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Spermathecal Cytology of *Ambystoma opacum* (Amphibia: Ambystomatidae) and the Phylogeny of Sperm Storage Organs in Female Salamanders

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ABSTRACT Sperm storage glands, spermathecae, were examined from mated female *Ambystoma opacum* during the breeding season. No differences occur in the spermathecal ultrastructure of individuals sacrificed prior to oviposition and those sacrificed within 3 days of removal from tended clutches of recently oviposited eggs. The simple tubuloalveolar glands produce two types of secretory vacuoles. Apical secretory vacuoles contain glycosaminoglycans for export into the lumen to bathe stored sperm, perhaps providing the chemical/osmotic environment necessary for sperm quiescence. The other type of secretory vacuole contains an unsaturated lipid that is produced for export into the connective tissue surrounding the spermathecae. The role of this secretion may involve the contraction of myoepithelial cells, resulting in sperm expulsion. Some sperm undergo degradation in the spermathecal epithelium, and an interepithelial leukocyte was observed in one specimen. Apical secretory vacuoles and sperm are absent from the spermathecae of a specimen sacrificed 62 days after removal from a tended egg clutch. This is the first report on the spermathecal cytology of a salamander from the Ambystomatidae, and comparisons with salamanders from other families provide a morphological basis for considering spermathecae polyphyletic within the Caudata.

The North American salamander family Ambystomatidae includes 27 species (Shaffer et al., '91). As with most other salamanders, female ambystomatids possess sperm storage glands, spermathecae, in their cloacal walls (Sever, '92a). Fertilization occurs during oviposition by release of sperm from the spermathecae as eggs pass through the cloaca (Boisseau and Joly, '75). Thus, in salamanders, sequestering sperm in spermathecae provides a means for internal fertilization as well as physical separation of mating and oviposition (Sever, '91a).

The ultrastructure of ambystomatid spermathecae has not been studied. Cytological reports exist for these organs in the Salamandridae (Dent, '70; Boisseau and Joly, '75; Brizzi et al., '89) and Plethodontidae (Pool and Hoage, '73; Davitt and Larsen, '88a; Sever, '91b, '92b). In salamandrids and plethodontids, sperm storage in spermathecae has been reported to extend from several months to over 2 years (Baylis, '39; Benson, '68; Marynick, '71; Boisseau and Joly, '75; Massey, '90; Sever, '91b, '92b).

Most ambystomatids are winter or spring breeders in temperate regions, but the marbled salamander, *Ambystoma opacum,* breeds in autumn (Bishop, '47; Minton, '72). During that time, males and females of *A. opacum* congregate at the borders of ephemeral or permanent ponds for courtship and mating. Oviposition may closely follow mating in nature; egg laying has been observed 1–14 days after matings in the laboratory (Noble and Brady, '33). Thus a shorter time period for storage of recently sequestered sperm probably occurs in females of *A. opacum* than that reported for salamandrids and plethodontids. The females tend their eggs under cover around the border of ponds until the nest is flooded by rising water levels, whereupon the eggs hatch (Noble and Brady, '33).

Sever ('91a) proposed that spermathecae and internal fertilization are synapomorphies for the suborder Salamandroidea. One purpose of this study is to determine if ultrastructure of the spermathecae of an ambystomatid provides morphological support for a hypothesis of monophyly for sperm storage

in salamanders. This study also seeks to ascertain if the spermathecal epithelium of *Ambystoma opacum* has functions similar to those reported for other salamanders. For example, after oviposition in the plethodontid *Eurycea cirrigera,* residual sperm are phagocytized by the spermathecal epithelium (Sever, '92b). In the present study, transmission electron microscopy was used to increase our knowledge of phylogeny, morphology, and function of sperm storage in salamanders by examining the ultrastructure of spermathecae removed from *A. opacum* collected during the breeding season before and after oviposition.

MATERIALS AND METHODS

The *Ambystoma opacum* used in this study were collected at a breeding pond in Morgan Monroe State Forest, Morgan County, Indiana. Four gravid females were collected from the border of the pond on September 20, 1991, prior to oviposition. They were returned to the laboratory, and two were sacrificed September 23, 1991, and two were sacrificed October 9, 1991. Four additional females were collected from the site on October 6, 1991, spent and tending clutches of eggs. Two of the spent females were sacrificed October 9, 1991, and the other two were maintained alive in the laboratory. Laboratory maintenance was initially at 18–20°C and local photoperiod, with specimens housed singly in 17 × 31 × 9 cm plastic containers supplied with wet paper towels and access to a variety of invertebrates for food. One of the salamanders was found dead in its cage on November 20, 1991, after last being observed alive on November 18. The other specimen was placed into simulated "hibernation" in a refrigerator at 5°C on December 6, 1991, and subsequently was sacrificed January 6, 1992.

Following sacrifice of the specimens in 10% MS-222, snout-vent length (SVL) was measured from the tip of the snout to the posterior end of the vent to the nearest 0.1 mm. The specimens were of 69.0–81.8 mm SVL. The right spermathecal area was excised from the cloaca and placed in 2.5% glutaraldehyde in Millonig's phosphate buffer at pH 7.4. The rest of the body was preserved in 10% neutral buffered formalin and retained in the senior author's possession at Saint Mary's College.

The left one-half of the spermathecal area subsequently was removed from two specimens (pre- and postovipository), and the tissue was frozen at −25°C, and 15 μm sections were cut with a AO Cryo-cut II cryostat. The presence of lipids in the sections was determined by staining with Sudan black B.

The spermathecal tissue fixed in glutaraldehyde was trimmed into 1.5 mm blocks, rinsed in Millonig's buffer, postfixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol (with en bloc staining in 2% uranyl acetate in 50% ethanol), cleared in propylene oxide, and embedded in an epoxy resin (EMBED-812; Electron Microscopy Science, Fort Washington, PA). Semithin sections (500 nm to 1 μm) for light microscopy were cut with glass knives, placed on microscope slides, and stained with toluidine blue. Ultrathin (60–70 nm) sections for electron microscopy were collected on uncoated copper grids and stained with solutions of uranyl acetate and lead citrate. Sections were cut using RMC XL1000 and RMC MT7 ultramicrotomes, and thin sections were viewed using a Hitachi H-300 transmission electron microscope.

RESULTS

The spermathecae of female *Ambystoma opacum* surround the dorsal and lateral portions of the anterior end of the cloaca (Fig. 1A). The spermathecae are simple tubuloalveolar glands with a narrow neck opening into the cloaca and an expanded distal end surrounded by a loose connective tissue sheath, the tunica propria (Fig. 1B). No differences were found in cytology of different portions of the spermathecae. The anatomy and secretory activity of the spermathecae are the same in individuals collected on September 20 and sacrificed on September 23 or October 9 prior to oviposition and in specimens collected October 6 while tending recently oviposited eggs and sacrificed October 9 (Fig. 1C,D). Spermathecal cells are irregular in shape, varying from squamous to columnar. The nucleus is basal and is oriented with the long axis of the cell. Due to the irregular shape of the epithelium, the apical cytoplasm is fluctuated (Fig. 1C,D).

