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# Sperm Storage in the Spermatheca of the Red-Back Salamander, *Plethodon cinereus* (Amphibia: Plethodontidae)

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*ABSTRACT* In northern Indiana, the mating season of *Plethodon cinereus* occurs after hibernation from March until June, when oviposition begins. During the mating season, a female stores sperm in its spermatheca, a compound tubular gland in the roof of the cloaca. The apical cytoplasm of the spermathecal epithelium is filled with large secretory vacuoles whose product is released while sperm are stored. Females induced to oviposit in June and July by injections of human chorionic gonadotropin (hCG) still retain much sperm 1 month after oviposition, but secretory vacuoles are absent in all specimens sacrificed in July and August. Instead, some sperm are embedded in the spermathecal epithelium with resultant spermiophagy involving lysosomes. A female sacrificed in September 2 months after oviposition possesses scant sperm, but spermiophagy alone does not seem extensive enough to account for the decrease in sperm numbers. Females sacrificed in October prior to hibernation lack sperm in their spermathecae; some secretory vacuoles are present, but they are not as numerous or as enlarged as in specimens collected in March and May. Inter- and intrafamilial differences in the cytology of sperm storage may not be phyletically informative at the family level but related to species-specific reproductive adaptations. J. Morphol. *234:*131–146, 1997. 

The red-back salamander, *Plethodon cinereus* (Green), is completely terrestrial, widespread, and often locally abundant in forested regions of the eastern United States and Canada (Conant and Collins, '91). The female cloacal anatomy at the light microscopy level was described by Kingsbury (1895) and Sever ('78a, '94a). They noted storage of sperm in a compound tubular gland, the spermatheca, in the dorsal roof of the cloaca prior to oviposition. Such glands are know from females in seven families of salamanders that comprise the suborder Salamandroidea (Sever, '91a). Fertilization in oviparous species within the suborder occurs during oviposition by release of sperm from the spermatheca onto eggs passing through the cloaca (Jordan, 1893; Boisseau and Joly, '75).

The study of the cytological relationships between sperm and cells associated with the spermatheca is best accomplished by electron microscopy. For a given species, the annual cycle of sperm storage requires examination of spermathecae from females: (1) sexually active but unmated, (2) mated, but prior to oviposition, (3) immediately after oviposition, and (4) from various periods following oviposition, extending into a period where the female is not sexually active.

*Plethodon cinereus* belongs to the family Plethodontidae, the largest family of salamanders with some 27 genera and more than 220 species (Frost, '85). Yet the only transmission electron microscopy (TEM) studies on the annual cycle of sperm storage among the Plethodontidae concern *Eurycea quadridigitata* (Pool and Hoage, '73) and *E. cirrigera* (Sever, '91b, '92; Sever and Brunette, '93). Davitt and Larsen ('88), however, used scanning electron microscopy (SEM) to study the spermatheca of *Plethodon larselli* prior to ovulation.

*Plethodon* is in the tribe Plethodontini, a genus that includes some 50 species lacking an aquatic larval stage, whereas *Eurycea* is a member of the tribe Hemidactylini and contains 25 species that have aquatic nests

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and larvae (Duellman and Trueb, '86). Other families in which the cycle of sperm storage has been studied by TEM are Ambystomatidae (Sever and Kloepfer, '93; Sever, '95; Sever et al., '95), Amphiumidae (Sever et al., '96b), Proteidae (Sever and Bart, '96), and Salamandridae (Dent, '70; Brizzi et al., '95; Sever et al., '96a). This study is a continuation of comparative work that extends such TEM analyses of sperm storage to new taxa that are selected with especial emphasis on their phylogenetic relationships and reproductive adaptations. *Plethodon cinereus* is the first plethodontine and the only fully terrestrial salamander in which the ultrastructure of the annual cycle of sperm storage has been described.

## MATERIALS AND METHODS

All specimens were collected in St. Joseph County, Indiana, under collecting permits issued by the Indiana Division of Wildlife. Some specimens (Table 1) were sacrificed within 24 hr of capture. Other specimens collected 17 May were isolated from males and from each other and maintained in the laboratory in plastic culture dishes, 7 cm in diameter, at 20–22°C and local photoperiod (Table 2). The dishes were lined with wet filter paper, and twice weekly several *Drosophila* were added for food. In an attempt to induce oviposition, the salamanders were injected with 0.5 ml human chorionic gonadotropin (hCG, 500 IU/ml, Sigma Chemical Co., St. Louis, MO) on 31 May. Those that failed to oviposit were reinjected on 28 June and 2 July (Table 2).

*TABLE 1. Specimens sacrificed immediately after collection.*[1]

| | | Ovarian follicles | | |
|---|---|---|---|---|
| Date | SVL | N | Dia | Sperm |
| 31 Mar | 48.5 | 9 | 2.5 | Y |
| 31 Mar | 48.0 | 14 | 1.9 | N |
| 31 Mar | 47.7 | 8 | 2.2 | Y |
| 3 May | 43.7 | 9 | 2.7 | Y |
| 17 May | 46.3 | 8 | 2.8 | N |
| 17 May | 43.9 | — | — | N |
| 17 May | 37.3 | — | — | N |
| 14 Aug | 48.0 | 12 | 1.2 | N |
| 14 Aug | 47.7 | 5 | 1.0 | N |
| 14 Aug | 46.7 | 6 | 1.0 | N |
| 18 Aug | 35.5 | 7 | 0.9 | N |
| 6 Oct | 50.5 | 13 | 1.4 | N |
| 6 Oct | 48.7 | 14 | 1.2 | N |
| 6 Oct | 45.9 | 11 | 1.3 | N |
| 6 Oct | 46.3 | 13 | 1.3 | N |

[1]Measurements are in mm.

*TABLE 2. Females collected 11 May and 17 May and maintained in the laboratory*[1]

| | | Oviposit | | | Follicles | |
|---|---|---|---|---|---|---|
| SVL | hCG | N | Date | Sacrifice | N | Size |
| 47.1 | 31 May | 4 | 3–4June | 4 June | 1 | 3.6 |
| 49.2 | 31 May | 3 | 3–4June | 4 June | 4 | 3.3 |
| 47.8 | 31 May | 5 | 4–5June | 4 July | 9 | 3.0 |
| 47.7 | 28 June | 1 | 2 July | 4 July | 4 | 2.8 |
| 41.0 | 28 June | 3 | 4 July | 4 July | 4 | 3.2 |
| 48.8 | 2 July | 0 | | 4 July | 9 | 3.1 |
| 49.7 | 2 July | 0 | | 4 July | 7 | 3.6 |
| 46.7 | 2 July | 0 | | 4 July | 7 | 3.3 |
| 46.4 | 28 June | 4 | 1 July | 4 Aug | 6 | 2.4 |
| 48.6 | 28 June | 8 | 1 July | 4 Sep | 17 | 0.8 |

[1]Date of hCG injection indicates last date; specimens also were injected on previous date(s). Measurements are in mm; all specimens had sperm in their spermathecae.

Specimens were killed by immersion in 10% MS-222, and snout-vent length (SVL) was measured from the tip of the snout to the posterior end of the vent. The spermathecal region was excised from freshly killed specimens and fixed for paraffin infiltration for light microscopy (LM, two specimens), or for embedding in epoxy resin for thin (LM) or ultrathin sections for transmission electron microscopy (TEM, 23 specimens). Carcasses of all specimens are stored in 10% neutral-buffered formalin (NBF) in the research collections at Saint Mary's College.

Prior to paraffin infiltration, the tissues were initially fixed in NBF, rinsed in water, dehydrated in ethanol, cleared in Histosol (National Diagnostics, Manville, NJ), and embedded in paraffin. Sections (10 μm) were cut with a rotary microtome and affixed to albuminized slides. Slides from one specimen were stained with 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). Slides from a second specimen were treated with the Gomori reaction (acid phosphatases). Procedures followed Kiernan ('90).

