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Sperm storage in females of the smooth newt (*Triturus v. vulgaris* L.): II. Ultrastructure of the spermathecae after the breeding season

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Abstract

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Sperm storage in cloacal spermathecae was studied in females of *Triturus v. vulgaris* from southern England killed at the end of the breeding season in June. This species mates and oviposits eggs in ponds from March to June. Included in the sample were 12 unmated females collected in terrestrial situations in March and mated in the laboratory. Some of these females oviposited viable eggs in the laboratory whereas others did not oviposit after mating. In addition, we examined five females with unknown mating histories that were collected from a breeding pond in June. We found that all of the specimens contained some stored sperm and were similar in spermathecal ultrastructure. The spermathecae exhibited characteristics of secretory epithelium at the end of a cycle, including irregular heterochromatic nuclei surrounded by scant cytoplasm, absence of organelles involved in synthetic activities, few secretory vacuoles, and wide intercellular canaliculi. Spermiophagy by the spermathecal epithelium was extensive. In contrast, spermathecae from females at the beginning of the breeding season as reported in our previous study were actively producing a PAS+ secretion and did not exhibit spermiophagy. Spermiophagy is a means of eliminating sperm prior to the next breeding season.

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Introduction

Females in the seven families of salamanders that constitute the suborder Salamandroidea possess sperm storage glands, spermathecae, in the walls of their cloacae (summarized by Sever 1991; Sever and Brizzi 1998). In the cosmopolitan family Salamandridae, spermathecae have been described at the light microscopy level for 22 of the 53 species (Sever 1992a) including *Triturus vulgaris*, the most widespread and abundant species of urodele in Europe (Griffiths 1996). Previous studies on the subspecies found in England, *T. v. vulgaris*, have concerned histology of the spermathecae at the light microscopy level throughout the year (Verrell and Sever 1988) and ultrastructure of the spermathecae during initiation of the breeding season in March (Sever *et al.* 1999). The only other salamandrids in which ultrastructure of the spermathecae has been described are *Salamandra salamandra* (Greven and Guex 1994) and *Salamandrina terdigitata* (Brizzi *et al.* 1989, 1995) from Europe and *Notophthalmus viridescens* (Dent 1970; Sever *et al.* 1996b) from North America.

In southern England, *Triturus v. vulgaris* has an extensive breeding season that can last four months. Individuals migrate from terrestrial hibernacula in late February and March and migrate to breeding ponds (Verrell and Halliday 1985). Breeding activity commences upon arrival of newts at the pond, but breeding continues into June (Verrell *et al.* 1986; Verrell and McCabe 1988). Newts leave the ponds in the summer months to assume a terrestrial existence until the next breeding season. In this paper we extend the

Table 1 Females collected from terrestrial situations in March, mated in the laboratory and killed on 16 June

		Oviposited	
Specimen	Mated	Dates	Eggs laid (hatching)
F1	19 March – 1 spermatophore 20 March – 1 spermatophore	28 April–30 May	133 (103)
F2	20 March – 1 spermatophore 21 March – 1 spermatophore	28 April–19 May	73 (71)
F3	24 March – 1 spermatophore 26 March – 1 spermatophore	6 May–28 May	56 (48)
F4	7 April – 1 spermatophore 8 April – 1 spermatophore	11 April–20 May	88 (68)
F5	7 April – 2 spermatophores 8 April – 1 spermatophore	14 April–12 May	33 (22)
F6	14 April – 1 spermatophore 16 April – 1 spermatophore	12 April–28 May	37 (37)
F7	24 March – 1 spermatophore		0
F8	27 March – 1 spermatophore		0
F9	2 April – 1 spermatophore 3 April – 1 spermatophore		0
F10	9 April – 2 spermatophores		0
F11	7 May – 1 spermatophore 8 May – 1 spermatophore		0
F12	9 May – 1 spermatophore 14 May – 1 spermatophore		0

observations of Sever *et al.* (1999) on sperm storage at the beginning of mating activity by reporting on the ultrastructure of the spermathecae of female smooth newts killed at the end of the breeding season.

