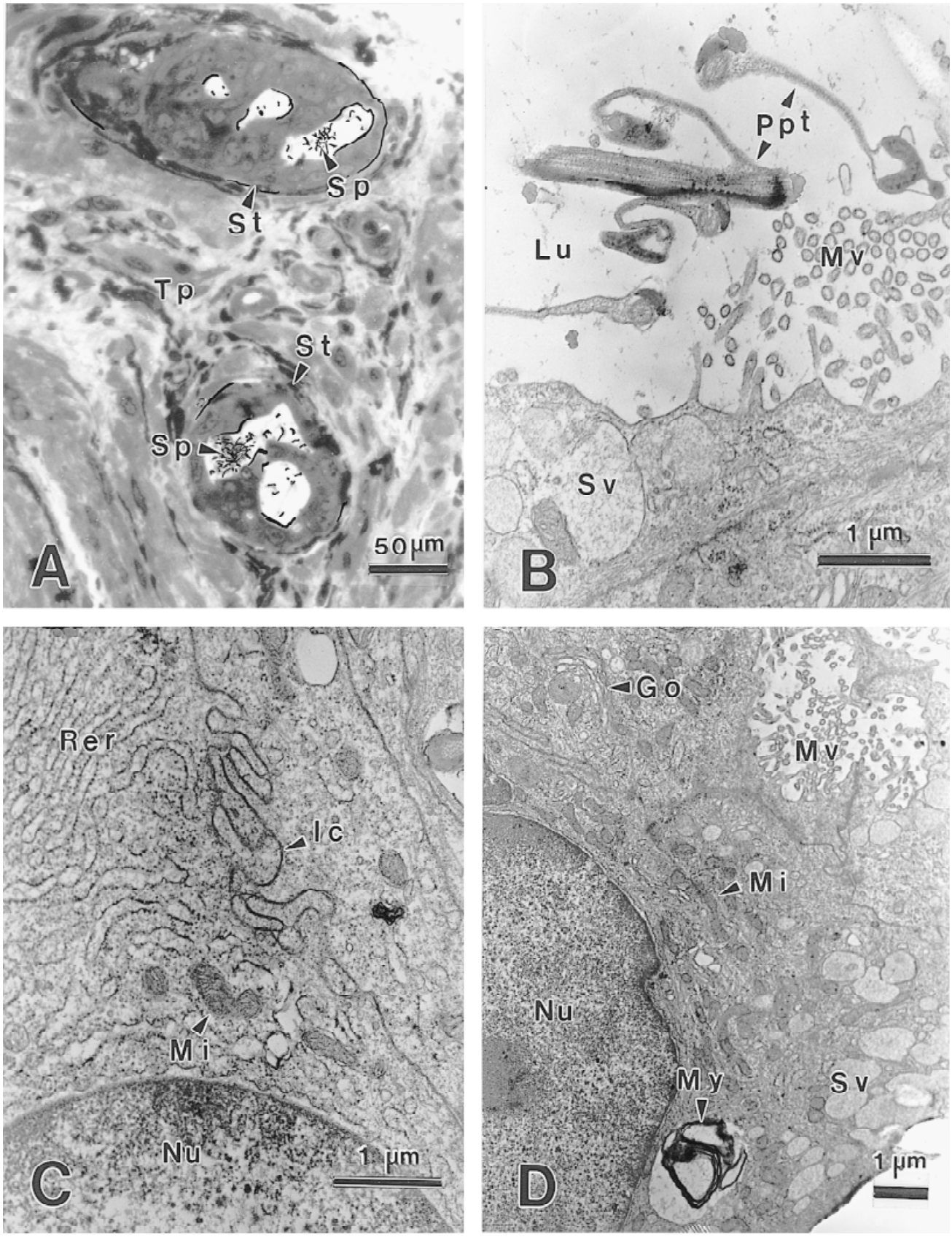
Duration of sperm storage and migration of sperm

Verrell and Sever (’88) reported sperm in the spermathecae of one of four individuals collected while immigrating to breeding ponds, and in all females collected in ponds from April through July. An aquatic female collected in August and fall-collected terrestrial females lacked stored sperm (Verrell and Sever, ’88). The sperm in the terrestrial female collected in March were interpreted by Verrell and Sever (’88) to be inviable sperm retained from the previous season, since sperm were sparse, and the sperm were PAS–, whereas freshly stored sperm are PAS+.

During this study, however, we found sperm in one of the four terrestrial females we collected in March, and sperm in this female were numerous and appeared normal in cytology. These factors plus the presence of oviducal eggs in this individual indicates that she had mated, perhaps even oviposited some eggs, and had left the breeding pond. Such “wandering behaviour” between water and land following arrival at a breeding pond has been reported in the newts *Notophthalmus viridescens* (Hurlbert, ’69) and *Triturus alpestris* (Joly and Miaud, ’89) as well as other urodeles (Pimental, ’60; Hasumi and Iwasawa, ’92).

The pond (Linford) where the mated terrestrial individual was collected had an unusually low water level in this particular breeding season, perhaps increasing the chances that a female would “wander” after arrival at the site. The specimens

Fig. 3. Spermathecae of female *Triturus v. vulgaris*. (A) and (B) are transmission electron micrographs of apocrine blebs containing secretory product from a female 48-mm SVL collected 4 March and sacrificed 10 March prior to mating. (**A**) Shows epithelium at the spermathecal orifice, and (**B**) shows the luminal border in a more distal portion of a spermathecal tubule. (**C**) Scanning electron micrograph of a longitudinal section through a spermathecal tubule showing the lumen and surrounding tissues. Female 48-mm SVL collected 9 March and sacrificed 10 March prior to mating. Ab, apocrine blebs; Ac, apical cytoplasm; Ci, cilia; Cl, cleavage line; Fm, flocculent material; Lu, lumen; Rb, red blood cell; St, spermatheca; Tp, tunica propria.


A
Sp
St
Tp
St
Sp
50 µm
B
Ppt
Lu
Mv
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D
Go
Mv
Mi
Nu
My
Sv
1 µm

Figure 4.

used in the mating experiments, however, were from a site (Newton Longville) where the pond was deep and aquatic habitat was plentiful; specimens were collected from off a road encircling the pond and appeared to be emigrating toward the site. Thus, although we cannot be certain absolutely, we believe none of the females used in the staged laboratory matings had previously mated in the current season.