Tissues prepared for plastic infiltration prior to sectioning for LM and TEM were 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, the tissue was rinsed in Millonig's buffer, postfixed in 2% osmium tetroxide, dehydrated in ethanol, cleared in propylene oxide, and embedded in an epoxy resin (EMBED-812; Electron Microscopy Sciences,

Fort Washington, PA). Semithin sections (0.5–1 μm) for light microscopy were cut with glass knives, placed on microscope slides, and stained with toluidine blue. Ultrathin sections (70 nm) for TEM were collected on uncoated copper grids and stained with solutions of uranyl acetate and lead citrate. These sections were cut with RMC XL1000 and RMC MT7 ultramicrotomes, and thin sections were viewed with a Hitachi H-300 transmission electron microscope. Terminology for sperm ultrastructure follows Picheral ('79).

## RESULTS

### *Breeding season*

Of seven specimens collected and sacrificed March–May, five have 9–14 vitellogenic follicles 1.9–2.8 mm mean diameter, and three of these specimens have abundant sperm in their spermathecae (Table 1). Specimens collected and sacrificed August and October have 5–14 follicles 0.9–1.2 mm mean diameter, and lack sperm in their spermathecae. These findings support those of Sever ('78a) that mating occurs in spring, the peak period of oviposition is early summer, and the onset of vitellogenesis for the next breeding season occurs in late summer and fall.

Injections with hCG of three females on 31 May and of four females on both 31 May and 28 June resulted in oviposition of eggs (Table 2). The females sacrificed within 2 days of oviposition and those sacrificed ~1 month after oviposition retain some eggs of ovipository size in their ovaries and abundant sperm in their spermathecae. A female that oviposited eight eggs on 1 July and was sacrificed 4 September contains numerous small follicles (indicative of a new cycle of vitellogenesis) and scant sperm in its spermatheca (Table 2). Thirteen females injected on 31 May, 28 June, and 2 July failed to oviposit any eggs and were sacrificed 4 July. The three of these examined for this study contain large ovarian follicles and abundant sperm in their spermathecae. Thus hCG is not highly effective in inducing oviposition in *Plethodon cinereus,* unlike some other species (Armstrong and Duhon, '89; Sever et al., '96a).

### *Spermathecal ultrastructure*

Sperm are present in two out of three females collected and sacrificed 31 March, but sperm are not as abundant as in a mated female collected and sacrificed 3 May (Figs. 1, 2). In the mated specimens from March, large secretory vacuoles are abundant in the apical cytoplasm (Fig. 1A), and the vacuoles consist of an electron-dense particle, 0.1–0.3 μm dia, surrounded by an irregular-shape mass of flocculent material, 0.7–2.5 μm dia (Fig. 1B). Previous work has shown that the secretion is alcian blue positive (AB+) at pH 2.5 and contains glycoproteins (Sever, '94b). Sperm are not associated closely with the epithelial border, appear normal in cytology, and small clusters exhibit the same orientation (Fig. 1A,C). The unmated female examined from March also possesses numerous secretory vacuoles in the apical cytoplasm (Fig. 1D). Various small particles and cellular debris occur in the lumina of all specimens, mated or not (Fig. 1A,C,D). Secretory vacuoles are associated with Golgi complexes (Fig. 1B). Intercellular canaliculi are narrow, labyrinthine, and possess numerous junctional complexes (Fig. 1B,D).

In the mated specimen collected and sacrificed 3 May prior to oviposition, the lumen is crowded with sperm, and portions of some sperm, especially the nuclei, are adjacent to or embedded in the spermathecal epithelium (Fig. 2A,C,D). Sperm in the lumen appear normal in cytology and random in overall orientation (Fig. 2A). No definitive indications of degeneration of the nuclei of embedded sperm are found (Fig. 2C), but the middle piece of the tail of an embedded sperm exhibits an unusual mottling pattern in the axial filament (Fig. 2D). The secretory vacuoles are less numerous and contain less flocculent material than in the March specimens and in an unmated vitellogenic female collected and sacrificed in May (Fig. 3A,B).

The unmated but vitellogenic female collected and sacrificed 17 May has the apical cytoplasm packed with large secretory vacuoles, some of the largest of which lack the central dense particle (Fig. 3A,B). A nonvitellogenic female collected and sacrificed on the same day has fewer secretory vacuoles, and many of these consist primarily of an electron-dense particle (Fig. 3C). In this specimen, the lumen is narrow, filled with debris, and lacks sperm (Fig. 3C). The epithelial border has elongate microvilli (Fig. 3C,D) that are absent in mated and unmated vitellogenic females from March and May (Figs. 1,2). Also, the apical cytoplasm contains rough endoplasmic reticulum (RER) and numerous vesicles and microfilaments (Fig. 3D).