Materials and Methods

Specimens were collected in the cities or vicinities of Oxford and Milton Keynes, UK. A total of 17 adult females (>40 mm snout–vent length, SVL) was examined specifically for this study. Twelve of these were collected in March 1997 at night in terrestrial situations and were assumed to be unmated females migrating to breeding ponds (Sever *et al.* 1999). The females were gravid as determined by their distended posterior trunk regions, through the ventral skin of which the outlines of large ovarian follicles could be discerned. The females were acclimated together in aquaria containing pond water and a gravel substrate. One or more matings with males from the same localities were staged in the laboratory from 19 March to 8 May (Table 1). Successful mating culminates at the end of a stereotyped courtship with a sperm-containing spermatophore cap deposited by the male lodged in the cloacal orifice of a female (Halliday 1974). In addition, five females with unknown reproductive histories were collected from ponds on 15 June 1997. All specimens were killed on 16 June 1997.

Specimens were killed by immersion in 5% benzocaine, and 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 midsagittally. The right half was fixed for paraffin infiltration for light microscopy (LM). The left half was fixed for embedding in epoxy resin for transmission electron microscopy (TEM). Carcasses of all specimens were preserved in neutral buffered formalin (NBF) and are housed in the research collections at Saint Mary's College.

For the paraffin procedure, tissues were initially fixed in NBF, rinsed in water, dehydrated in ethanol, cleared in toluene, 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 haematoxylin-eosin (general histology) or with alcian blue 8GX at pH 2.5 (AB, primarily for carboxylated glycosaminoglycans) followed by the periodic acid–Schiff's procedure (PAS, for neutral carbohydrates and sialic acids). Procedures followed Kiernan (1990).

Tissue for TEM was trimmed into 1 mm blocks and 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. After initial fixation, tissues were rinsed in Millonig's buffer, postfixed in 2% osmium tetroxide, dehydrated through a graded series of ethanol, cleared in propylene oxide, and polymerized in an epoxy resin (Embed 812, Electron Microscopy Sciences, Port Washington, PA). Plastic sections were cut with an RMC MT7 ultramicrotome (Research and Manufacturing Co., Tucson, AZ) and DiATOME (DIATOME LTD, Biel, Switzerland) diamond knives. Semi-thin sections (0.5–1 mm) for LM 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 a solution of uranyl acetate and lead citrate. Ultra-thin sections were viewed with a Hitachi H-300 transmission electron microscope (Nissei Sangyo America, Mountain View, CA). Terminology for sperm ultrastructure follows Picheral (1979).

Results

All 17 females, whether used in staged matings or wild-caught from ponds in June, have sperm in their spermathecae. The amount of sperm cannot be reliably quantified or relative amounts of sperm even reliably reported, but none of the specimens have spermathecae 'full' or crowded with sperm. Histochemically, the spermathecae show little reaction to the carbohydrate treatments utilized. A weak PAS+ reaction (neutral carbohydrates) occurs in the apical spermathecal epithelium of some specimens, but no spermathecae exhibit positive reactions with AB (carboxylated glycosaminoglycans).

The ultrastructure of the spermathecae is similar in all specimens examined, whether they were: (1) killed after ovipositing less than 100 (Fig. 1) or more than 100 fertile eggs (Fig. 2); (2) mated once or twice but did not oviposit (Fig. 3); or (3) were captured from a pond at the end of the breeding season and had an unknown reproductive history.

Cytoplasm in the epithelial cells is scant, and the nuclei fill most of the cells (Figs 1A, 2A, 3A). The nuclei are irregular and heterochromatic. Intercellular canaliculi are wide (Figs 1A, 2A) and often labyrinthine (Fig. 3C). Small groups of secretory vacuoles occur in the apical cytoplasm of some cells (Figs 1C, 2C, 3B), but other cells appear to lack secretory vacuoles. When present, secretory vacuoles contain a flocculent material and usually have a central or eccentric electron-dense particle (Fig. 2C). Mitochondria are sparse, and organelles (Golgi complexes, rough endoplasmic reticulum, RER) associated with the production of secretory products as noted by Sever *et al.* (1999) were not observed.