If so, our findings provide information on the length of time necessary for sperm to migrate from a spermatophore cap into the spermathecae in female smooth newts. After picking-up a spermatophore cap, the cap remains visible in the cloacal orifice for 20–45 min, during which time the sperm mass is drawn into the cloaca, probably by muscular contractions (Hardy and Dent, '86), and is no longer externally visible (Halliday, '74). We sacrificed a female, presumably unmated (but see above), 1 hr after mating. A mass of sperm was present just inside the cloacal orifice, but sperm also were present in the spermathecae. Sperm, therefore, can migrate from the sperm cap into the spermathecal tubules within 1 hr of mating. A mass of sperm still was present inside the cloacal orifice of a female sacrificed 12 hr after mating, but none was present in a female sacrificed 24 hr after mating.

Thus, in *Triturus v. vulgaris*, more than 12 hr may be necessary for all sperm (if not inadvertently expelled with urinary and digestive waste materials) to migrate into the spermathecae, but the process can be completed within 24 hr. The only other observations in urodeles on sperm migration from the spermatophore cap to the spermathecae were made on the red-spotted newt, *Notophthalmus viridescens*, by Hardy and Dent ('86). In that species, sperm migration into spermathecal tubules also occurs within 1 hr of insemination, and only small, scattered groups of sperm are found in the cloacal lumen 24 hr after attachment of the sperm cap to the cloacal lips (Hardy and Dent, '86).

Evidence for spermiophagy

We did not find frequent associations between sperm and the spermathecal epithelium, although a few sperm nuclei were observed embedded in the apical cytoplasm using both TEM (Figs. 6C, 6D and 10C) and SEM (Fig. 7). In only one instance did we observe definite indications of sperm degeneration (Fig. 10C). These findings are in contrast to observations on the closely related Italian newt, *Triturus italicus* (Sever and Brizzi, unpublished), the spectacled salamander, *Salamandrina terdigitata* (Brizzi et al., '95), and the North American red-spotted newt, *Notophthalmus viridescens* (Sever et al., '96a), in which extensive spermiophagy occurs by the spermathecal epithelium. It will be interesting to discover whether spermiophagy is more extensive in *T. vulgaris* collected from later in the breeding season, or from post-breeding females.

Spermiophagy also has been reported in the plethodontids *Eurycea cirrigera* (Sever, '92b) and *Plethodon cinereus* (Sever, '97) and the ambystomatid *Ambystoma opacum* (Sever and Kloepfer, '93). In these species and in *N. viridescens*, spermiophagy occurs from the beginning of the sperm storage period, but this does not seem to be the case for either *T. italicus* (Sever and Brizzi, unpublished) or *S. terdigitata* (Brizzi et al., '95), in which spermiophagy occurs only at the end of the breeding season. In several species in which the ultrastructure of the annual cycle of sperm storage has been studied, no evidence of spermiophagy has been found. These species are the ambystomatid *A. tigrinum* (Sever, '95), the amphiumid *Amphiuma tridactylum* (Sever et al., '96b), and the proteid *Necturus beyeri* (Sever and Bart, '96).

Spermathecal secretions

The secretory product was being actively produced and released in females sacrificed both before and after mating. As noted by Verrell and Sever ('88), the product is PAS+ and AB–, indicating the presence of neutral carbohydrates and the absence of glycosaminoglycans. Much variability occurs among salamanders in the carbohydrate histochemistry of the spermathecae, but most species possessing simple spermathecae have AB+ secretions (Sever, '94). The significance of this character is unknown, but it is clear that one type of secretion (either AB+ or PAS+) predominates in most species.

Fig. 4. Spermathecae containing sperm from a 48-mm SVL female *Triturus v. vulgaris* collected on land 5 March and sacrificed 10 March. A light micrograph of a semi-thin plastic section stained with toluidine blue is illustrated in (A), and (B), (C), and (D) are electron micrographs. (**A**) Sections through spermathecae containing sperm. (**B**) Apical epithelium and adjacent lumen. (**C**) Supranuclear area of spermathecal epithelium. (**D**) Apical epithelium containing a mylenic figure (My). Go, Golgi complex; Ic, intercellular canaliculi; Lu, lumen; Mi, mitochondria; Mv, microvilli; My, mylenic figure; Nu, epithelial cell nucleus; Ppt, principle piece of the tail; Rer, rough endoplasmic reticulum; Sp, sperm; St, spermathecae; Sv, secretory vacuoles; Tp, tunica propria.