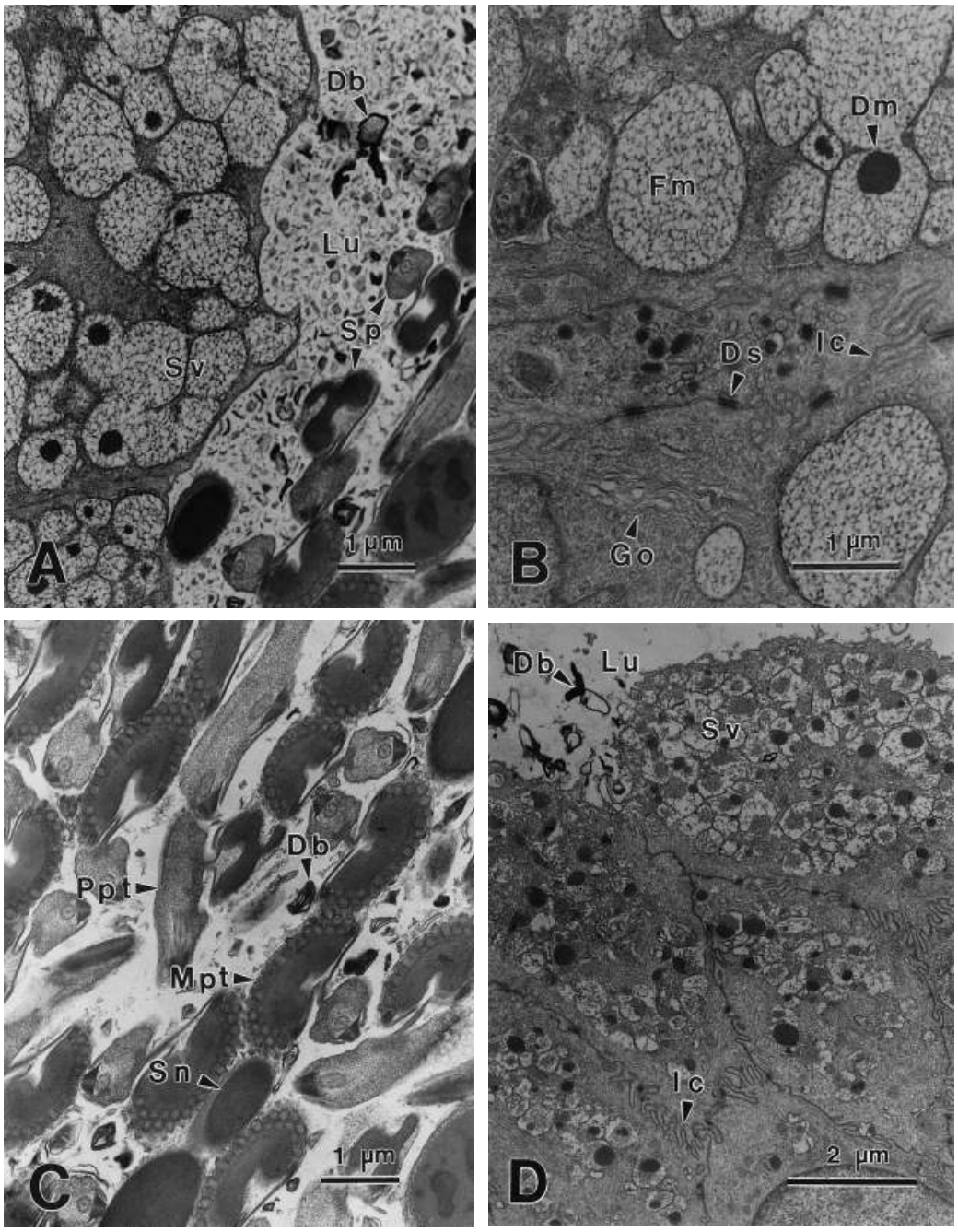


Fig. 1. Ultrastructure of the spermathecae of female *Plethodon cinereus* collected and sacrificed 31 March. **A.** Apical cytoplasm and sperm in the lumen of a 48.5 mm SVL specimen with 8 ovarian follicles 2.2 mm mean dia. **B.** Same specimen as A, showing supranuclear cytoplasm and secretory vacuoles. **C.** Same specimen as A, showing sperm in the lumen. **D.** Apical cytoplasm of a 48.0 mm SVL female with 14 ovarian follicles 1.9 mm mean dia and lacking sperm. Db, debris; Dm, dense material; Ds, desmosome; Fm, flocculent material; Go, Golgi complex; Ic, intercellular canaliculi; Lu, lumen; Mpt, middle piece of the tail; Ppt, principle piece of the tail; Sn, sperm nucleus; Sp, sperm; Sv, secretory vacuoles.

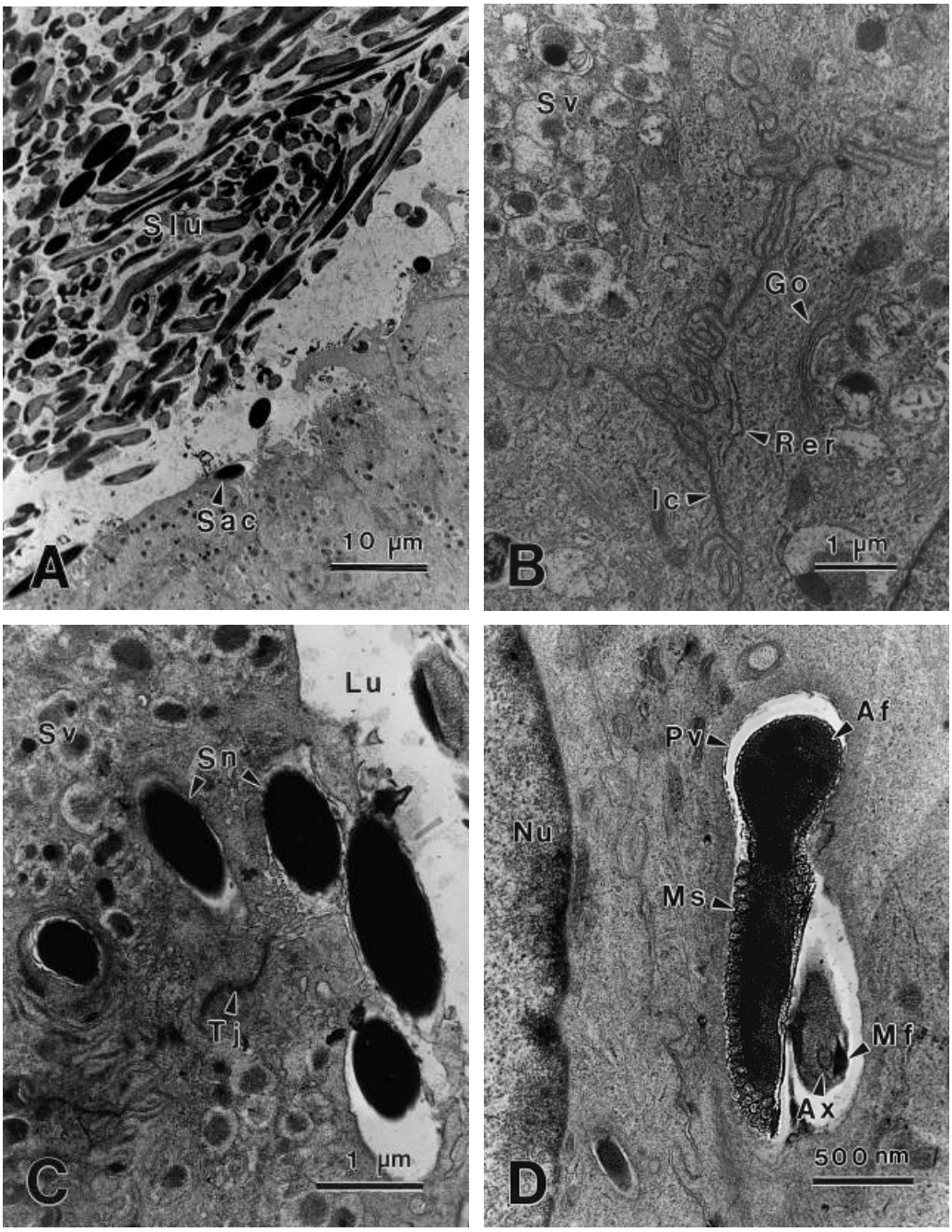


Fig. 2. Ultrastructure of the spermatheca of a mated female *Plethodon cinereus* 43.7 mm SVL with 9 ovarian follicles 2.7 mm mean dia collected and sacrificed 3 May prior to oviposition. **A.** Apical cytoplasm and sperm in the lumen. **B.** Supranuclear cytoplasm. **C.** Sperm nuclei embedded in the apical cytoplasm. **D.** Middle piece of the tail of a sperm cell embedded deep into the spermathecal epithelium. Af, axial fiber; Ax, axoneme; Go, Golgi complex; Ic, intercellular canaliculi; Lu, lumen; Mf, microfilaments; Ms, mitochondrial sheath; Nu, epithelial cell nucleus; Pv, phagocytic vacuole; Rer, rough endoplasmic reticulum; Sac, sperm associated with the apical cytoplasm; Slu, sperm in the lumen; Sn, sperm nucleus; Sv, secretory vacuoles; Tj, tight junction.