Two distinct types of secretory vacuoles are produced for export from the spermathecal epithelium (Figs. 1–3). One is found in the apical cytoplasm, and these vacuoles in paraffin sections are periodic acid-Schiff (PAS)-negative and alcian blue-positive at pH 2.5 (Sever, '92b), indicating the presence of sulfated or carboxylated acidic mucosubstances (glycosaminoglycans; GAGs). These membrane-bound vacuoles are variable in size, but the largest ones are 1.0–1.2 μm in diameter and contain a flocculent material as

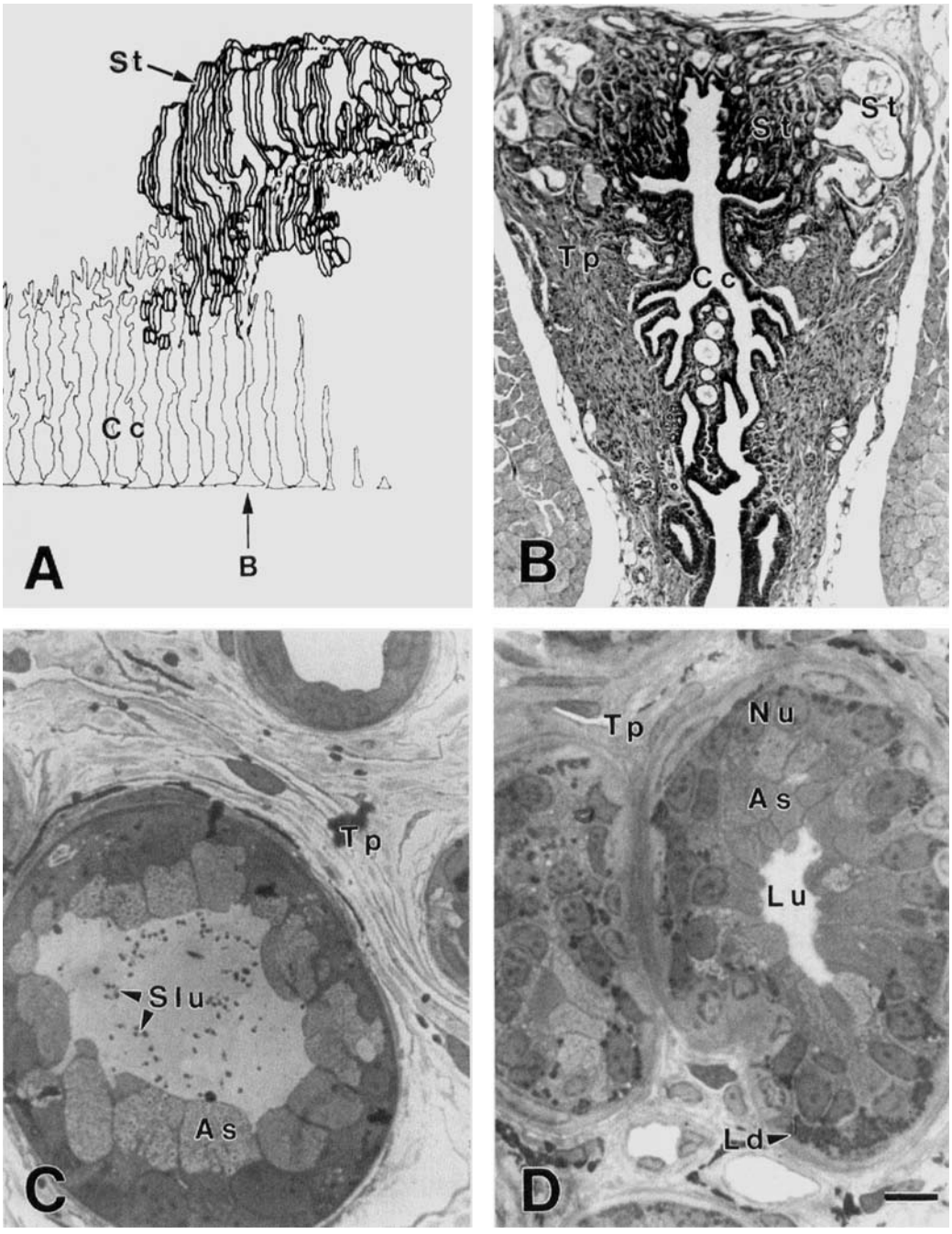

Fig. 1. *Ambystoma opacum*. Overview of the spermathecae and surrounding structures in females. Specimen used for A and B is a female sacrificed after mating and before oviposition and used by Sever ('92a). **A:** Three-dimensional reconstruction of the spermathecae (St) and cloacal cavities (Cc). Right lateral views with sections rotated 120° clockwise; anterior is to the right, and the distance between sections of the cloacal walls is ~120 μm. **B:** Transverse paraffin section stained with hematoxylin-eosin through the area labeled B in A. **C:** Plastic thick section through the spermathecae of a specimen collected September 20 and sacrificed October 9 prior to oviposition. **D:** Plastic thick section through the spermathecae of a specimen collected October 6 while tending eggs and sacrificed October 9. Sections in C and D stained with toluidine blue. Bar = 250 μm for B and 15 μm for C and D. As, apical secretion; Cc, cloacal cavities; Ld, lipid droplets; Lu, lumen; Nu, nucleus of spermathecal epithelial cell; Slu, sperm in the lumen; Tp, tunica propria.