The most striking feature is the cytological evidence for extensive spermiophagy (Figs 1B,C, 2, 3D). Sperm embedded in the cytoplasm of epithelial cells are obviously undergoing degradation as indicated by their abnormal conformations, fragmentation and loss of electron density (Figs 1C, 2C, 3D). Vacuoles containing degenerating sperm constitute phagosomes (Sever 1992b). Phagosomes observed in the spermathecal epithelium of *T. vulgaris* have not been found in association with any specific organelles; some phagosomes, however, occasionally are found in the proximity of secretory vacuoles (Fig. 2C).

Sperm free in the lumen appear normal in cytology (Figs 1A, 3A). The orientation of unembedded sperm in the lumen is random (Fig. 1A), and sperm do not appear to be crowded or tangled (Figs 1A, 2A, 3A). Some sperm in the lumen are embedded in cellular debris (Fig. 2A), and such sperm often appear abnormal (Fig. 3D). The debris could result from apocrine blebs forming from the fluctuating luminal border noted in some regions of the spermathecal epithelium. Degenerating sperm often are found in apical projections of the spermathecal epithelium (Figs 1B, 2B) that appear identical to portions of cells that apparently are cleaved by an apocrine process into the lumen (Fig. 3D).

Discussion

Comparisons with females from early in the breeding season

The epithelia of spermathecae of females examined by Sever *et al.* (1999) from March possess abundant cytoplasm, rounded euchromatic nuclei, narrow intercellular canaliculi, and an abundant apical secretory product that is PAS+ and AB–. Mitochondria, Golgi complexes and RER are abundant, and the secretory product is released by an apocrine process at the onset of mating. Extensive spermiophagy does not occur in mated females killed in March (Sever *et al.* 1999).

In contrast, the females killed in June show characteristics of secretory epithelium at the end of a cycle, such as the large nucleus: cytoplasm ratio, the absence of organelles involved in synthetic activities, few secretory vacuoles and wide intercellular canalciuli (Krstic 1979; Fawcett 1986). The cleaving of portions of apical cytoplasm or perhaps entire cells into the lumen may indicate some programmed sloughing of the epithelium.

Verrell and Sever (1988) reported sperm in the spermathecae of all females collected in ponds from April to July, and that aquatic females collected in August and terrestrial females collected in autumn lack stored sperm. One way that females apparently rid their spermathecae of sperm from a past breeding season is through spermiophagy by the spermathecal epithelium. Sever and Hamlett (1998) proposed that spermiophagy is the inevitable result when sperm become trapped in the spermathecal epithelium.

Sperm free in the lumen of the females killed in June, however, still appeared normal in cytology. Perhaps ultimately these sperm will reach the epithelium and become phagocytized, or perhaps they will degrade in the lumen (Sever and Brizzi 1998), or simply leak out of the spermathecae, as noted for unused sperm in the vas deferens of the salamander *Ambystoma macrodactylum columbianum* (Zalisko and Larsen 1989). No evidence exists for the storage of viable sperm from one breeding season to next in *Triturus v. vulgaris* (Sever *et al.* 1999). Sever and Brizzi (1998) urge caution in accepting reports of long-term effective sperm storage (>6 months) in the spermathecae of salamanders.

Comparative biology of spermiophagy

Spermiophagy by the spermathecal epithelium has been reported in plethodontid salamanders in the genus *Desmognathus*