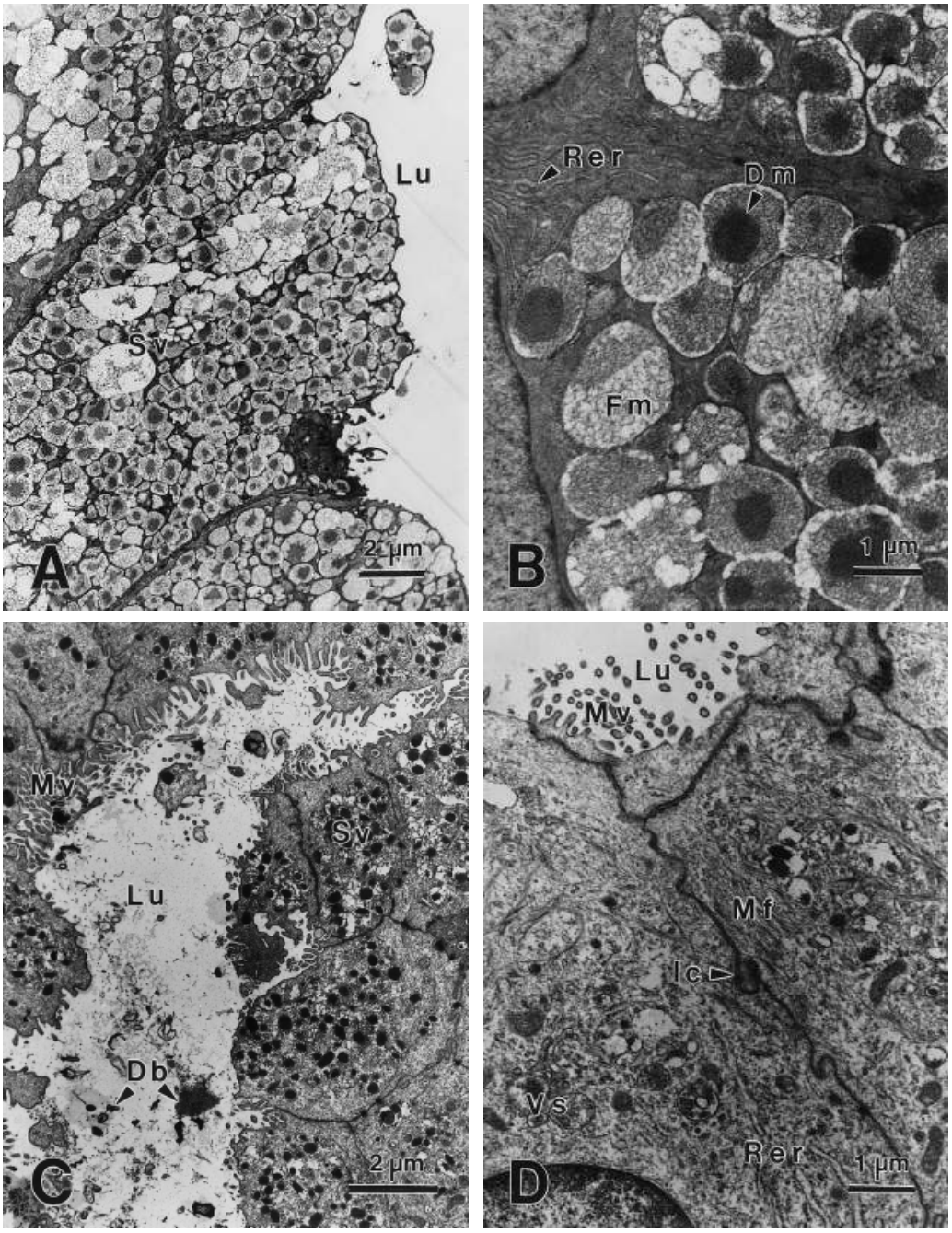


Fig. 3. Ultrastructure of the spermathecae of unmated female *Plethodon cinereus* collected and sacrificed 17 May. **A.** Apical cytoplasm of a 46.3 mm specimen with 8 ovarian follicles 2.8 mm mean dia. **B.** Same specimen as A, supranuclear cytoplasm. **C.** Apical cytoplasm and lumen of a nonvitellogenic female, 37.3 mm SVL. **D.** Same specimen as C, supranuclear cytoplasm. Db, debris; Dm, dense material; Fm, flocculent material; Ic, intercellular canaliculi; Lu, lumen; Mf, microfilaments; Mv, microvilli; Rer, rough endoplasmic reticulum; Sv, secretory vacuoles; Vs, vesicles.

The female collected 17 May, injected with hCG on 31 May, and sacrificed on 4 June after ovipositing three eggs has numerous sperm left in the lumen, some of which are associated with the apical epithelium (Fig. 4A). The cytoplasm has many small secretory vacuoles consisting primarily of electron-dense particles (Fig. 4A). In these regards, this specimen is similar to the mated individual sacrificed 3 May prior to oviposition (Fig. 2). However, the female sacrificed 4 June after oviposition has numerous, elongate microvilli (Fig. 4A), and the cytoplasm has many small vesicles and phagocytic vacuoles that contain debris and myelinic bodies (Fig. 4B). The female sacrificed on 4 July after ovipositing 5 eggs on 4–5 June also still possesses numerous sperm that appear normal in cytology in the lumen, and some sperm are found embedded in the cytoplasm (Fig. 4C). Secretory vacuoles are absent in the apical cytoplasm, which contains numerous small opaque vesicles that may be primary lysosomes (Fig. 4C). Occasionally, granules containing osmiophilic material, perhaps remnants of digested material, are found and interpreted to be secondary lysosomes (Fig. 4D). Also, a lipid droplet occurs in the epithelium of the July-sacrificed specimen, the only observation of lipids in any specimen (Fig. 4D, inset).

The two females injected with hCG on 28 June and sacrificed on 4 July after oviposition had been isolated from males since at least 17 May, but they still possess numerous sperm in the widened lumina of their spermathecae (Fig. 5A). The luminal sperm appear normal in cytology, and clusters of sperm show similar orientations (Fig. 5A). Both specimens have some sperm embedded deeply in epithelium, associated with clusters of heterogeneous vacuoles (Fig. 5B), which, because of their association with phagocytic vacuoles and sperm degeneration (Fig. 5C,D), are considered to be primary lysosomes. As reported in a previous study (Sever, '92), the vacuolated space around embedded sperm initially is reduced, and a fine fibrous meshwork occurs around the sperm (Fig. 5D). As the sperm degenerates and collapses inward, the filamentous material disappears, forming a secondary lysosome in which fragments of osmiophilic material persist (Fig. 5C). The secretory vacuoles characteristic of specimens from March–June are absent.

Three females that failed to oviposit after injection with hCG on 31 May, 31 June, and 2 July were sacrificed 4 July. All have full clutches of vitellogenic eggs (7–9 follicles, 3.1–3.6 mm mean dia) in the ovaries, and sperm in the spermathecae (Table 2). One of these females examined by TEM has normal-appearing sperm in the lumen and a lack of secretory vacuoles in the cytoplasm, similar to the females that oviposited in July and were sacrificed at the same time (Fig. 5E). Spermathecal tissue from the other two females after paraffin-preparation for light microscopy was stained with AB 8GX, pH 2.5 for carboxylated glycosaminoglycans and counterstained with PAS for neutral carbohydrates, or stained with the Gomori procedure for acid phosphatase, an indicator of lysosome activity. An AB+ reaction is scattered and most intense in the epithelium of the common tube, but PAS+ activity is uniform. In slides stained with the Gomori procedure, black-staining particles indicative of a positive reaction occur in the cytoplasm.

Two females injected with hCG on 31 May, 31 June, and 2 July were sacrificed approximately 1 month (4 August) and 2 months (4 September) after ovipositing 4 and 8 eggs, respectively, on 1 July (Table 2, Fig. 6). In the specimen sacrificed 4 August, portions of sperm cells occasionally are found embedded in endocytic vacuoles along the luminal border (Fig. 6A). Sperm still are relatively numerous in the lumen, and portions of the tail appear normal in cytology (Fig. 6A,B). However, the plasma membranes around sperm nuclei in the lumen are often crenated, detached, or disrupted (Fig. 6B), indicating sperm degradation in the lumen. In the specimen sacrificed in September, the lumen is narrowed and virtually devoid of sperm, with portions of only one or two cells appearing in some sections (Fig. 6C,D). The cytoplasm of both the August and September specimens lacks secretory vacuoles and contains numerous small vesicles (Fig. 6A,D).

