Sperm Storage in the Oviduct of the Internal Fertilizing Frog *Ascaphus truei*

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ABSTRACT This study provides the first descriptions of sperm storage at the tissue and cellular levels in a female frog or toad. Oviducal anatomy was studied by light and electron microscopy in *Ascaphus truei* from north coastal California. *Ascaphus truei* is one of the few species of anurans in which fertilization is internal. Unlike other anurans with internal fertilization, however, mating in *A. truei* consists of a unique combination of amplectic and copulatory mechanisms that we term "copulexus." Posterior to a short, aglandular infundibular region, the oviduct possesses: 1) a proximal, convoluted ampullary region where intrinsic tubular glands secrete gelatinous envelopes around eggs; 2) a middle ovisac region where fertilization occurs; and 3) a distal oviducal sinus formed by medial junction of the ovisacs. Sperm storage tubules (SSTs) occur in the anterior portions of the ovisacs and consist of simple tubular glands. SSTs and the rest of the oviducal lining stain positively with the periodic acid-Schiff's procedure for neutral carbohydrates and this reaction is especially intense in reproductively active females. Sperm were found in the SSTs of gravid females as well as some nonvitellogenic females. The sperm are in orderly bundles in the SSTs, and although occasionally sperm nuclei were embedded in the epithelium, no evidence for spermiophagy was found. Oviducal sperm storage in *A. truei* is homoplastic, with closest structural similarities to squamate reptiles. Oviduct/sperm design constraints appear to limit the options for expression of features associated with oviducal sperm storage. J. Morphol. 248:1–21, 2001.

KEY WORDS: Anura; *Ascaphus truei*; reproduction; sperm storage; ultrastructure

The tailed frog *Ascaphus truei* Stejneger (1899) is the sole member of the family Ascaphidae and is generally considered the sister taxon of all other anurans (Ford and Cannatella, 1993). *Ascaphus truei* is associated with cold, clear mountain streams in disjunct populations in the Cascade Mountains west to the coast from southern British Columbia to northwest California, in the Blue Mountains of southwestern Washington and northeastern Oregon, and in the Rocky Mountains of northern Idaho and western Montana (Metter, 1968). Of the nearly 5,000 species of anurans, *A. truei* is the only species known to engage in copulation that includes intromission. The male possesses a "tail" that, when engorged, forms a sulcus for passage of sperm and is inserted in the cloaca of the female (Noble, 1925; Noble and Putnam, 1931; Slater, 1931). Copulation has been assumed to be an adaptation that ensures fertilization in fast-moving water (Stebbins and Cohen, 1995).

The presence of sperm in the lumen of the oviducts and in oviducal glands of female *Ascaphus truei* was first reported by Noble (1925). Noble (1925) apparently examined sections of the oviduct prepared for light microscopy but the histology of the oviduct and stored sperm was not described in any detail. Metter (1964a,b) suggested periods of 1–2 years of sperm storage in female *A. truei* and briefly described the gross anatomy of the oviduct and location of sperm storage, but once again did not provide any histological details. In this article we provide the first descriptions using light and electron microscopy of the oviduct and sperm storage in *A. truei*, the only frog in which female sperm storage is known to occur.

MATERIALS AND METHODS

All specimens of *Ascaphus truei* were collected from the North Fork Mad River watershed on lands owned by the Simpson Timber Company in western Humboldt County, California, in the north coast redwood (*Sequoia sempervirens*) zone (Diller and Wallace, 1994). Although *A. truei* is not listed under either the federal or state of California endangered species acts, it is considered of special concern due to its habitat requirements and sensitivity to land management activities. Thus, we sought to minimize the number of animals collected for this study. Permits for the collection of specimens were granted

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TABLE 1. Specimens utilized in this study[1]

		Follicles				Sperm[2]		
Date	SVL	N	Range	Mean	SE	Am	Ov	Os
10 April	47.4	47	2.3–3.0	2.6	0.07	0	0	0
10 April	47.1	67	2.3–2.9	2.6	0.06	0	0	0
10 April	42.3	25	0.8–0.9	0.8	0.01	0	0	0
18 June	52.5	64	2.0–3.7	2.8	0.06	0	+	+
18 June	49.4	71	0.5–1.6	1.2	0.03	0	+	0
12 July	48.1	15	1.1–1.5	1.4	0.04	0	+	0
12 July	47.2	63	2.8–3.9	3.4	0.08	0	+	0
7 Nov	43.2	70	1.0–1.6	1.3	0.05	0	0	0
7 Nov	38.5	42	0.6–0.8	0.6	0.02	0	0	0

[1]All measurements are in mm.
[2]Absence (0) or presence (+) of sperm in the ampulla (Am), ovisac (Ov), or oviducal sinus (Os).

to L.V. Diller from the California Department of Fish and Game.