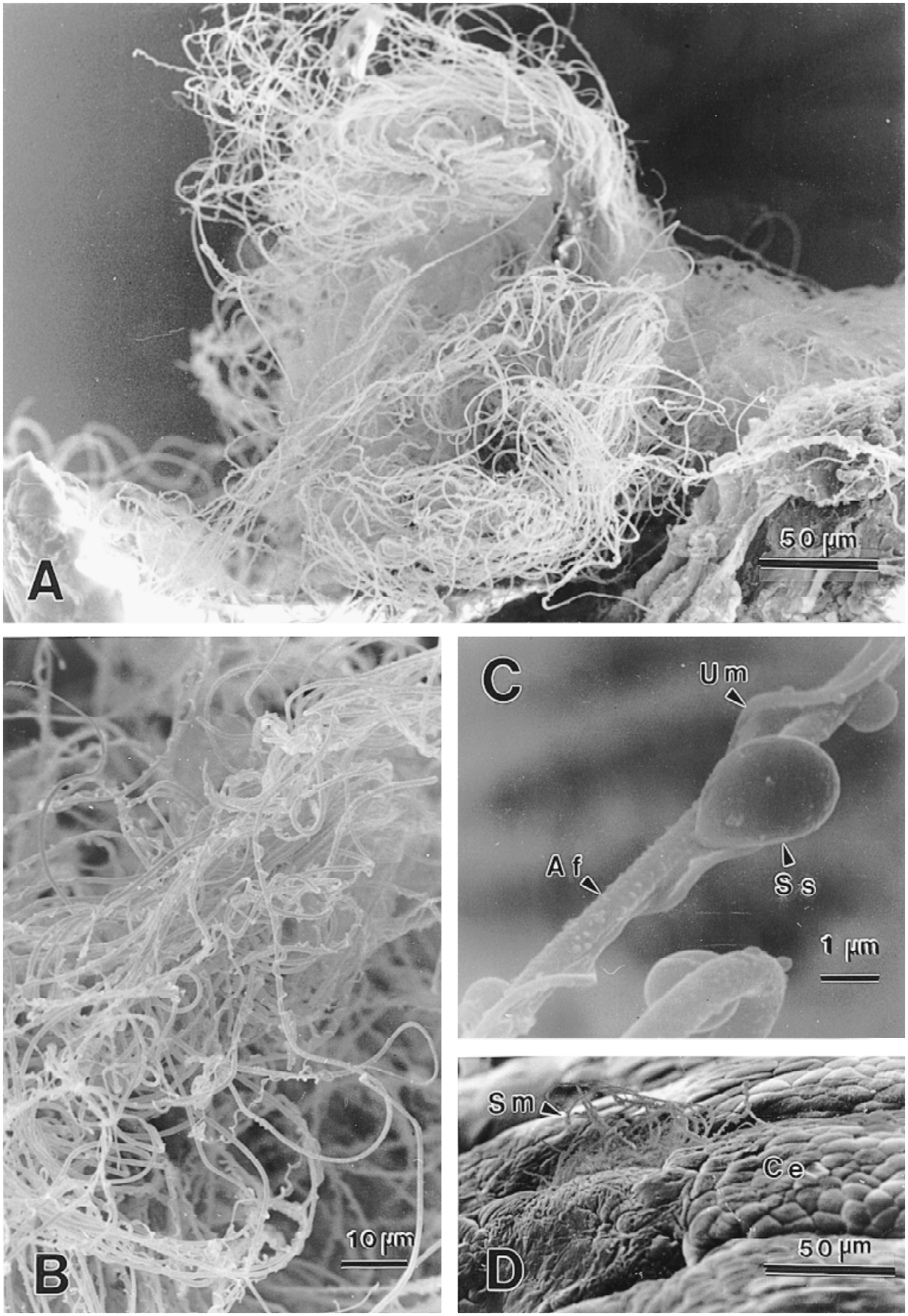

Figure 5.

The secretory vacuoles in TEMs contain a flocculent material with an occasional eccentric electron-dense particle. In most other species, the vacuoles usually contain a mixture of flocculent material and electron-dense particles, the latter of which may be centrally located, as in *Ambystoma opacum* (Sever and Kloepfer, '93), or eccentric, as in *A. tigrinum* (Sever, '95). In *Eurycea cirrigera* (Sever, '91b) and *Notophthalmus viridescens* (Sever et al., '96a), however, the secretory vacuoles are uniformly electron-dense. No consistent relationship occurs between the ultrastructural appearance of the secretory vacuoles and their carbohydrate histochemistry.

Although some have hypothesized a "nutritive" function for spermathecal secretions (Benson, '68; Dent, '70; Boisseau and Joly, '75; Davitt and Larsen, '88a; Greven and Guex, '94), we can find no cytological evidence for such a process. Hardy and Dent ('86) found that sperm were inactive during storage in the spermathecae of *Notophthalmus viridescens*. If sperm are quiescent, they are not likely to be expending energy. We agree with Sever and Kloepfer ('93) that the secretions probably provide the environment for maintaining sperm quiescence during storage. The "debris" associated with stored sperm in SEMs presumably represents secretory products.

The only other urodeles in which an apocrine mode of secretion has been proposed for spermathecal secretions are the plethodontid *Plethodon larselli* (Davitt and Larsen, '88a) and the amphiuma, *Amphiuma tridactylum* (Sever et al., '96b). In other salamanders, the exportation of secretory product is merocrine (Sever, '97).

Appearance of stored sperm and implications for polyspermy

Davitt and Larsen ('88a) illustrated densely packed, parallel clusters of sperm in the spermathecae of *Plethodon larselli* prior to ovulation, but a random orientation of sperm after induced oviposition. Sever ('97), in *P. cinereus*, noted that small clusters of stored sperm exhibit the same orientation, and this condition seems evident in electron micrographs of spermathecal sperm in most other species as well (cf., Sever, '91b; Sever and Kloepfer, '93). In *Triturus vulgaris*, however, the sperm in the sperm cap are arrayed in tangled masses (Fig. 5), and this condition also was observed in the spermathecal tubules (Fig. 9). In the specimen sacrificed during oviposition, disordered masses of sperm were found external to the spermathecal orifices (Fig. 10D).

Except for salamanders in the family Hynobiidae, urodele eggs have no block to polyspermy; many sperm may enter an egg although only one will eventually be involved in fertilization (Elinson, '86). Waights ('98) found in a sample of 30 freshly oviposited eggs that four contained no sperm, one contained 54, and another had 100. In the remaining 24 eggs, there were 1–20 sperm (mean 4.2). The sperm were observed either singly or as disorderly masses on the surface of the freshly oviposited eggs (Waights, '98). The tangled masses of sperm found external to the spermathecal orifices in the *Triturus vulgaris* sacrificed during oviposition indicate that sperm may be released in such masses onto eggs as they pass through the cloaca, and possibly sperm penetrate eggs in this state.

Presence of spherical structures

Kuramoto ('95, '97) reported the association of spherical structures with the cytoplasmic droplets in some sperm from the reproductive tracts of males of *Hynobius stejnegeri* and *H. tsuensis*. The significance of these spherical bodies was not discussed, and we can find no mention of these structures associated with the sperm of any other salamander. On some sperm in a detached spermatophore cap within the cloaca of a female *Triturus vulgaris* (Fig. 5C), we found spherical structures, but we did not find a cytoplasmic droplet. Cytoplasmic droplets are relatively larger, irregular masses, and only a single cytoplasmic droplet is associated with a given sperm cell (Murphy et al., '73). Wortham et al. ('82) reported that cytoplasmic droplets usually were found on sperm taken from the vas deferens of ambystomatid and plethodontid salamanders, but were not found on sperm of the salamandrid *Notophthalmus viridescens*. Other references on the sperm of salamandrids also fail to mention a cytoplasmic droplet (Picheral, '79; Kuramoto, '95; Kuramoto and Tanaka, '97).