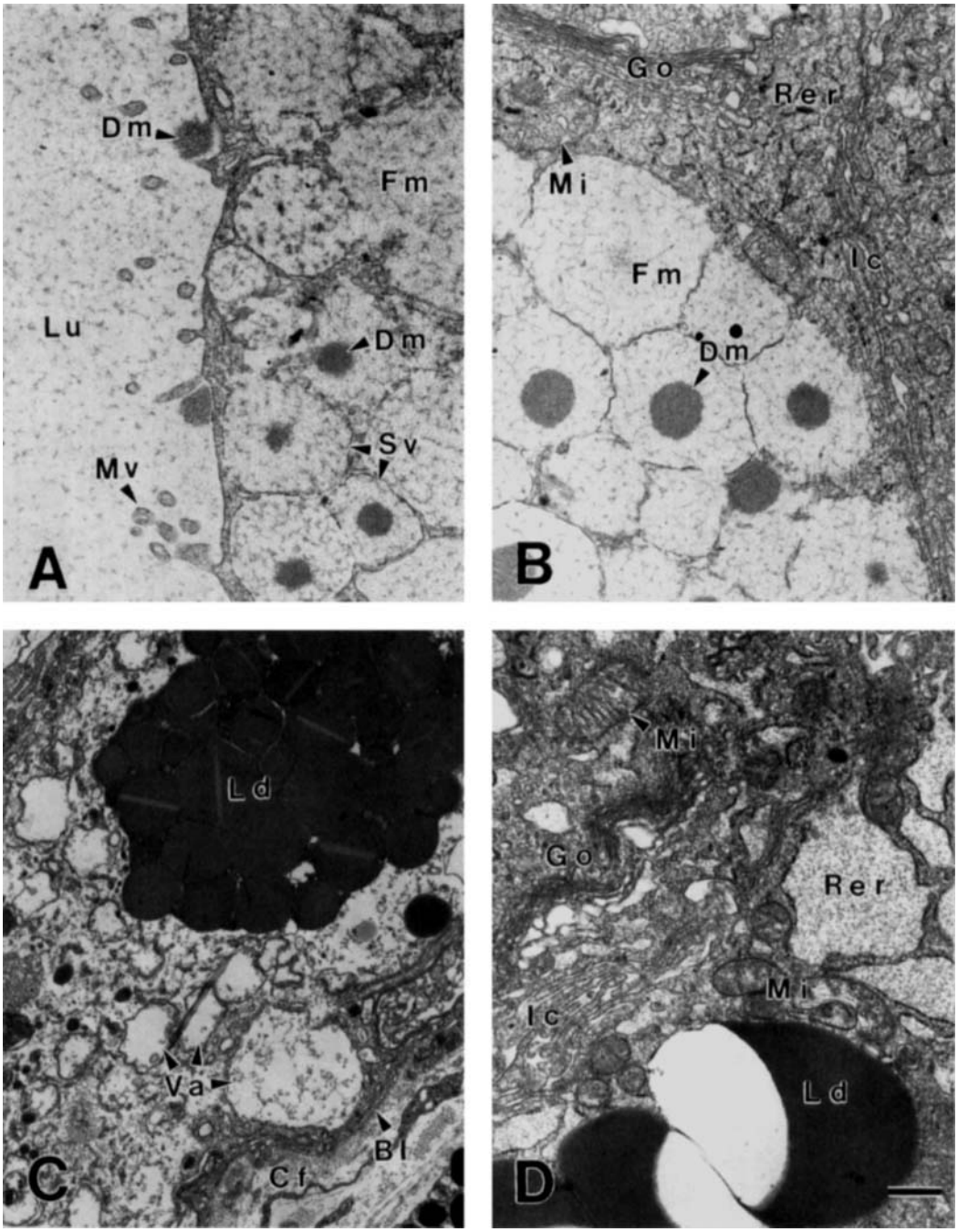

Fig. 2. *Ambystoma opacum*. Secretory products in the spermathecal epithelium of females. A and B show the apical product containing glycosaminoglycans in secretory vacuoles (Sv) consisting of a flocculent material (Fm) with a central electron-dense material (Dm). C and D show electron-dense, largely basal, lipid droplets (Ld). **A:** Luminal border of a specimen collected September 20 and sacrificed October 9 prior to oviposition. Note presence of flocculent material and the electron-dense material in the lumen as well as in cytoplasmic secretory vacuoles. **B:** Same specimen as in A, showing organelles associated with basal regions of the apical product. **C:** Basal portion of a spermathecal epithelial cell from a specimen collected September 20 and sacrificed September 23 prior to oviposition, showing an aggregation of lipid droplets. **D:** Specimen collected October 6 while tending eggs and sacrificed October 9, showing organelles associated with lipid droplets. The association with enlarged cisternae of rough endoplasmic reticulum may indicate that the droplets contain lipoproteins. Bar = 570 nm for A, 440 nm for B, 715 nm for C, and 520 nm for D. Bl, basal lamina; Cf, collagen fibers; Dm, dense material; Fm, flocculent material; Go, Golgi apparatus; Ic, intercellular canaliculus; Ld, lipid droplets; Lu, lumen; Mi, mitochondria; Mv, microvilli; Rer, rough endoplasmic reticulum; Sv, secretory vacuoles; Va, vacuoles.