Females of *Ascaphus truei* were collected during four periods: 8–15 June, 5–6 July, 24 October 1999, and 22–29 March 2000. Specimens were collected at night from along the border of streams. The frogs were shipped to Saint Mary's College where the specimens were killed within a week of receipt (Table 1) and oviducal tissue was removed and prepared for microscopic examination.

Specimens were killed by immersion in 10% MS-222 (3-aminobenzoic acid ethyl ester), and snout–vent length (SVL) was measured from the tip of the snout to the posterior end of the cloacal orifice. The reproductive tracts and cloacae were removed from freshly killed specimens and prepared for light microscopy (LM) and transmission electron microscopy (TEM). Carcasses of all specimens were preserved in 10% neutral buffered formalin (NBF) and are housed in the research collections at Saint Mary's College.

For LM examination, some tissues were initially fixed in 10% NBF, rinsed in water, dehydrated in ethanol, cleared in toluene, and embedded in paraffin or glycol methacrylate (JB-4 Plus; Electron Microscopy Sciences, Fort Washington, PA) plastic resin. Paraffin sections (10 μm) were cut with a rotary microtome and affixed to albuminized slides. Alternate paraffin slides from each specimen were stained with hematoxylin-eosin (general histology), brilliant indocyanine 6B (BB, for proteins), and Alcian blue 8GX at pH 2.5 (AB, for primarily carboxylated glycosaminoglycans), followed by the periodic acid-Schiff's procedure (PAS, for neutral carbohydrates and sialic acids). Sections (2 μm) from tissues embedded in JB4 were stained with methylene blue and basic fuchsin. Sections also were cut from tissues frozen at −60°C after NFB fixation. The frozen sections (15 μm) were cut with an AO Cryo-Cut II (American Optics, Buffalo, NY) and stained with Sudan black (SB). Procedures followed Dawes (1979), Humason (1979), and 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 and 3.7% formaldehyde in cacodylate buffer, pH 7.2. After initial fixation, tissues were rinsed in distilled-deionized water, 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). Plastic sections were cut with an RMC MT7 ultramicrotome (Research and Manufacturing Co., Tucson, AZ) and DiATOME (Biel, Switzerland) diamond knives. Semithin 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 solutions of uranyl acetate and lead citrate. Ultrathin sections were viewed with a Hitachi H-300 transmission electron microscope (Nissei Sangyo America, Mountain View, CA).

RESULTS

Regions of the Oviduct

Oviducal terminology, except where noted, follows Sever et al. (1996a) and Wake and Dickie (1998). Regions of the oviduct of *Ascaphus truei* are illustrated in Figures 1 and 2. The ostial opening leads into the infundibulum, which is a narrow, thin-walled tube that extends from posterior to the transverse septum to the anterior border of the kidney. The infundibulum connects to the ampulla, which is lateral to the anterior portion of the kidneys. The ampulla is thick-walled, sinuous, and contains exocrine glands involved in the deposition of outer gelatinous egg envelopes (Wake and Dickie, 1998). Distal to the ampulla is the straight ovisac, called the "uterus" by Metter (1964b). In the anterior portion of the ovisac are exocrine glands that serve as sperm storage tubules (SSTs). The ampulla and the ovisac are the only regions of the oviduct that possess intrinsic tubular exocrine glands (Fig. 2). The two ovisacs join medially dorsal to the urinary bladder to form an *oviducal sinus* (Fig. 1). The only other anuran in which an oviducal sinus has been noted is the viviparous African bufonid *Nimbaphrynoides occidentalis* by Xavier (1973). This area is clearly anterior to the cloaca, which is dorsal to the pubic symphysis. The oviducal sinus is not "urogenital," because the Wolffian ducts and urinary bladder empty into the cloaca, along with wastes from the colon.

Reproductive Condition of the Sample

Mating in this population has been observed in the field only during May, although males with highly developed secondary sex characters have been observed in June and July. In addition, males captured and brought into captivity in April were