Cytoplasmic droplets are an indicator of imma-

Fig. 5. (A) to (C) are scanning electron micrographs of sperm from a 48-mm SVL female *Triturus v. vulgaris* collected 9 March and sacrificed 11 March, 1 hr after mating. (**A**) Mass of sperm just inside the cloacal orifice. (**B**) Detail of sperm mass showing disorderly arrays of individual sperm. (**C**) Multiple spherical droplets on a sperm tail. (**D**) Scanning electron micrograph showing mass of sperm just inside the cloacal orifice of a 45-mm SVL female *T. vulgaris* sacrificed 12 hr after her last mating. Af, axial fiber; Ce, cloacal epithelium; Sm, sperm mass; Ss, spherical structure; Um, undulating membrane.

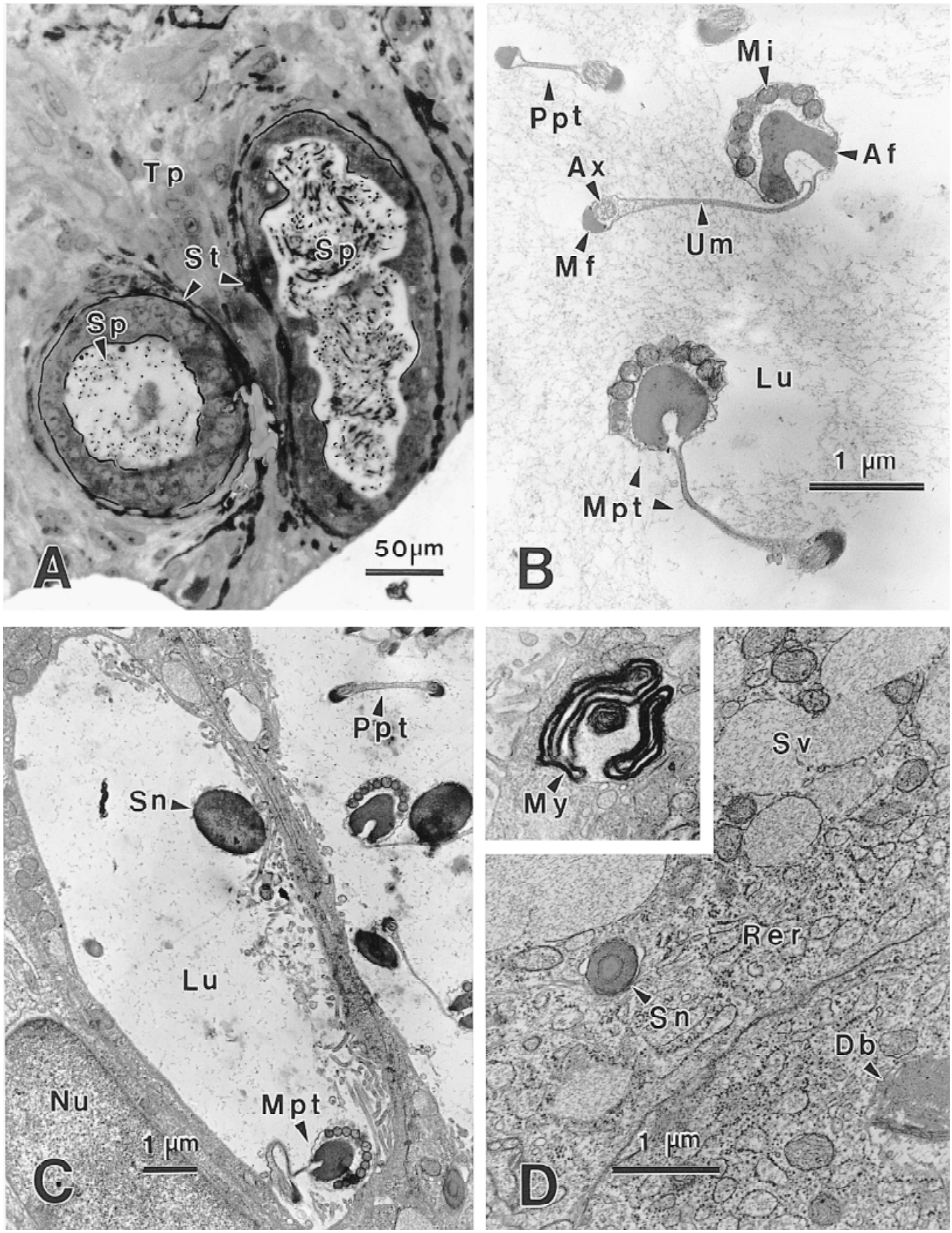
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Figure 6.

ture sperm (Cummins, '73; Bedford, '79; Picheral, '79). In mammals, cytoplasmic droplets are present on sperm after detachment from Sertoli's cells and are subsequently lost during sperm maturation in the epididymis (Bedford, '78, '79; Kaplan et al., '84; Hermo et al., '88). As indicated above, cytoplasmic droplets have been reported on sperm from the vas deferens of various hynobiids, ambystomatids, and plethodontids (Murphy et al., '73; Picheral, '79; Wortham et al., '82; Kuramoto, '95, '97). Several reports indicate that the droplets are shed in the spermatophore cap (Russell et al., '81; Zalisko et al., '84). Davitt and Larsen ('88b), however, reported that *in Rhyacotriton olympicus* the cytoplasmic droplets are lost during storage of sperm in the spermathecae, and the droplets subsequently are phagocytized by the spermathecal epithelium.

The significance of our finding spherical structures (but no cytoplasmic droplet) on some sperm is unknown and requires further study. If, however, the spherical structures also are an indicator of sperm immaturity, the presence of such sperm in the detached spermatophore cap could have consequences for considerations of sperm competition, some other aspects of which are considered next.

Implications for sperm competition

Parker ('70) listed four conditions necessary for sperm competition to occur. These are (1) individual females are inseminated by more than one male; (2) females can store sperm, at least for the duration of the period over which matings with different males occur; (3) sperm remain viable during the storage period; and (4) sperm are stored and used efficiently by females. Conditions 1–3 have all been studied in urodeles, but very little is known about condition 4 (Halliday, '98).