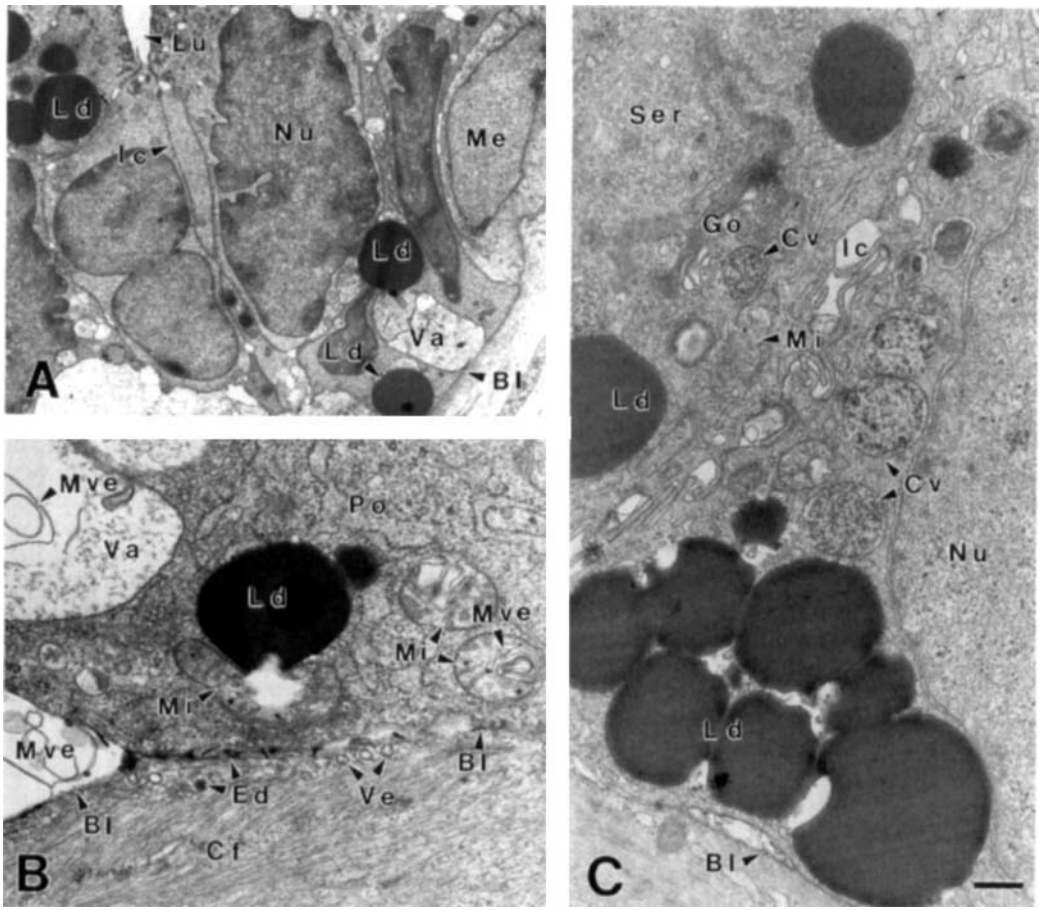

Fig. 3. *Ambystoma opacum.* Production of the lipid secretion in the spermathecal epithelium and its diffusion into the surrounding connective tissue in females. **A:** Specimen collected September 20 and sacrificed October 9 prior to oviposition. Overview of the spermathecal epithelium containing lipid droplets (Ld). **B:** Specimen collected October 6 while tending eggs and sacrificed October 9, showing a lipid droplet associated with mitochondria (Mi), polyribosomes (Po), basal lamina (Bl) with vesicles (Ve), and multivesicular structures (Mv) within vacuoles (Va) and mitochondria. Note the presence of electron-dense material (Ed), perhaps diffusing lipid material, associated with the basal lamina. **C:** Same specimen as in B, showing the association of lipid droplets with the secretory apparatus. Note the presence of smooth endoplasmic reticulum (Ser) on the luminal (immature) side of the Golgi apparatus and condensing vacuoles (Cv) on the stromal (mature) side. Bar = 1.8 μm for A, 345 nm for B, and 606 nm for C. Bl, basal lamina; Cf, collagen fibers; Ed, electron-dense material; Go, Golgi apparatus; Ic, intercellular canaliculus; Ld, lipid droplets; Lu, lumen; Me, myoepithelial cell nucleus; Mi, mitochondria; Mve, multivesicular structures; Nu, nucleus of spermathecal epithelial cell; Po, polyribosomes; Va, vacuoles; Ve, vesicles.

well as a central, electron-dense particle 300–450 nm in diameter (Fig. 2A,B). Both the flocculent material and the electron-dense material also are present in the lumen (Fig. 2A). The secretion is presumably merocrine, since the apical epithelial border remains intact. Associated with the basal border of the secretory vacuoles are Golgi complexes and cisternae of rough endoplasmic reticulum (Rer; Fig. 2B). The presence of Rer could indicate combination of GAGs with proteins, forming proteoglycans. Ninhydrin-Schiff tests for proteins in paraffin sections were negative (Sever, '92b), but proteins may have been removed by solutions used in specimen preparation.

The other type of secretory vacuole is an electron-dense droplet that in frozen sections cut for light microscopy stains positively with Sudan black B for lipids. The extreme osmio-

philic appearance at the ultrastructural level is indicative of highly unsaturated lipids. These lipid droplets are extremely variable in size, with droplets from 700 nm to 3.2 μm often in the same cell. Sometimes, the droplets form large aggregates in the basal regions of epithelial cells (Fig. 2C). Less densely staining or clear areas are present in some droplets, perhaps due to lack of penetration by osmium tetroxide during fixation (Fig. 2C,D). The lipid droplets are associated with smooth endoplasmic reticulum (Ser), Golgi complexes, cisternae of rough endoplasmic reticulum, and whorls of polyribosomes (Figs. 2D, 3). The association with ribosomal structures may indicate that the droplet contains lipoprotein.

Although lipid droplets sometimes occur in apical regions (Fig. 3A), the droplets are most numerous along the basal border, often adjacent to the plasmalemma and associated basal lamina, vacuoles, and vesicles (Fig. 3). The cytological evidence suggests that the lipid droplets are produced for export into the tunica propria. Smooth endoplasmic reticulum exists on the immature, apical face of the Golgi complexes, and condensing vacuoles are oriented on the mature, basal face of the complexes (Fig. 3C). Electron-dense material, perhaps representing diffusion of the lipid product, is evident in the basal lamina, along with vacuoles, vesicles, and multivesicular structures that may be involved in transport of product (Fig. 3B). Basal mitochondria are also frequently seen in close contact with lipid droplets, and multivesicular structures occur in the matrix of some of these mitochondria (Fig. 3B).

Vesicles also are seen along the apical border, and they apparently are released into the lumen, where they may coalesce into multivesicular structures (Fig. 4A,B). These vesicles appear empty, and their significance is unknown. Structures consisting of tightly packed, concentric membranes are frequently encountered in the cytoplasm. These inclusions often are associated with microfilaments or intercellular canaliculi, indicating movement within or between cells, but the function of concentric membrane structures remains to be determined. As was mentioned above, membranous structures are found in some basal mitochondria (Fig. 3B), and mitochondria therefore may be involved in production of the concentric membrane structures.

Clusters of elongate mitochondria frequently are numerous in areas of the apical cytoplasm where secretory vacuoles are absent (Fig. 4D). Mitochondria in such fields are characterized by narrow, tubular cristae and electron-dense intramitochondrial granules, and Ser can be found in the surrounding cytoplasm (Fig. 4E).

Sperm are present in the lumina of spermathecae of all six animals sacrificed September 23 and October 9. Lumina of some glands in each specimen are empty, however, and none of the glands is crowded with sperm. Oviposition results in no apparent difference in the relative number or appearance of sperm. Sperm in the lumen are randomly oriented and are surrounded by the flocculent secretory product (Fig. 5A,B). Sperm occur beyond the border of microvilli associated with the luminal border of the epithelial cells (Fig. 5B). Sperm generally appear normal in cytology, although structures that may represent fragments of degraded sperm are sometimes seen. The plasma membrane around the nucleus is irregular but intact (Fig. 5C). Associated with the tail of some sperm are structures that may represent portions of cytoplasmic droplets, including concentric membrane structures (Fig. 5D,E).

A few sperm are embedded in the spermathecal cytoplasm of two specimens sacrificed prior to oviposition, and these sperm apparently are undergoing degradation as indicated by loss of the mitochondrial sheath around the axial fiber of the middle piece of the tail (Fig. 6). Embedded sperm are found so rarely in *Ambystoma opacum* that the mechanism of spermiophagy cannot be elucidated fully, but cytologically they resemble degenerating sperm of *Eurycea cirrigera* (Sever, '92b). The small, membrane-bound, circular organelles associated with embedded sperm are interpreted as primary lysosomes, and more irregular aggregations of dense material represent secondary lysosomes and their condensation products (Fig. 6A). Sperm initially are enclosed in phagocytic vacuoles, since the sperm appear most normal when embedded in vacuoles, and such vacuoles are associated with the apical regions of the glands (Fig. 6B). The vacuolated space then is replaced by a filamentous material, forming a phagosome (Fig. 6B) that subsequently becomes associated with primary lysosomes (Fig. 6A).

The specimen collected October 6 with an egg clutch and found dead on November 20 showed evidence of much secretory activity in its spermathecae, but this specimen may have been dead several hours before preserva-