Specimens of unknown reproductive history during the previous summer were collected and sacrificed in August and October. None of the four specimens from each month contain sperm in their spermathecae, and all possess follicles of a size to indicate onset of a wave of vitellogenesis that could lead to mature follicles for the next breeding season (Table 1). Some secretory vacuoles, consisting primarily of the electron-dense particles, occur in the spermathecal epithelium of these specimens, and these secretory vacuoles are relatively more numerous in specimens sacrificed in October (Fig. 7). In specimens from

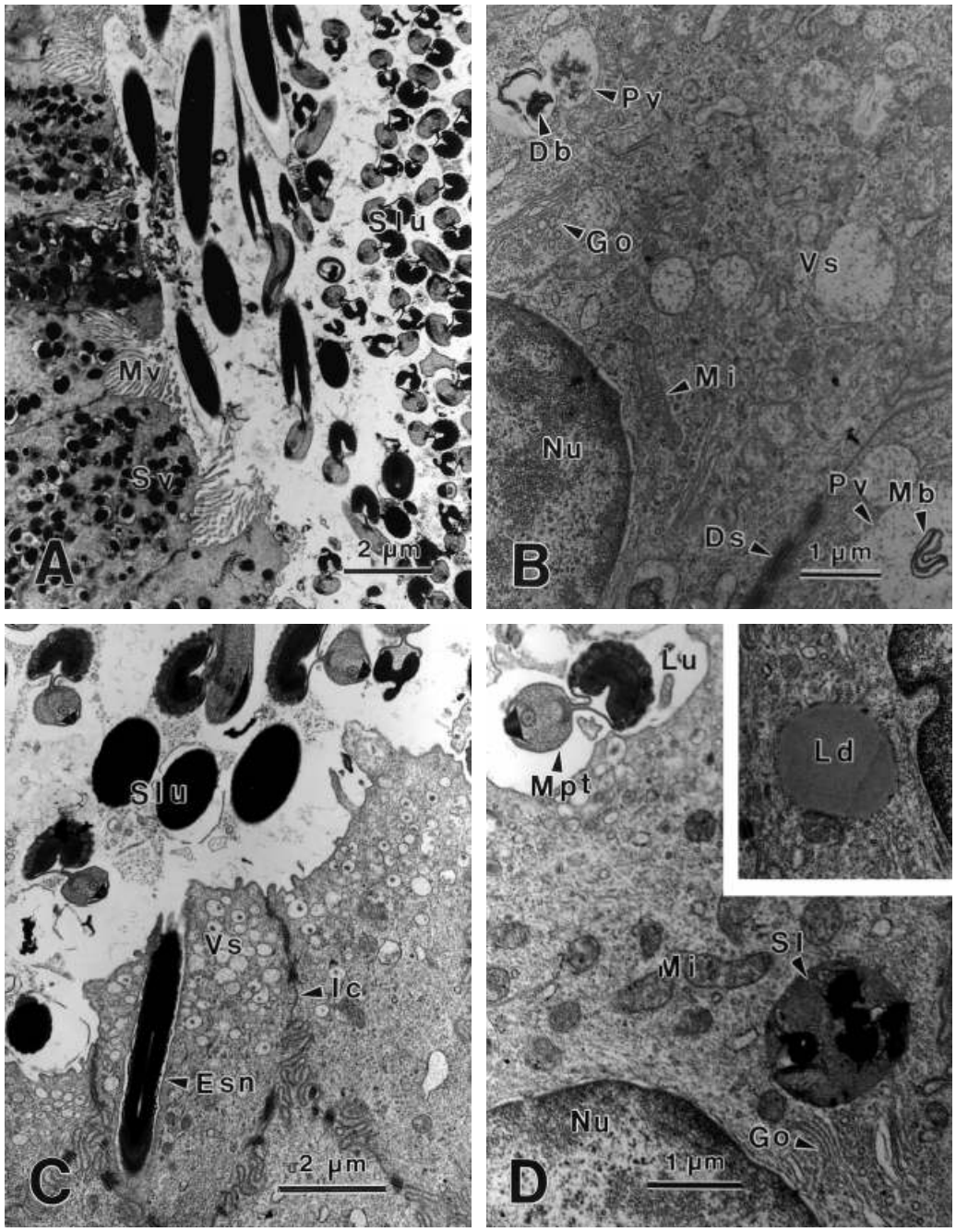


Fig. 4. Ultrastructure of the spermathecae of female *Plethodon cinereus* induced to oviposit following injections of hCG on 31 May. **A.** Specimen 49.2 mm SVL sacrificed 4 June after ovipositing 3 eggs on 3–4 June, showing apical cytoplasm and sperm in the lumen. **B.** Same specimen as A, supranuclear cytoplasm. **C.** Specimen 47.8 mm SVL sacrificed 4 July after ovipositing 5 eggs 4–5 June, showing apical cytoplasm and sperm in the lumen. **D.** Same specimen as C, supranuclear cytoplasm. Db, debris; Ds, desmosome; Esn, embedded sperm nucleus; Go, Golgi complex; Ic, intercellular canaliculi; Ld, lipid droplet; Lu, lumen; Mb, myelinic body; Mi, mitochondria; Mpt, middle piece of the tail; Mv, microvilli; Nu, epithelial cell nucleus; Pv, phagocytic vacuole; Sl, secondary lysosome; Slu, sperm in the lumen; Sv, secretory vacuoles; Vs, vesicles.