In many urodeles, an individual female can become inseminated by more than one male by two means (Halliday and Verrell, '84; Verrell, '89). First, she may respond positively, on different occasions, to the courtship behaviour of different males. Second, she may become multiply inseminated during a single mating encounter, by both the male who initiates courtship and a male who sexually interferes in the encounter. A number of studies (reviewed by Halliday, '98) suggest that, in *Triturus*, females typically mate with several males as a result of both of these phenomena. For example, Rafinski ('81), using electrophoretic information, has reported high levels of multiple paternity in *T. alpestris*.

For newts in Britain the breeding season is prolonged. In both *Triturus cristatus* (Verrell and Halliday, '85a) and *T. v. vulgaris* (Verrell and Halliday, '85b; Verrell et al., '86), there is much variation among individuals in terms of how long they spend in water, with some being present in a pond for as long as 9 months. Mating activity, however, is confined largely to a 3-month period in the spring.

For *Triturus v. vulgaris* breeding in Britain, data from a number of studies (Halliday, '74, '76; Verrell and Halliday, '85b; Verrell et al., '86; Hosie, '92; Waights, '96) enable us to build up a picture of how mating dynamics change over the course of the breeding season (Halliday, '98). The primary determinant of the long duration of breeding activity in this species is the mode of egg-laying. Females lay their eggs individually, each carefully wrapped in a leaf; this is a time-consuming process and a female may need 3 months to lay her entire clutch (Baker, '92). This prolonged oviposition period appears to have selected for an early initiation of breeding activity in female smooth newts who, unlike many amphibians, migrate to water at the same time as, and, occasionally slightly before, males (Verrell and Halliday, '85b). Females become highly receptive of matings within a few days of their arrival and, in the laboratory, mate with several males over a few days (Hosie, '92). At this time, the secondary sexual characters of the males, notably the dorsal crest, are not fully developed (Griffiths and Mylotte, '88). After a few days, females begin to lay their eggs and, although they continue to mate during egg-laying (Pecio, '92), they become less receptive to males (Hosie, '92). Field observations suggest that competitive interactions among males are rare at the beginning of the breeding season, but become more common later, while females are egg-laying (Verrell and McCabe, '88). Indeed, at the very beginning of the season, mating competition may be

Fig. 6. Spermathecae of 48-mm SVL female *Triturus v. vulgaris* collected 9 March and sacrificed 11 March, 1 hr after mating. A light micrograph of a semi-thin plastic section stained with toluidine blue is shown in (A), and (B) to (D) are transmission electron micrographs. (**A**) Sections through spermathecae containing sperm. (**B**) Sperm in the lumen. (**C**) Luminal border, showing sperm associated with apical cytoplasm. (**D**) Detail of spermathecal epithelium; inset shows mylenic figure (My). Af, axial fiber; Ax, axoneme; Lu, lumen; Mi, mitochondria; Mf, marginal fiber; Mpt, middle piece of the tail; My, mylenic figures; Nu, epithelial cell nucleus; Ppt, principle piece of the tail; Rer, rough endoplasmic reticulum; Sn, sperm nucleus; Sp, sperm; St, spermathecae; Sv, secretory vacuoles; Tp, tunica propria, Um, undulating membrane.