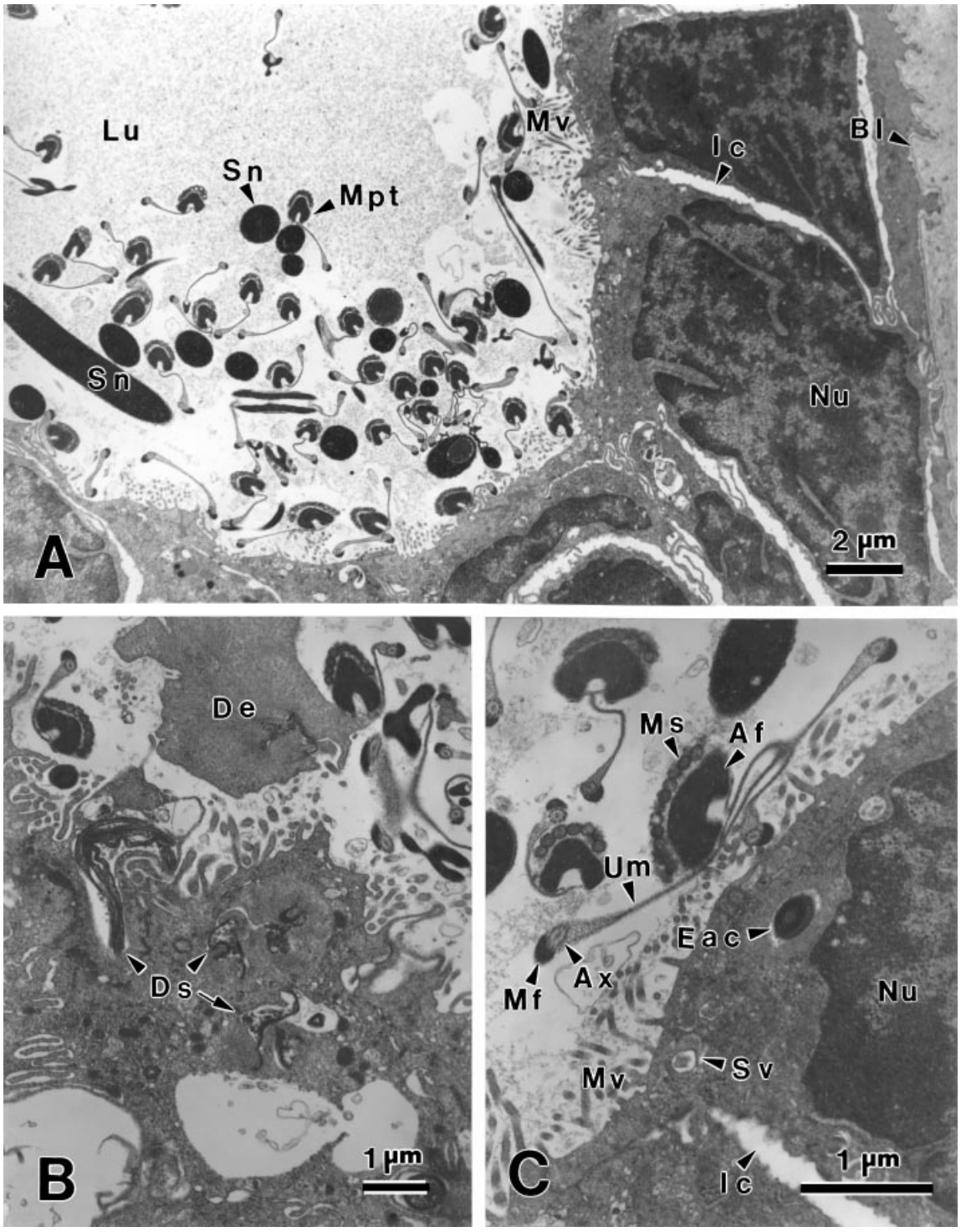

Fig. 1—Transmission electron micrographs of spermathecae of a laboratory-mated *Triturus v. vulgaris* killed on 16 June after picking up single spermatophores on 14 April and 16 April and ovipositing 37 eggs (all viable) 12 April–28 May. —**A**. Spermathecal epithelium and adjacent lumen containing sperm. —**B**. Apical cytoplasm showing degenerating sperm in the epithelium and desquamated epithelium in the lumen. —**C**. Apical cytoplasm illustrating embedded sperm as well as normal appearing sperm in the lumen. Abbreviations: Af, axial fibre; Ax, axoneme; Bl, basal lamina; De, desquamated epithelium; Ds, degenerating sperm; Eac, embedded acrosome; Ic, intercellular canaliculi; Lu, lumen; Mf, marginal fibre; Mpt, middle piece of the tail; Ms, mitochondrial sheath; Mv, microvilli; Nu, nucleus of an epithelial cell; Sn, sperm nucleus; Sv, secretory vacuoles; Um, undulating membrane.