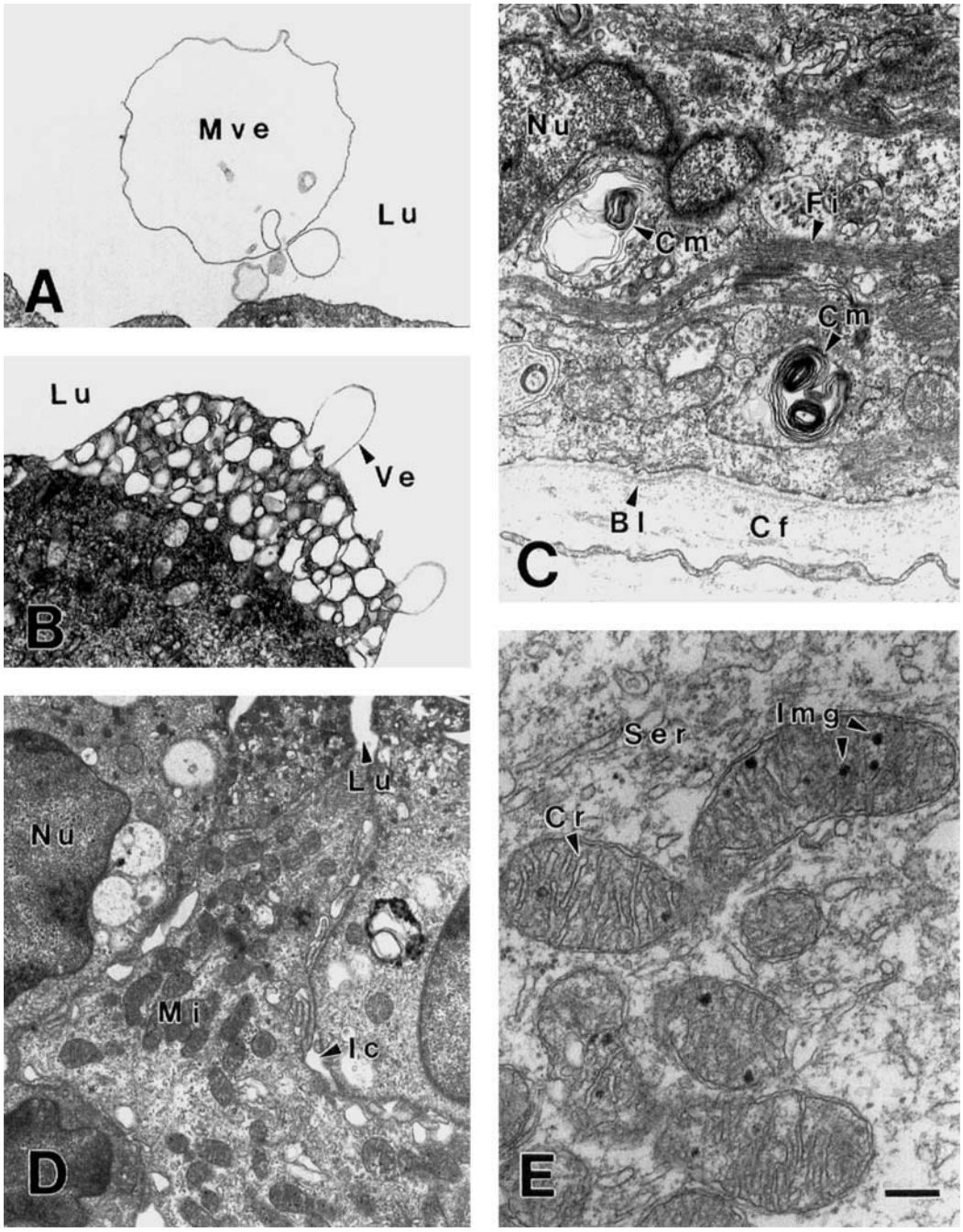

Fig. 4. *Ambystoma opacum*. Other secretory and cytoplasmic structures noted in the spermathecal epithelium of females. **A:** Specimen collected September 20 and sacrificed September 23 prior to oviposition, showing multivesicular structures (Mve) in the lumen. **B:** Same specimen as in A, showing the budding of vesicles (Ve) along the luminal border. **C:** Same specimen as in A and B, showing cytoplasmic inclusions composed of tightly packed concentric membranes (Cm) and associated microfilaments (Fi). **D:** Specimen collected September 20 and sacrificed October 9 prior to oviposition, showing abundance of elongate mitochondria (Mi) in the apical cytoplasm. **E:** Detail of mitochondria from the specimen shown in C, showing tubular cristae (Cr), intramitochondrial granules (Img), and surrounding matrix of smooth endoplasmic reticulum (Ser). Bar = 500 nm for A, 715 nm for B, 667 nm for C, 795 nm for D, and 190 nm for E. Bl, basal lamina; Cf, collagen fibers; Cm, concentric membranes; Cr, mitochondrial cristae; Fi, microfilaments; Ic, intercellular canaliculus; Img, intramitochondrial granules; Lu, lumen; Mi, mitochondria; Mve, multivesicular structures; Nu, nucleus of spermathecal epithelial cell; Ser, smooth endoplasmic reticulum; Ve, vesicles.