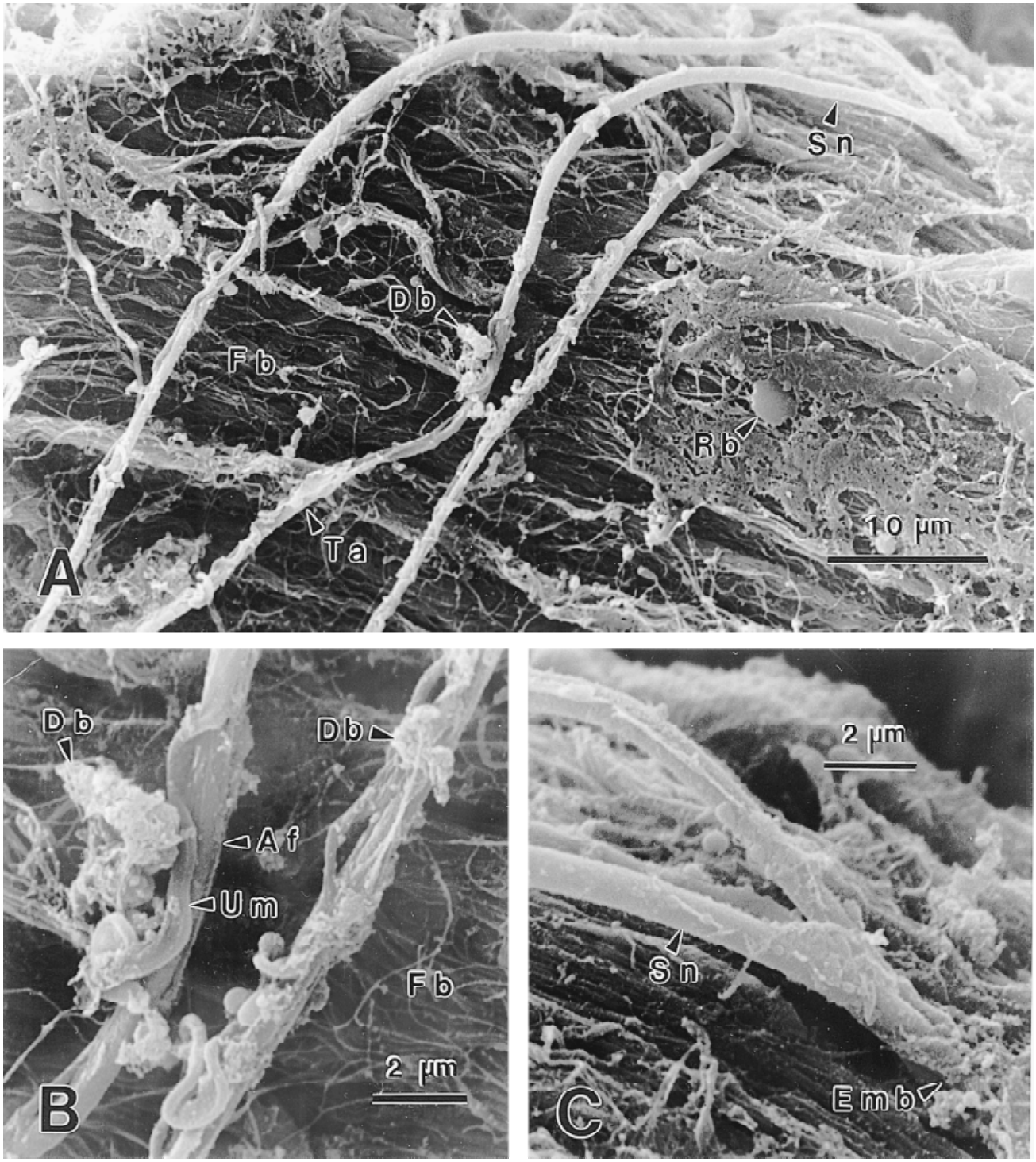

Fig. 7. Scanning electron micrographs of sperm in the spermathecae of a 45-mm female *Triturus v. vulgaris* sacrificed 12 hr after double matings (10 minutes apart). (**A**) Three sperm cells in relation to the luminal lining of a spermatheca. (**B**) Detail of the tails of two sperm with associated debris. (**C**) Nuceli of two sperm embedded (Emb) in the spermathecal epithelium. Af, axial fiber; Db, debris; Emb, embedded sperm nuclei; Fb, fibrous material; Sn, sperm nucleus; Rb, red blood cell; Ta, sperm tail; Um, undulating membrane.

more intense among females than among males. At this time, females intrude upon courting pairs and "steal" sperm (Waights, '96). The prolonged breeding season of *Triturus* thus provides ample opportunity for females to multiply mate. Furthermore, there is evidence that they combine multiple mating with sperm competition in an adaptive mating strategy (Halliday, '98). In a laboratory study, females of *T. v. vulgaris* remated selectively with males whose dorsal crest was better developed than that of their previous mate (Gabor and Halliday, '97), a strategy that, if there is a last-male paternity advantage, enables females to improve the "quality" of their successive mates.

An alternative, or additional, reason why female newts mate several times during the breeding season is to replenish their sperm supplies, either because sperm deteriorate and die over time, and/or because they are not used efficiently. At present, we know little about these issues. Potentially, however, females apparently need mate only once. A sample of 300 *Triturus v. vulgaris* spermatophores analyzed by Waights ('98) contained 30,000–120,000 sperm, which should be sufficient to fertilize a full clutch, which rarely exceeds 300 eggs (Baker, '92).

Sperm competition has been investigated extensively in *Triturus* by Rafinski and Pecio. In *T. alpestris*, a paternity study, using males from different populations that can be identified by electrophoretic markers, revealed considerable variation, with the second male fathering 15–95% of a female's progeny but overall, a second-male advantage in a majority of families (Rafinski, pers. com.). Pecio (pers. com.) has investigated paternity by mating females sequentially with males of different species, *T. vulgaris* and *T. montandoni*. There is a tendency for the second male to have a mating advantage, though this is tempered by a tendency for conspecific sperm to be more likely to fertilize a female's eggs. Overall, these data suggest that there is a last-male advantage in *Triturus*. Finally, a possibility that emerges from the present study is that the spaghetti-like tangles in which smooth newts apparently remain from pick-up to egg-laying is an adaptation that reduces the effectiveness of competition from the ejaculates of rival males.

Phylogenetic considerations

The presence of cloacal sperm storage glands is considered a synapomorphy for the seven families of salamanders that comprise the suborder Salamandroidea (Sever, '91a). The ultrastructure of sperm storage has been studied in species representing five of the seven families. Some of these studies, such as the present one, deal only with specimens from certain stages of maturity or reproductive activity (Joly, '60, '66; Dent, '70; Pool and Hoage, '73; Greven and Guex, '94), whereas others report the complete annual cycle of sperm storage, extending into the post-breeding period when sperm generally disappear (Brizzi et al., '89, '95; Sever, '91b, '92b, '95, '97; Sever and Brunette, '93; Sever and Kloepfer, '93; Sever and Bart, '96; Sever et al., '95, '96a,b). More studies covering the entire cycle and using the same protocol are necessary to enable interspecific comparisons leading to phylogenetic hypotheses (Sever, '97).

However, if the presence of sperm storage is a synapomorphy for the Salamandroidea, we are dealing with a character that evolved in the common ancestor to the seven families in the suborder,

Figures 8, 9 and 10 on following pages.

Fig. 8. Spermathecae of a 43-mm SVL female *Triturus v. vulgaris* sacrificed 4 days after mating. A light micrograph of a semi-thin plastic section stained with toluidine blue is shown in (A), and (B) to (D) are transmission electron micrographs. (**A**) Sections through spermathecae containing sperm. (**B**) Overview of spermathecal epithelium and adjacent luminal and stromal areas. (**C**) Apical cytoplasm of the spermathecal epithelium. (**D**) Numerous embedded mylenic figures. Ac, apical cytoplasm; Ic, intercellular canaliculi; Lu, lumen; Me, melanin granules; Mpt, middle piece of the tail; Mv, microvilli; My, mylenic figures; Nu, epithelial cell nucleus; Sn, sperm nucleus; Sp, sperm; St, spermathecae; Sv, secretory vacuoles; Tp, tunica propria.

Fig. 9. Scanning electron micrographs of sperm in the spermathecae of a 54-mm SVL female *Triturus v. vulgaris* sacrificed 7 days after mating. (**A**) Sperm just inside a spermathecal orifice. (**B**) Nucleus of a single sperm cell. (**C**) Detail of a tangled mass of sperm and debris. The collagen fibers became associated with the sperm as a result of the dissection of the spermathecal walls. Ac, acrosome; Af, axial fiber; Cf, collagen fibers; Db, debris; Lu, lumen; Rb, red blood cell; Sn, sperm nucleus; So, spermathecal orifice; Sp, sperm; Um, undulating membrane.

Fig. 10. Spermathecae of a 42-mm female *Triturus v. vulgaris* sacrificed after laying 11 eggs 4 days after mating. A light micrograph of a semi-thin plastic section stained with toluidine blue is shown in (A); (B) and (C) are transmission electron micrographs; and (D) is a scanning electron micrograph. (**A**) Sections through spermathecae possessing sperm. (**B**) Sperm in the lumen and adjacent spermathecal epithelium. (**C**) Apical spermathecal cytoplasm showing inclusions, including portions of a degraded sperm cell (Ds). (**D**) Mass of sperm external to a spermathecal orifice. Ac, apical cytoplasm; Af, axial fiber; Ax, axoneme; Ce, cloacal epithelium; Db, debris; Ds, degraded sperm; Ic, intercellular canaliculi; Lu, lumen; Mf, marginal filament; Mi, mitochondria; Mpt, middle piece of the tail; Mv, microvilli; Nu, epithelial cell nucleus; Ppt, principle piece of the tail; Sm, sperm mass; Sp, sperm; St, spermathecae; Tp, tunica propria; Um, undulating membrane.