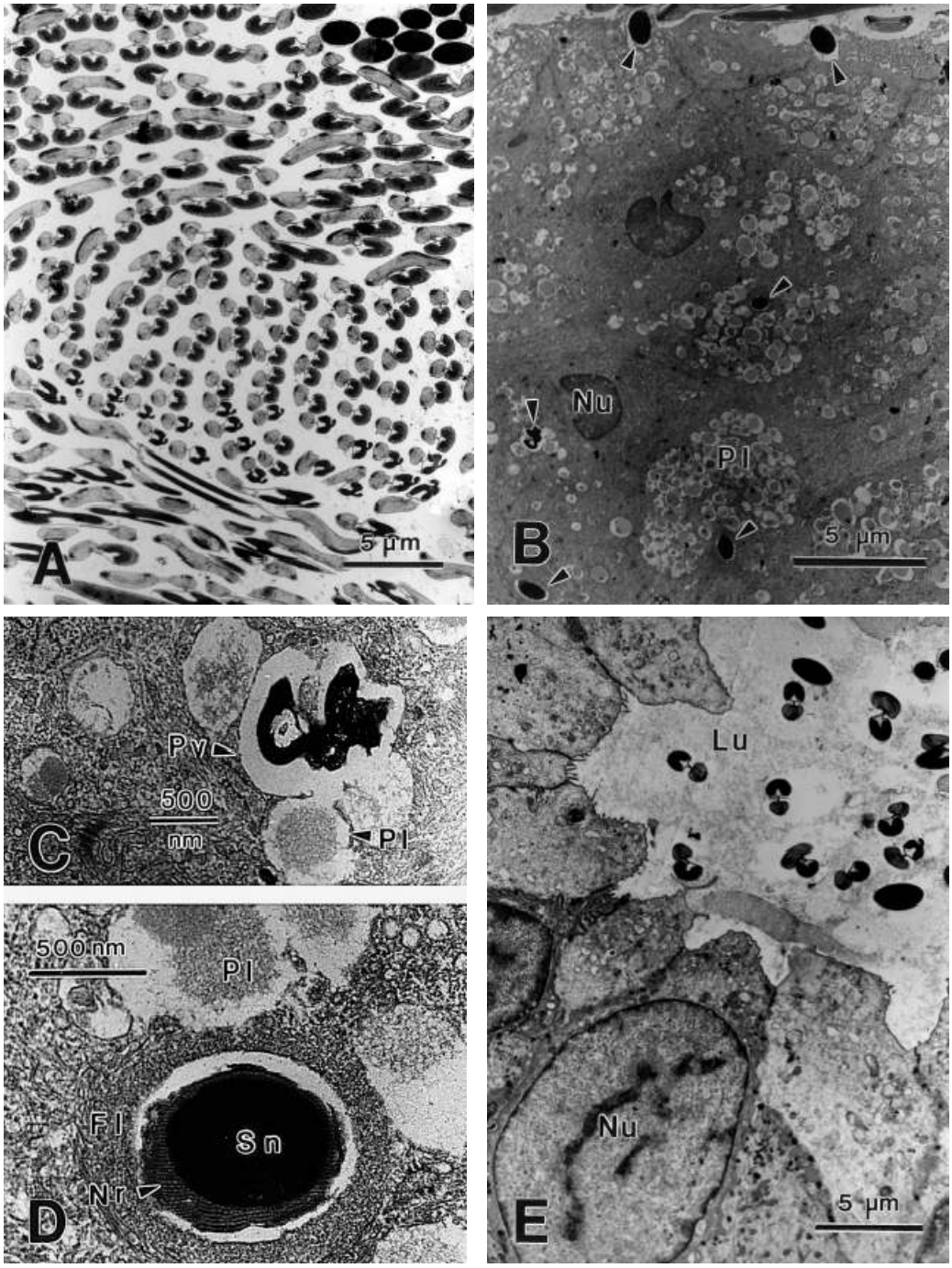


Fig. 5. Ultrastructure of the spermathecae of female *Plethodon cinereus* injected with hCG on 31 May, 28 June, and 2 July, and sacrificed 4 July. **A.** Specimen 47.7 mm SVL that oviposited 1 egg on 2 July, showing sperm in the lumen. **B.** Specimen 41.0 mm SVL that oviposited 3 eggs on 4 July. Unlabeled arrows indicate sperm embedded in the cytoplasm. **C.** Same specimen as B, showing connection between lysosomes and phagocytic vacuole containing a portion of a degraded sperm cell, an early stage in the transformation of a primary lysosome into a secondary lysosome. **D.** Same specimen as B, showing degrading sperm nucleus embedded in the cytoplasm. Note disruption of the nuclear ridge (Nr) and the meshwork of fine filamentous material (Fl) that encases phagocytic vacuoles during early stages of sperm degeneration. **E.** Specimen 48.8 mm SVL that did not oviposit despite presence of sperm and 9 ovarian follicles 3.1 mm mean dia. Fl, filamentous meshwork; Lu, lumen; Nr, nuclear ridge; Nu, nucleus of an epithelial cell; Pl, primary lysosome; Pv, phagocytic vacuole; Sn, sperm nucleus.

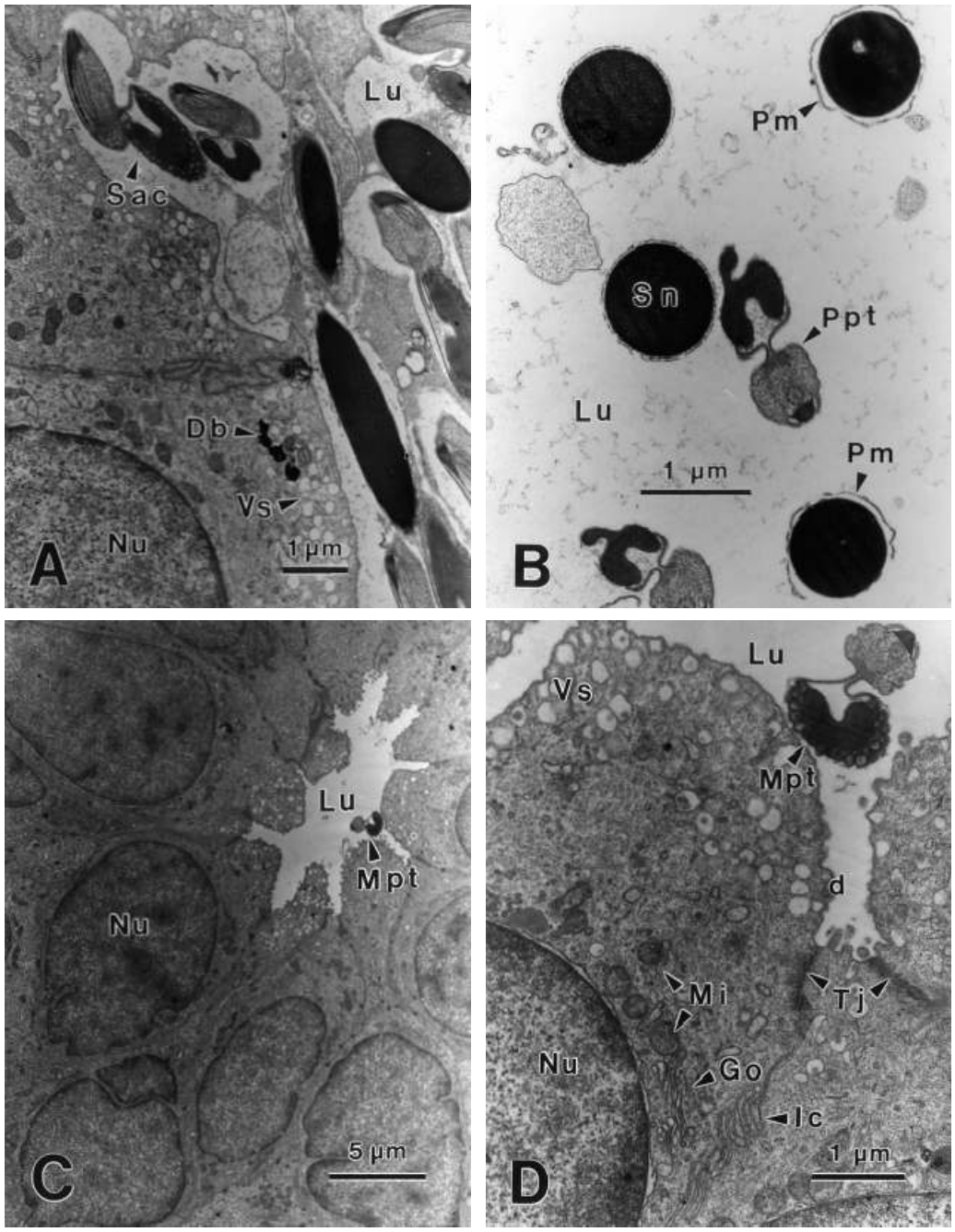


Fig. 6. Ultrastructure of the spermathecae of female *Plethodon cinereus* injected with hCG on 31 May, 28 June, and 2 July, and sacrificed 1–2 months after oviposition. **A.** Specimen 46.4 mm that oviposited 4 eggs on 1 July and was sacrificed 4 August, showing the apical cytoplasm and sperm. **B.** Same specimen as A, showing sperm in the lumen. Note disrupted plasma membranes (Pm) of sperm nuclei. **C.** Specimen 48.6 mm SVL that oviposited 8 eggs 1 July and was sacrificed 4 September, showing an overview of a spermathecal tubule. **D.** Same specimen as C, showing apical cytoplasm and the middle piece of a sperm cell in the lumen. Db, debris; Go, Golgi complex; Ic, intercellular canaliculi; Lu, lumen; Mi, mitochondria; Mpt, middle piece of the tail; Nu, nucleus of an epithelial cell; Pm, plasma membrane; Ppt, principle piece of the tail; Sac, sperm associated with the apical cytoplasm; Sn, sperm nucleus; Tj, tight junction; Vs, vesicles.

both months, luminal areas are narrow, and Golgi, RER, and microfilaments occur in the supranuclear cytoplasm (Fig. 7B,D). Microvilli are especially numerous and elongate in the specimens from October (Fig. 7C).

## DISCUSSION

### *Breeding season*

In such a wide-ranging species, considerable variation in the mating season and period of effective sperm storage may occur. Breeding seasons of salamanders in northern Indiana are influenced by the harsh winters, during which generally freezing temperatures preclude surface activity of poikilothermic animals during much of the period from November through February (Minton, '72). Werner ('69) claimed that initiation of the spermatogenic cycle of *Plethodon cinereus* in the spring is highly dependent on the temperature. Thus the cycle can vary from year to year at a locality or between localities within a given year. Some literature exists that indicates an autumnal "false breeding season" occurs in which males and females of *P. cinereus* engage in courtship and mating, but the females do not oviposit eggs. This phenomenon also has been documented extensively in northern populations of the newt, *Notophthalmus viridescens* (reviewed by Sever et al., '96a).