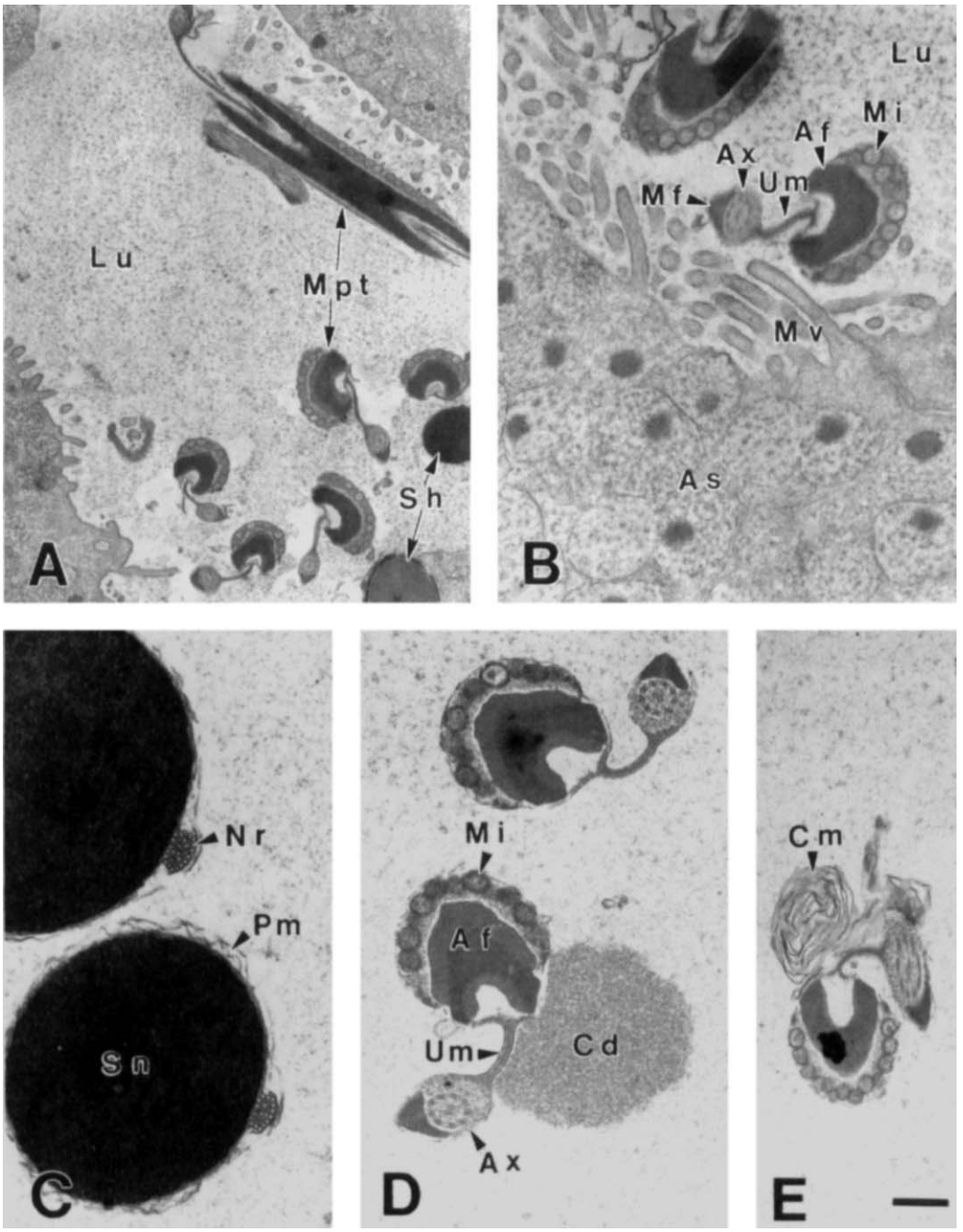

Fig. 5. *Ambystoma opacum*. Sperm in the lumina of spermathecae of females. **A:** Lumen and apical spermathecal cytoplasm of a female collected October 6 while tending eggs and sacrificed October 9. Note that sperm are not all in the same orientation. **B:** Same specimen as in A, showing details of sperm cytology and adjacent spermathecal epithelium. The flocculent material from the apical secretion (As) surrounds the sperm in the lumen. **C:** Specimen collected September 20 and sacrificed October 9 prior to oviposition, showing nuclear region of the head of luminal sperm cells. Note that the plasma membrane (Pm), although fluctuated, is intact. **D:** Same specimen as in C, showing what may be the remanents of a cytoplasmic droplet (Cd) associated with the undulating membrane (Um) of the middle piece of the tail. **E:** Same specimen as in C and D, showing an structure composed of concentric membranes (Cm) associated with the middle piece of the tail. Bar = 885 nm for A, 463 nm for B, 178 nm for C, 333 nm for D, and 476 nm for E. Af, axial fiber of a spermatozoon; As, apical secretion; Ax, axoneme of a spermatozoon; Cd, cytoplasmic droplet; Cm, concentric membranes; Lu, lumen; Mf, microfilament; Mi, mitochondria; Mpt, middle piece of the tail of a spermatozoon; Mv, microvilli; Pm, plasma membrane; Sh, head of a spermatozoon; Um, undulating membrane of a spermatozoon.

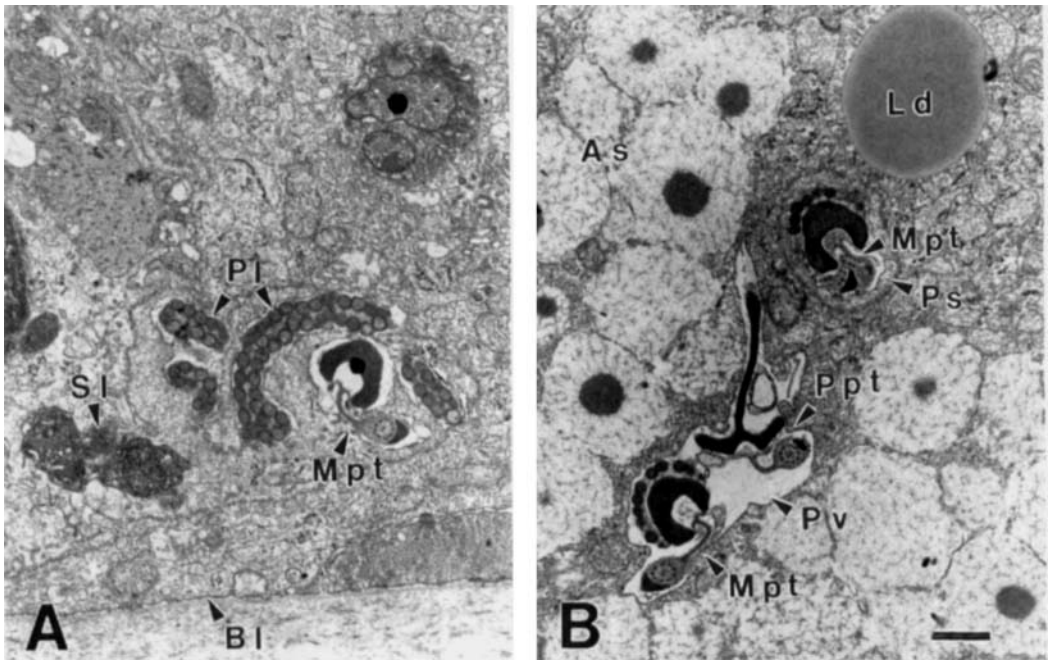

Fig. 6. *Ambystoma opacum.* Spermiophagy by the spermathecal epithelium of a female collected September 20 and sacrificed October 9 prior to oviposition. **A:** Basal epithelium, showing primary (Pl) and secondary (Sl) lysosomes and an embedded portion of the middle piece of the tail of a spermatozoon (Mpt). **B:** Apical secretion and lipid droplet associated with portions of embedded sperm cells. Bar = 595 nm for A and 583 nm for B. As, apical secretion; Bl, basal lamina; Ld, lipid droplets; Mpt, middle piece of the tail of a spermatozoon; Pl, primary lysosome; Ppt, principle piece of the tail of a spermatozoon; Ps, phagosome; Pv, phagocytic vacuole; Sl, secondary lysosome.

tion, so an accurate assessment of its spermathecal anatomy is precluded. However, no sperm are apparent in the spermathecae of this specimen.

The specimen collected October 6 while tending her recently oviposited eggs and sacrificed January 6, 62 days after collection, has a markedly different spermathecal cytology. Cytoplasm is scant, since the euchromatic nuclei are extremely large, occupying most of the cells (Fig. 7A,B). As opposed to the case in the specimens sacrificed in September and October, nucleoli are prominent, and intercellular canaliculi are wide. The only evidence of secretory activity is scattered lipid droplets, which are not as electron dense as those in females from previous months. The lipid droplets are associated with the nuclei and a small amount of Ser (Fig. 7C). A leukocyte (either a macrophage or a neutrophil) occurs among the spermathecal epithelial cells in sections of one tubule (Fig. 7D). Interepithelial leukocytes were not observed in any other specimen.

DISCUSSION

The secretory activity of the spermathecal epithelium of female *Ambystoma opacum* differs in several respects from that reported for other salamanders. In other species (Dent, '70; Boisseau and Joly, '75; Brizzi et al., '89; Sever, '91b), only an apical secretion for export into the lumen has been reported, and this product consists of uniformly electron-dense granules rather than vacuoles composed largely of flocculent material. Despite differences in appearance of the apical secretory vacuoles, the consensus from various studies is that the product is a complex carbohydrate. Some variation occurs, however, in whether this substance is neutral, negatively charged, or a mixture of different carbohydrates.