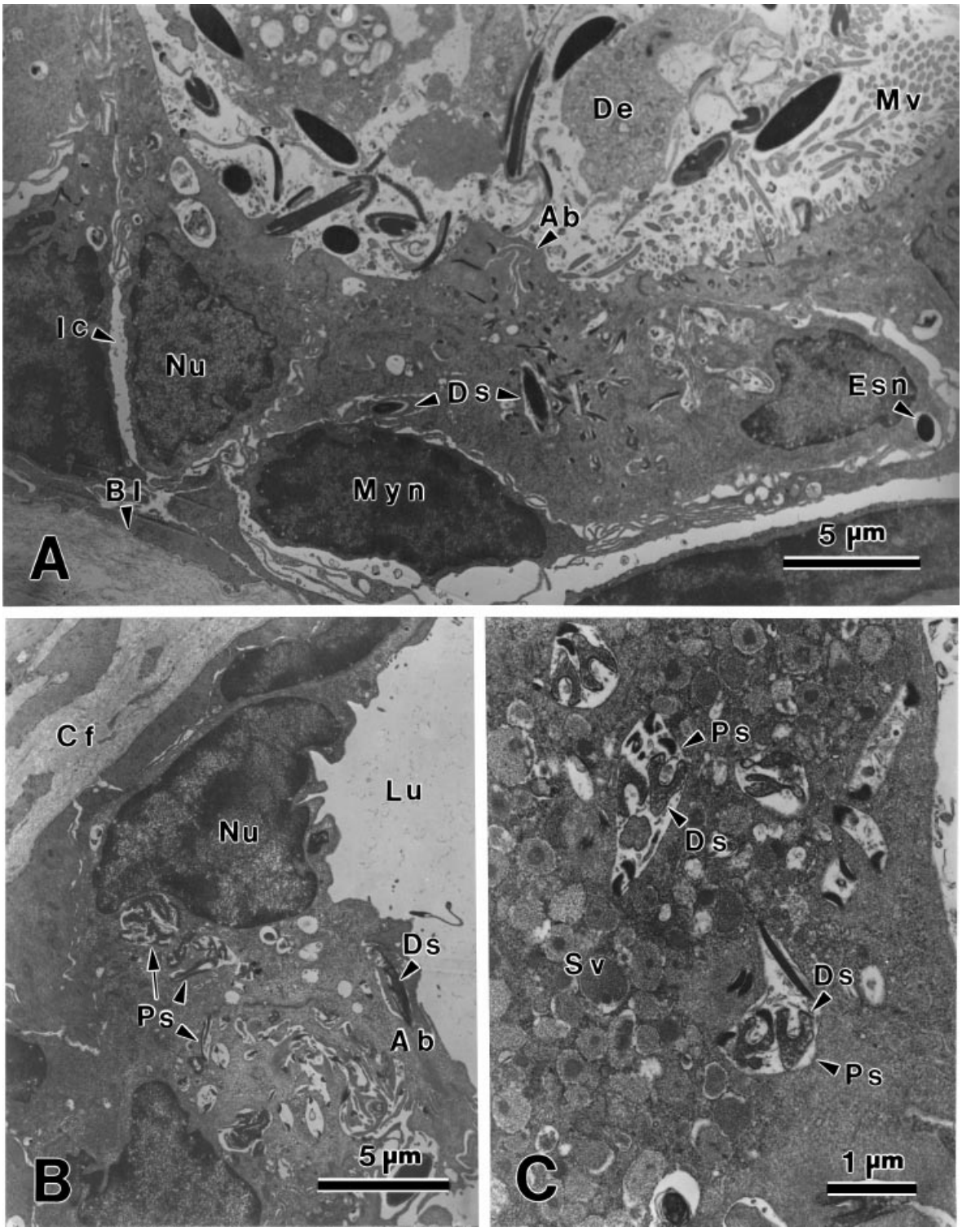

Fig. 2—Transmission electron micrographs of spermathecae of a laboratory-mated *Triturus v. vulgaris* killed on 16 June after picking up single spermatophores on 19 March and 20 March and ovipositing 133 eggs (103 viable) 28 April–30 May. —**A**. Spermatheca containing degenerating sperm in the epithelium and the adjacent lumen containing sperm and desquamated epithelium. —**B**. Spermathecal epithelium with phagosomes. —**C**. Detail of phagosomes in the spermathecal epithelium. Abbreviations: Ab, apocrine bleb; Bl, basal lamina; Cf, collagen fibers; De, desquamated epithelium; Ds, degenerating sperm; Esn, embedded sperm nucleus; Ic, intercellular canaliculi; Lu, lumen; Mv, microvilli; Myn, myoepithelial cell nucleus; Nu, nucelus of an epithelial cell; Ps, phagosomes; Sv, secretory vacuoles.