Hood ('34) reported that all 55 male *Plethodon cinereus* collected 7 October near Rochester, New York, carried spermatophores, but sperm were lacking in spermathecae of females. A subsequent collection on 16 October revealed that 5 of 25 females contained sperm in their spermathecae. In Maryland, Sayler ('66) reported spermatozoa in the vasa deferentia of male *P. cinereus* from September to May, and that most females with follicles $>$ 1.3 mm dia possessed sperm in their spermathecae from October to the time of oviposition in June. Sayler ('66), using only light microscopy, could not find sperm in spent females. Werner ('67), using specimens from localities in southern Michigan within 30 km of those used in the present study, found spermatophores in the cloacae of three of 30 mature females examined in 13 October. The last date spermatophores were found in the cloacae the following spring was 1 May. Werner ('67) found a continuous egg-laying period for the species from June–August. Finally, on 15 October in Virginia, Gergits and Jaeger ('90) observed 10 instances of courtship behavior of *P. cinereus* in the field, four of which resulted in insemination.

Sever ('78b) found that male *Plethodon cinereus* from Indiana possessed sperm in their vasa deferentia and hypertrophied cloacal glands in October and April collections, but lacked sperm and active cloacal glands in collections from June and August. Preovipository females from April and June collections contained sperm in their spermathecae, but other females from June, August, and October collections lacked sperm (Sever, '78a). Although mating could occur prior to hibernation (males possess sperm in their vasa deferentia), neither Sever ('78a) nor the current study found any evidence of mating in the fall and thus storage of sperm over winter. A similar situation was found in another plethodontid, *Eurycea cirrigera,* which is sympatric with *P. cinereus* in the southern half of Indiana (Sever, '91b). Considering the reports of fall matings of *P. cinereus* in other locales, however, it is possible that such activity occurs to some extent in northern Indiana as well.

Thus the current study found support for a period of sperm storage lasting 3–4 months, but if mating does occur in some females prior to hibernation, sperm could be stored for a much longer period, perhaps 9 months (October–June). However, whether such sperm could survive that period or be used in fertilization is not known. In the current study, mated specimens examined from May and June had relatively more sperm than those sacrificed in March, indicating repeated matings may occur from the first occasion of female receptivity until the onset of oviposition. The conditions for sperm competition are present, and paternity may be influenced by the order of insemination and duration of storage (Tilley and Hausman, '76; Labanick, '83; Halliday and Verrell, '84; Houck and Schwenk, '84; Houck et al., '85; Verrell, '88).

In other species, periods of effective sperm storage in spermathecae of female salamanders have been reported from several days (*Ambystoma*) to several years (*Salamandra*) (e.g., Baylis, '39; Marynick, '71; Trauth, '83; Brizzi et al., '89; Pecio, '92), and this topic has been reviewed rather extensively in several recent papers (Sever, '95; Sever et al., '96a,b). As noted by Sever ('95), the reports of long-term sperm storage ($>$6 months) are not based upon an ultrastructural analysis of the annual cycle of sperm

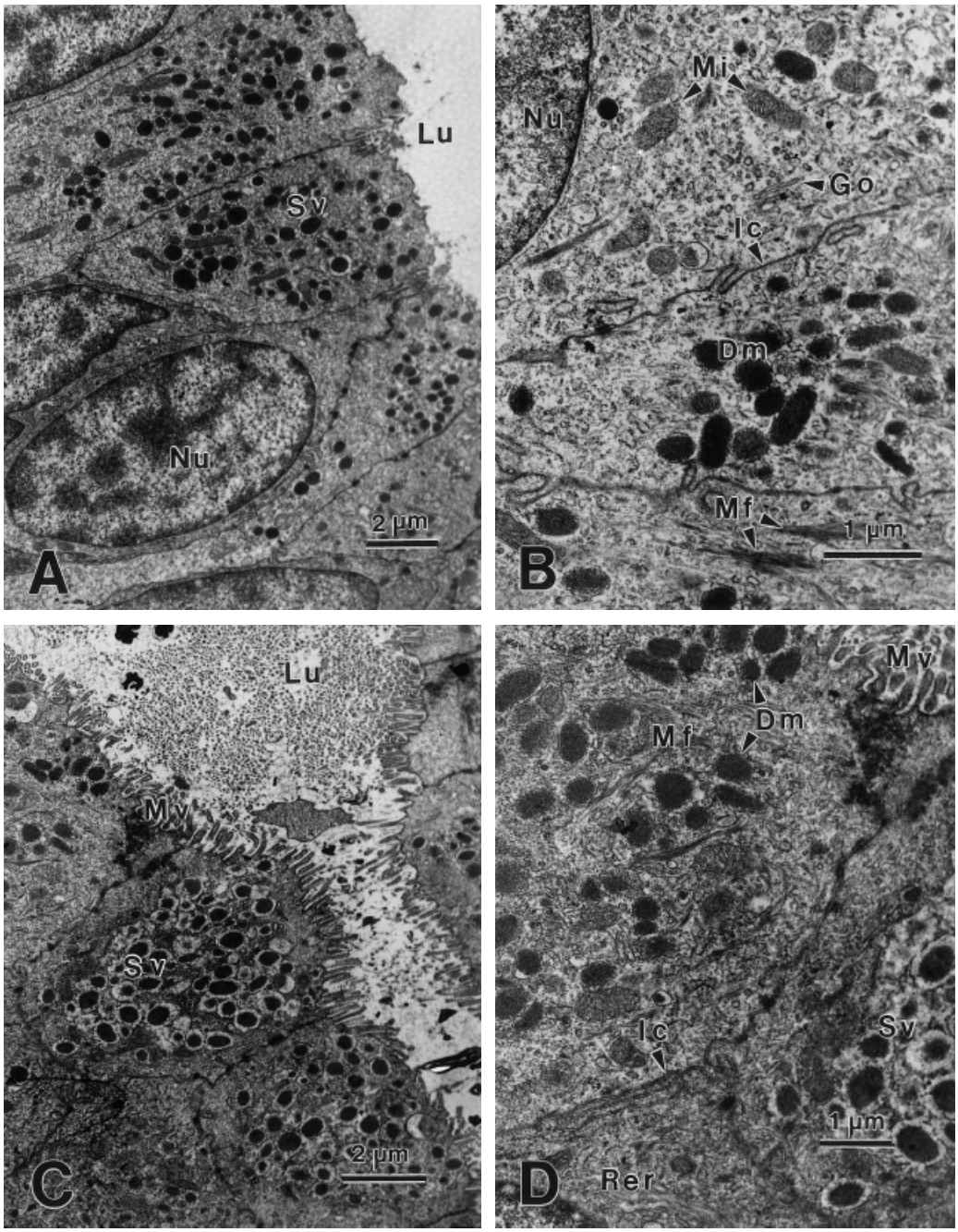


Fig. 7. Ultrastructure of the spermathecae of female *Plethodon cinereus* collected and sacrificed after the breeding season. **A.** Apical cytoplasm of a specimen 35.5 mm SVL collected and sacrificed 14 August and containing 7 ovarian follicles 0.9 mm mean dia. **B.** Same specimen as A, showing supranuclear cytoplasm. **C.** Apical cytoplasm and lumen of a specimen 50.5 mm SVL collected and sacrificed 6 October and possessing 13 follicles 1.4 mm mean dia. **D.** Same specimen as C, showing supranuclear cytoplasm. Dm, dense material; Go, Golgi complex; Ic, intercellular canaliculi; Lu, lumen; Mf, microfilaments; Mv, microvilli; Mi, mitochondria; Nu, nucleus of an epithelial cell; Rer, rough endoplasmic reticulum; Sv, secretory vacuoles.

storage and have not been experimentally verified.