The apical secretion in *Ambystoma opacum* is PAS-negative, contraindicating neutral carbohydrates, and is Alcian blue-positive at pH 2.5, indicating GAGs. Boisseau and Joly ('75) found both PAS-positive and

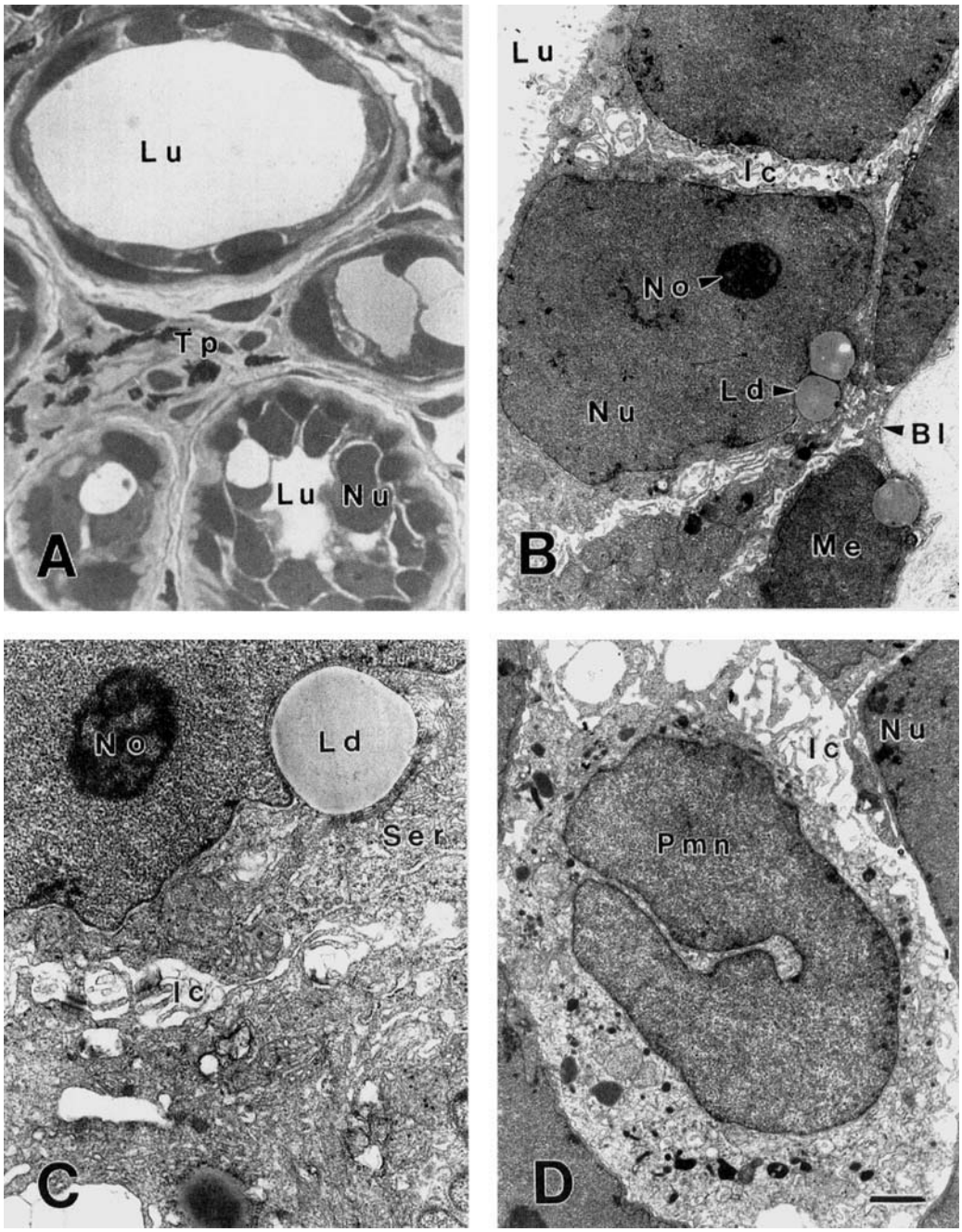

Fig. 7. *Ambystoma opacum*. Spermathecae of a female collected October 6 while tending eggs and sacrificed January 6, 62 days after collection. **A:** Plastic thick section stained with toluidine blue through spermathecae and surrounding connective tissue (Tp). **B:** Overview of spermathecal epithelium and myoepithelium. Note large nuclei (Nu), prominent nucleoli (No), scant cytoplasm, and broad intercellular canaliculi (Ic). **C:** Detail of lipid droplet (Ld) and associated cytological structures. **D:** An interepithelial leukocyte (Pmn), probably a macrophage or a neutrophil. Bar = 15 μm for A, 1.75 μm for B, 555 nm for C, and 133 nm for D. Bl, basal lamina; Ic, intercellular canaliculi; Ld, lipid droplets; Lu, lumen; Me, myoepithelial cell nucleus; No, nucleolus; Nu, nucleus of spermathecal epithelial cell; Pmn, nucleus of leukocyte; Tp, tunica propria.

low-pH Alcian blue-positive reactions in *Salamandra* and *Pleurodeles* but only PAS-positive reactions in *Taricha* and *Hydromantes*. For *Eurycea quadridigitata,* Pool and Hoage ('73) reported that the secretion is PAS-negative and toluidine blue-positive (an indicator of highly carboxylated carbohydrates), but their results with Alcian blue were "inconclusive." Brizzi et al. ('89), for *Salamandrina terdigitata,* found reactions were strongly PAS-positive, Alcian blue-positive, and toluidine blue-positive and also weakly ninhydrin-Schiff-positive for proteins. Brizzi et al. ('89) suggested the presence of proteoglycans (GAGs linked with proteins), and the association of Rer with the apical secretory vacuoles in *A. opacum* indicates the possibly of proteoglycans as well. In an abstract, Davitt and Larsen ('90) reported that "the plethodontid salamander" has several different types of secretory cells that possess PAS-positive and Alcian blue-positive material. Sever ('91b) reported PAS-positive and Alcian blue-negative apical secretions in *Eurycea cirrigera.* Dent ('70), for *Notophthalmus viridescens,* and Pool and Hoage ('73), for *E. quadridigitata,* reported glycogen in the epithelium.

The function of the apical secretion is unknown. GAGs are abundant in the matrix of connective tissues surrounding collagen fibers, where they bind both appropriately charged ions and water (Holtzman and Novikoff, '84). Some GAGs and proteoglycans form viscous fluids, such as those in joint capsules (Holtzman and Novikoff, '84). GAGs also are known from various male cloacal glands involved in sperm transport (Sever, '91a, '92a), and the cap of the spermatophore that holds sperm during insemination contains PAS-positive and Alcian blue-positive material (Zalisko et al., '84).

Several authors have proposed that the apical secretion provides nourishment for sperm during their storage in the lumen (Dent, '70; Boisseau and Joly, '75), but the mechanism by which sperm could use GAGs and proteoglycans for nutritive purposes has not been elucidated. Sperm are immobile during their storage in the spermathecae (Hardy and Dent, '86a), and we suggest that, rather than providing nutrition, the apical secretion simply provides the chemical/osmotic environment necessary for sperm quiescence.