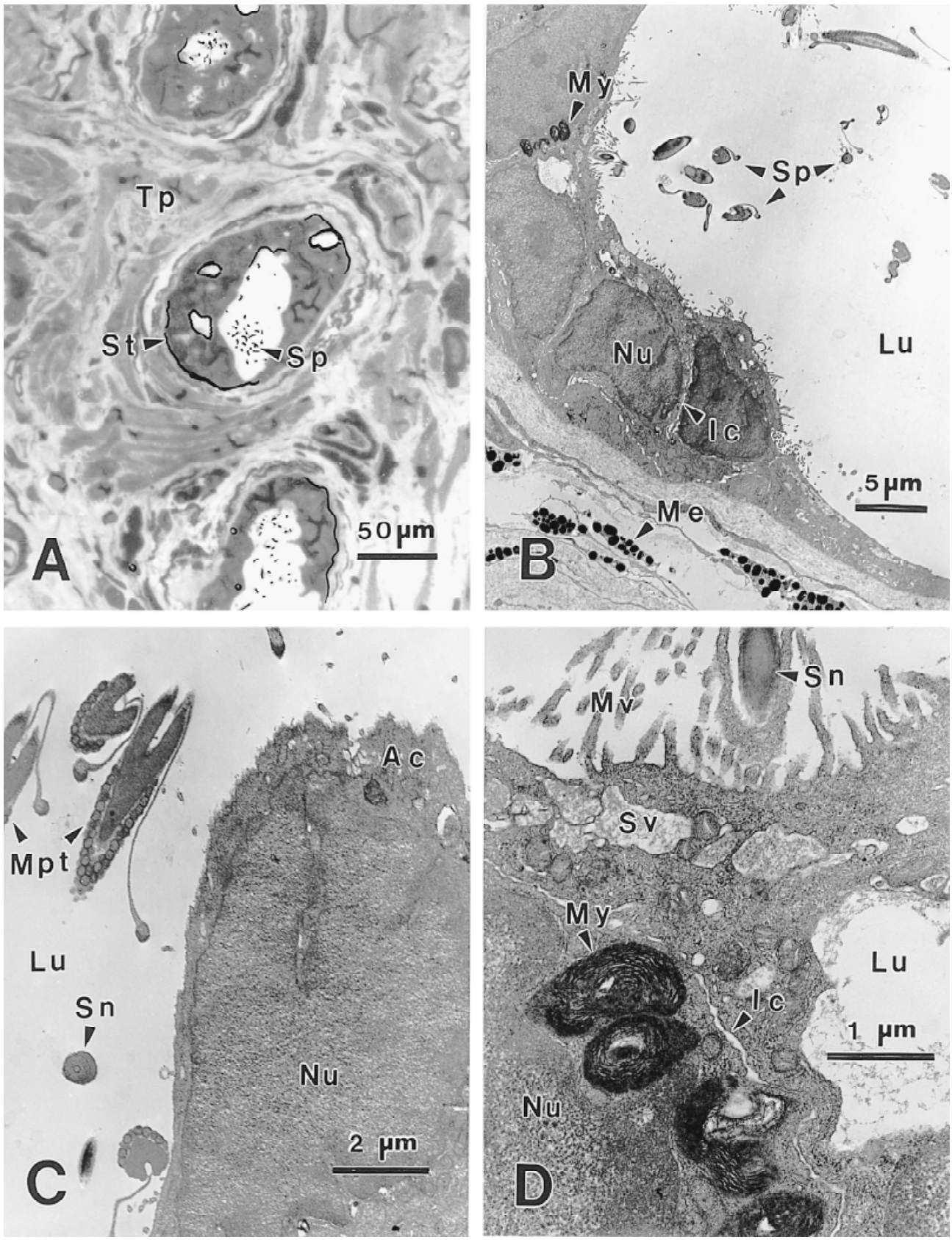
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Figure 8.