*Spermathecal cytology*

Two types of spermathecae occur in salamanders. In all families except the Plethodontidae, the spermathecae consist of numerous simple tubuloalveolar glands opening separately into the roof of the cloaca, designated "simple spermathecae" by Sever and Brunette ('93). Thus each separate tubule is a "spermatheca," and collectively the glands in any one female are spermathecae (Kingsbury, 1895). In the Plethodontidae, the spermathecae are compound alveolar glands, called "complex spermathecae" by Sever and Brunette ('93). In most plethodontids, distal bulbs connect to narrow neck tubules that lead into a common tube opening into the middorsal wall of the cloaca. Therefore, plethodontids possess a single spermatheca (one compound gland) in their cloacae as opposed to nonplethodontids, which have numerous simple spermathecae. In members of the *Plethodon cinereus* group (including *P. serratus* and *P. richmondi*), the neck tubules and distal portions are more elongate and tubular than in other plethodontids (Sever, '94a,b). Sever ('78a) described the gross appearance of the spermatheca of *P. cinereus* as resembling "a small stout cord that is in a tight oblong coil."

In common with some other *Plethodon,* the distal portions of the spermatheca of *P. cinereus* are asymmetrically arranged dorsomedial to the cloaca (the common tube is medial but remaining portions lie to the right or left of the midline) (Sever, '78a, '94a,b). Melanophores are abundant in the tissue surrounding the spermatheca, so that its location can be ascertained grossly by presence of a black area in the roof of the cloaca (Sever, '78a). The common tube is stratified epithelium, and the more distal regions are simple epithelium but can have a highly fluctuated appearance when hypertrophied (Sever, '94a).

The literature on the ultrastructure of the spermathecae of salamanders using TEM has been reviewed a number of times recently (cf. Sever, '95; Sever et al., '96a,b), and here I emphasize comparisons with the other plethodontids that have been examined with this methodology, *Eurycea quadridigitata* (Pool and Hoage, '73) and *E. cirrigera* (Sever, '91b, '92; Sever and Brunette, '93). In both *Eurycea,* the secretory vacuoles have a uniform electron density instead of the mixed density (flocculent shell around a dense core) found in *Plethodon cinereus.* However, secretory vacuoles of uniform density are known from only one nonplethodontid, *Notophthalmus viridescens* (Sever et al., '96a), whereas vacuoles of mixed density are known from a variety of other salamanders, including *Ambystoma opacum* (Sever and Kloepfer, '93), *A. tigrinum* (Sever, '95), *Salamandrina terdigitata* (Brizzi et al., '95), *Amphiuma tridactylum* (Sever et al., '96b), and *Necturus beyeri* (Sever and Bart, '96). In both *P. cinereus* and *E. cirrigera,* the synthesis of the glycoprotein secretory product follows a rather standard process, including peptide production by RER and packaging of vacuoles by the Golgi complexes (Krstić, '79; Sever, '91a), but in *E. quadridigitata,* a more complex process involving mitochondrial crystals, Golgi complexes, and smooth endoplasmic reticulum occurs (Pool and Hoage, '73). Release of the product in plethodontids occurs during sperm storage; the glands are depleted of secretory vacuoles following oviposition, and production of additional secretory material does not occur until start of the next vitellogenic cycle.

Degradation of sperm embedded in the cytoplasm has been reported for all three plethodontids and was studied most extensively in *Eurycea cirrigera* (Sever, '92; Sever and Brunette, '93). In that species, secretory vacuoles are found only in the epithelium of the common tube and neck tubules, and the epithelium of the distal bulbs is strictly phagocytic. *Plethodon cinereus* does not show regionalization of secretory and phagocytic activity. The amount of spermiophagic activity observed in *P. cinereus* does not seem sufficient to account for the removal of the vast quantities of sperm remaining after oviposition. Perhaps most sperm remaining "leak out" gradually or are expelled by myoepithelial contractions, as reported for sperm remaining in the vas deferens of *Ambystoma macrodactylum* after the breeding season (Zalisko and Larsen, '89).

No evidence was found that the epithelium provides a "nourishing function" for embedded sperm (Benson, '68). All ultrastructural evidence leads to the conclusion that embedded sperm undergo degeneration. The function of the secretion bathing sperm in the lumen is unclear, but it could be involved in sperm attraction, maintenance, capacitation, expulsion, and/or degradation, and the secretions could serve differ-

ent functions in different species (Hardy and Dent, '86, '87; Sever, '95). It is interesting to note, however, that all ultrastructural studies done so far on salamander spermathecae have found depletion of secretory product after oviposition and degradation of sperm remaining in the lumen prior to the start of the next breeding cycle.

The only other ultrastructural study on the spermatheca of *Plethodon* used SEM to study sperm storage in *P. larselli* prior to ovulation, using untreated females and those injected with pregnant mare gonadotropin, PMSG, which induces ovulation (Davitt and Larsen, '88). In the untreated females, the sperm are in parallel arrays, and one type of epithelial surface with short microvilli is present. Portions of the apical cytoplasm containing secretory material are "occasionally" observed free in the lumen (forming apocrine blebs), and some secretory vesicles also occur in the lumen. Both the blebs and the secretory vesicles are in contact with sperm, and other sperm are embedded in intact epithelial cells. After administration of PMSG, two types of cell surfaces are present. Distally, the epithelium has long microvilli and contains numerous apocrine blebs. Proximally, the gland contains large spherical vesicles and fewer blebs. Sperm are more random in orientation, and some are near the opening to the common tube. Davitt and Larsen ('88) concluded that at onset of ovulation, cellular changes occur within the epithelium of the spermatheca and secretions are released into the lumen. These secretions may be responsible for reactivation of the sperm just prior to their discharge into the cloaca.

In *Plethodon cinereus,* long microvilli occur in females sacrificed after oviposition but also in nonvitellogenic females and those beginning a new vitellogenic cycle. Microvilli are not as elongate in females just prior to or after mating, or in females sacrificed 1–2 months after oviposition. When sperm are numerous, groups of them show similar orientations, especially toward the center of the lumen. Only one type of epithelial cell is present in *P. cinereus* rather than the two types found in *P. larselli.* Differences in cell surfaces can be more easily observed by SEM, however, and it is possible some of the features described for *P. larselli* by Davitt and Larsen ('88) occur in *P. cinereus* as well. The apocrine mode of secretion described for *P. larselli* does not seem to occur in *P. cinereus.* The accumulation of secretory vacuoles along the luminal border (Figs. 1,3) and their subsequent disappearance without loss of apical cytoplasm (Figs. 2,4) leads to the conclusion that the release process is merocrine, as is known for most other salamanders (Sever, '91b, '95; Sever and Kloepfer, '93; Sever and Bart, '96; Sever et al., '96a; Brizzi et al., '95). An apocrine mode of secretion, however, was recently reported for *Amphiuma tridactylum* (Sever et al., '96b).

In summary, some differences between the spermathecal cytology of *Plethodon cinereus* and other salamanders were found. Other than the possession of complex spermathecae, however, none of these can be correlated directly with phylogeny, and *P. cinereus* did not have any unique adaptations that could be related to terrestrial breeding. Examination of additional plethodontids, especially species representing the subfamily Desmognathinae and the tribe Bolitoglossini within the subfamily Plethodontinae, would be welcome. Studies on members of the families Dicamptodontidae and Rhyacotritonidae, two small groups of salamanders endemic to the northwestern Pacific coast of the United States, also are necessary so that we may have data on representatives from all families in which females store sperm. A comparative analysis that will lead to hypotheses concerning the evolution of sperm storage in salamanders still awaits more detailed studies that consider the complete annual cycle. These studies take time; resolution of the questions concerning the significance of variability in sperm storage mechanisms is a goal still to be achieved.

According to parsimony analysis, however, sperm storage evolved prior to the splitting of the major families in the suborder Salamandroidea (Sever, '91a, '94b) and thus before interfamilial differences in other aspects of reproductive biology were established. Therefore, sperm storage is an ancient trait, and some of the differences in mechanisms may not be phyletically informative but related to species-specific reproductive adaptations in which a considerable amount of homoplasy no doubt occurs.

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