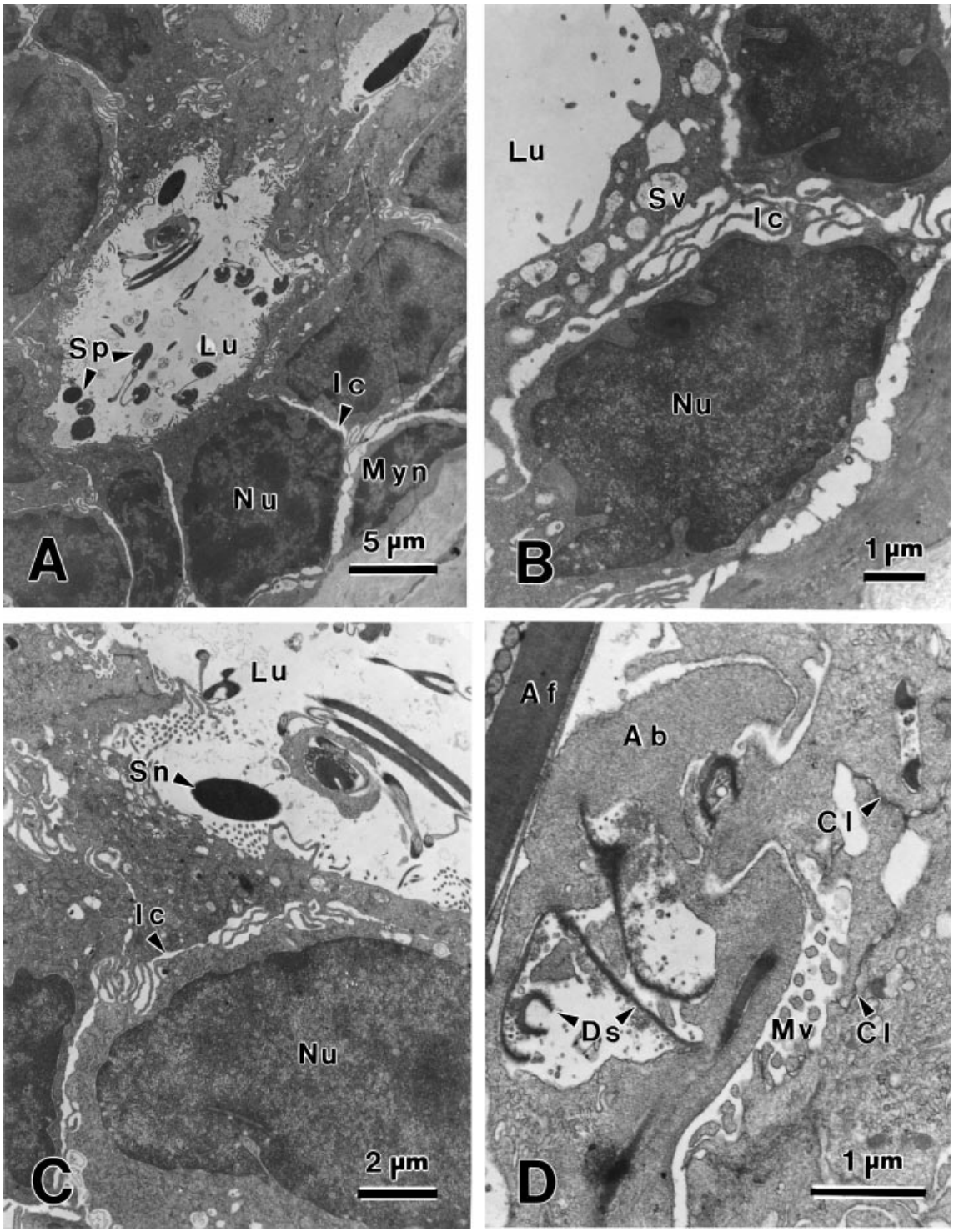

Fig. 3—Transmission electron micrographs of spermathecae of a laboratory-mated *Triturus v. vulgaris* killed on 16 June after picking up single spermatophores on 2 April and 3 April, ceasing mating activity and not ovipositing. —**A**. Spermatheca with luminal sperm. —**B**. Spermathecal epithelium illustrating irregular nuclei, scant cytoplasm and wide intercellular canaliculi. —**C**. Normal appearing sperm adjacent to the apical spermathecal epithelium. —**D**. Degenerating sperm associated with an apocrine bleb. Abbreviations: Ab, apocrine bled; Af, axial fibre; Cl, cleavage line; Ds, desquamated epithelium; Ic, intercellular canaliculi; Lu, lumen; Myn, myoepithelial cells nucleus; Mv, microvilli; Nu, nucleus of an epithelial cell; Sn, sperm nucleus; Sp, sperm; Sv, secretory vacuoles.

(Sever and Hamlett 1998) and in *Eurycea cirrigera* (Sever 1992b) and *Plethodon cinereus* (Sever 1997); in the ambystomatid *Ambystoma opacum* (Sever and Kloepfer 1993); and in the salamandrids *Salamandrina terdigitata* (Brizzi *et al.* 1995) and *Notophthalmus viridescens* (Sever *et al.* 1996b). In other species in which spermathecal ultrastructure has been studied, no evidence of spermiophagy by the epithelium has been found, i.e. *A. tigrinum* (Sever 1995); *Amphiuma tridactylum* (Sever *et al.* 1996a); and *Necturus beyeri* (Sever and Bart 1997). Joly (1960) proposed that sperm embedded in the spermathecal epithelium of *Salamandra salamandra* could be receiving nourishment, but others consider this unlikely (Sever and Kloepfer 1993; Sever and Brizzi 1998; Sever and Hamlett 1998). Eventual sperm degeneration in the lumen, however, has been reported in all species in which ultrastructure has been used to study the annual cycle of sperm storage in salamanders (Sever and Brizzi 1998).

In *Eurycea cirrigera* and *Notophthalmus viridescens*, both of which have mating seasons of two to four months, spermiophagy occurs at the beginning of the sperm storage season, and continues throughout the breeding season. The situation in *Salamandrina terdigitata*, however, is more similar to that observed in *Triturus vulgaris*. Little phagocytic activity occurs early in the breeding season, but after oviposition, intense spermiophagy results in a sharp and massive destruction of residual sperm and cellular debris (Brizzi *et al.* 1995).

Determination of the reproductive and phylogenetic significance of spermiophagy as well as other spermathecal characters still requires the examination of the annual cycle of sperm storage in a larger and more diverse assemblage of species. As noted by Sever and Brizzi (1998), sperm storage is an ancient trait, evolving in the common ancestor of the seven families in the Salamandroidea, and thus the diversity currently observed in spermathecal characters may not be phyletically informative but related to other species-specific reproductive habits. Examination of the spermathecal cycle in additional populations within the extensive range of *Triturus vulgaris* therefore will be of special interest to see if spermathecal characters are conserved over a wide geographical area or if differences occur that can be related to regional variations in reproductive cycles.

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