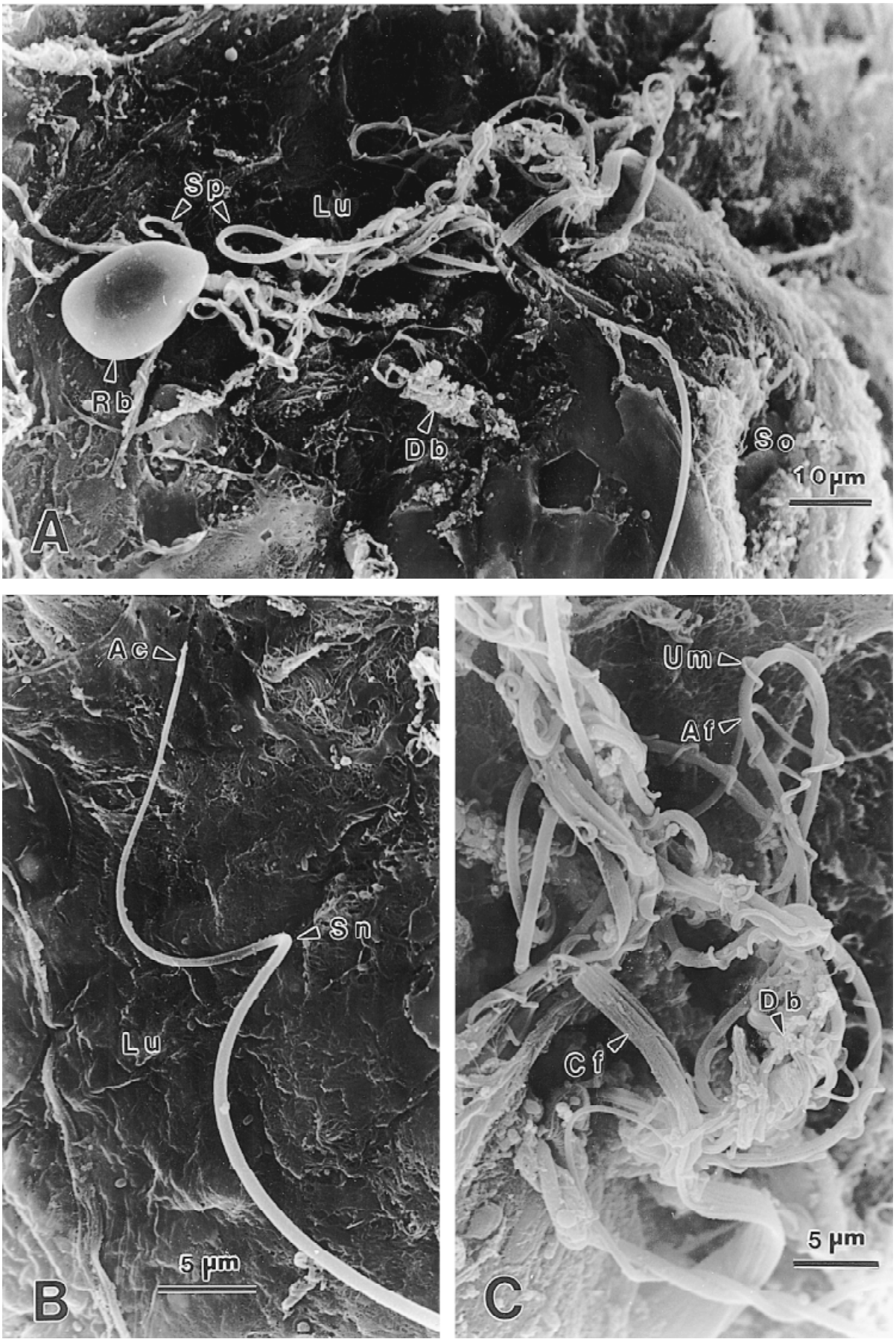

Figure 9.

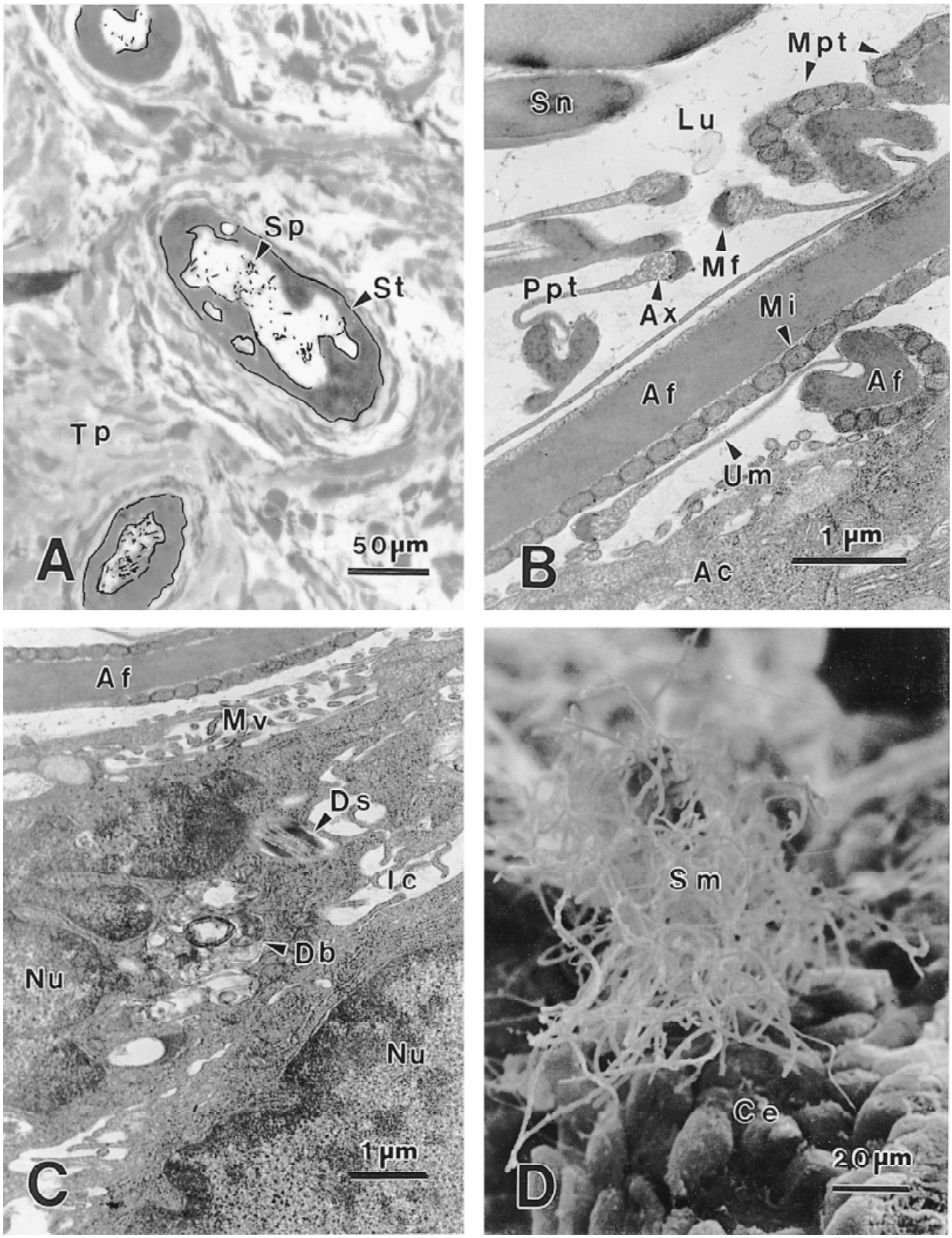
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Figure 10.

and thus prior to the many other morphological and reproductive adaptations that characterize these families (Sever, '97). Some characters, such as duration of sperm storage, obviously have ecological significance and must have co-evolved with other breeding adaptations. Careful analyses of such characters may lead to phyletic hypotheses. For example, sperm storage for a short period (a few hours or a few days) logically may be hypothesized to have evolved before storage for several months (Sever, '95). However, the phyletic significance of other characters, such as carbohydrate histochemistry, may be forever lost in antiquity, obscured by subsequent adaptations, atavisms, and/or convergence.

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