No lipid secretion, or indeed any secretion, produced for export into the surrounding connective tissue has been reported previously in salamander spermathecae. The male vas deferens also is a site for sperm storage, however, and Zalisko and Larsen ('90) reported the presence of lipid droplets in the epithelium of the vas deferens of *Ambystoma macrodactylum*. The lipid droplets in male *A. macrodactylum* are produced in the connective tissue surrounding the vas deferens and migrate into the epithelium, where they serve as an endogenous energy source (Zalisko and Larsen, '90). The lipid droplets in female *Ambystoma opacum* clearly are produced by the spermathecal epithelium, and the association with Rer indicates that the product actually is a lipoprotein. Some lipid droplets are associated with mitochondria, and perhaps have a role in endogenous energy production (such as in triglyceride metabolism), but the basal nature of droplet formation and the presence of dense material in the basal lamina constitute evidence that the lipid material is exported into the surrounding tunica propria. Demonstration of this mode of secretion is difficult, since lipids, lacking unit membranes, are secreted not by exocytosis (membrane fusion) but by a direct and outward diffusion across plasma membranes.

The chemical composition of the lipid is under further study. The extreme osmiophilic appearance of the droplets indicates a high degree of unsaturation. If the droplets are not composed completely of lipoproteins, other intriguing possibilities are that the droplets contain steroids or prostaglandins. Prostaglandins probably mediate contraction of the spermathecal myoepithelium during sperm expulsion (Hardy and Dent, '87).

Sperm are not as abundant or evenly distributed in the spermathecal tubules of *Ambystoma opacum* as reported for the plethodontid *Eurycea cirrigera* (Sever, '91b, '92b). The spermathecae of *E. cirrigera,* as with those of other plethodontids, are compound alveolar glands. The alveoli and neck tubules connect to a single common tube that opens onto the roof of the anterior end of the cloaca. In contrast, the spermathecae of *A. opacum* are numerous, simple glands whose pores occupy a wide area of the roof and lateral walls of the cloaca. Hardy and Dent ('86b) proposed that passive sperm are drawn into the female cloaca by contractions of the smooth muscle of the cloaca and that sperm subsequently move thigmotactically along the cloacal epithelium. Upon encountering the openings of the spermathecae, sperm are carried through the pores by thigmotaxis (Hardy and Dent, '86b). Perhaps the greater concentration of sperm in the spermathecae of *E. cirrigera* is a consequence of the great difference in number of possibilities for se-

questering sperm; i.e., in *A. opacum,* pores are numerous so that sperm are more widely scattered, while, in *E. cirrigera,* all sperm enter one orifice.

Some structures associated with sperm in the spermathecal lumen of *A. opacum* may be cytoplasmic droplets or fragments of such droplets. Russell et al. ('81) reported that cytoplasmic droplets in *Ambystoma texanum* become detached in the spermatophore. Davitt and Larsen ('88b) stated that, in *Rhyacotriton olympicus,* cytoplasmic droplets are shed in the spermathecae and are phagocytosed by the spermathecal epithelium.

No evidence of phagocytosis of cytoplasmic droplets was found in the current study, and, indeed, only infrequently were sperm found embedded in the epithelium and undergoing degradation in *A. opacum.* In contrast, the distal bulbs of the spermathecae of *Eurycea cirrigera* are highly modified for intense spermiophagy after oviposition (Sever, '92b; Sever and Brunette, '93). These differences could result from the amount of sperm present in the spermathecae. The spermathecae of *A. opacum* are simple tubules from which excess sperm may simply "leak out" gradually after oviposition and/or be expulsed by myoepithelial contractions, as has been reported for sperm remaining after the breeding season in the vas deferens of *A. macrodactylum* (Zalisko and Larsen, '89).

For sperm to leak out or be expelled from the distal bulbs of *Eurycea cirrigera,* they need to pass down neck tubules and a common tube. Thus perhaps spermiophagy by the distal bulbs is a more advantageous way to dispose of remaining sperm in *E. cirrigera.* Sperm are quiescent while in the spermathecae, and some energy expenditure for myoepithelial contractions would be necessary to expulse the sperm forcefully. In *A. opacum,* lipid droplets are still being exported into the surrounding connective tissue after oviposition, and perhaps these droplets are involved in myoepithelial contraction, as either an energy source or a prostaglandin that triggers sperm discharge (Hardy and Dent, '87). Lipid droplets are uncommon in the spermathecal epithelium of *E. cirrigera* (Sever, '91b, '92b).

Interepithelial leukocytes have not been reported before from the spermathecae of salamanders. However, invasion of phagocytic leukocytes has been reported as a method of sperm removal in the female reproductive tracts of many species of mammals, including hamsters (Yanagimachi and Chang, '63), bats (Mori and Uchida, '80), rabbits (Moyer et al., '65), and cats (Murakami et al., '85). The specimen of *A. opacum* in which the leukocyte was observed lacked sperm in the spermathecae, which were in an inactive secretory state as well. Thus the role of leukocytes in the spermathecal epithelium of *A. opacum* remains to be determined.

Some of the differences between the spermathecal morphology of *A. opacum* and other salamanders are of interest from a phylogenetic standpoint. Morphological studies at the gross and light microscopy levels indicate that the glands responsible for internal fertilization in the Salamandroidea are homologous and evolved in ancestor common to the suborder (Sever, '91a). Molecular studies, on the other hand, support the hypothesis that internal fertilization and the structures involved have evolved twice and/or have been secondarily lost in the ancestors to some families (Larson, '91). When morphological and molecular characters are combined, the result is a hypothesis of monophyly for internal fertilization in salamanders (Hillis, '91; Larson and Dimmick, '93), but this hypothesis is not particularly robust since it is largely a consequence of considering the cloacal glands homologous (Larson and Dimmick, '93).

The spermathecae of *Ambystoma opacum* and *Eurycea cirrigera* may share a developmental program only to the limited degree that the numerous tubules of *A. opacum* and the common tube of *E. cirrigera* arise from invaginations of the cloacal lining. It is difficult to conceive a common ancestral embryonic condition from which the opposing states of many tubules (ambystomatids) vs. a common tube (plethodontids) could develop. Differences in development and ultrastructure between the spermathecae of *A. opacum* and *E. cirrigera* may be evidence for the independent origin of sperm storage glands in ambystomatids and plethodontids.

The spermathecae in the Salamandridae, like those of the Ambystomatidae, consist of numerous simple tubules around the dorsal and lateral walls of the cloaca. The condition of possessing numerous tubules may result from convergent development paradigms and not from continuity with a common ancestor possessing this character state. Differences occur in secretory products between the salamandrids examined so far and *Ambystoma opacum.* Homology, therefore, cannot be assumed between the spermathecae of salamandrids and ambystomatids.

Thus the presumptive cloacal tissue has the capability for gland development, and selective pressures for sperm storage could

have resulted in glands for this purpose arising independently in different lineages. Based on female spermathecal morphology, the hypothesis of polyphyly for sperm storage organs in salamanders remains viable.

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