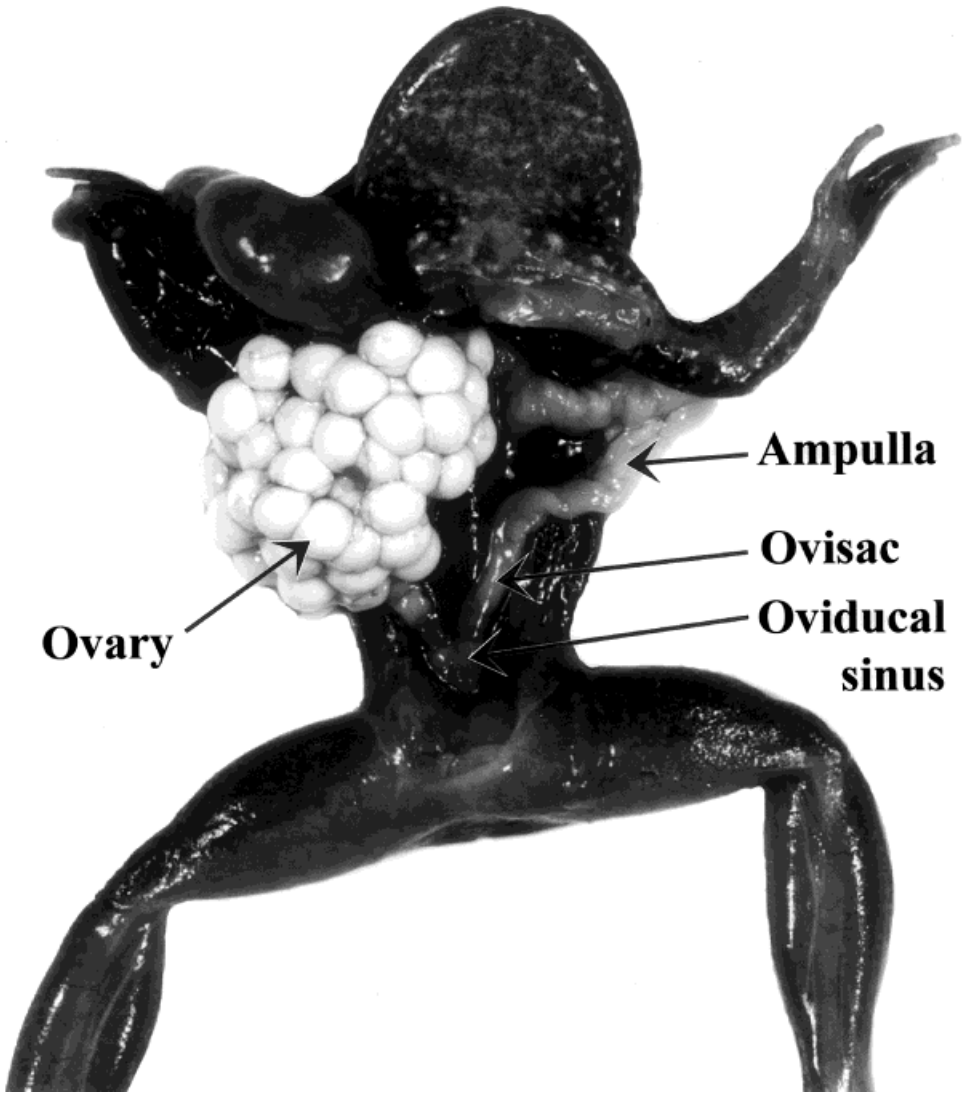

Fig. 1. Female *Ascaphus truei*. Oviduct of a 52.5 mm SVL specimen sacrificed 18 June. Right ovary removed.

observed to grasp females in amplexus and unsuccessfully attempt copulation.

Two of the four females collected in June and July for this study have large, vitellogenic eggs (2.8–3.4 mm mean dia.; Table 1), and one female was killed while in amplexus and copulation with a male. The combination of amplectic and copulatory mechanisms resulting in internal fertilization in *Ascaphus truei* is unique among anurans, and we term this act "copulexus." The ampullae of these females are hypertrophied and highly convoluted. These females are considered to be in active reproductive condition for the current breeding season. The other two females have small ovarian follicles (1.2–1.4 mm mean dia.) and their ampullae are relatively narrow and unconvoluted. These females clearly are not in the same stage of active reproductive readiness as the two females with large ovarian follicles. All four females from June and July, however, have sperm in their SSTs, and the female in copulexus also has sperm in the oviducal sinus (Table 1).

The two females examined from the November collection appear reproductively inactive, with small ovarian follicles (0.6–1.3 mm mean dia.) and narrow, unconvoluted oviducts. Two of the females sacrificed in April have numerous vitellogenic follicles nearly as large (2.6 mm mean dia.) as those of gravid females from June and July (Table 1). These females also possess hypertrophied ampullary regions of the oviduct. The other female from the April sample has small ovarian follicles (0.8 mm mean dia.) and regressed oviducts. None of the females sacrificed in November and April possess sperm in their oviducts.

Females Sacrificed in Summer in Active Breeding Condition

As noted above, two females sacrificed in summer have large ovarian follicles (2.8–3.4 mm mean dia.) and are considered reproductively active in a postmating and preovulatory condition. One of the fe-

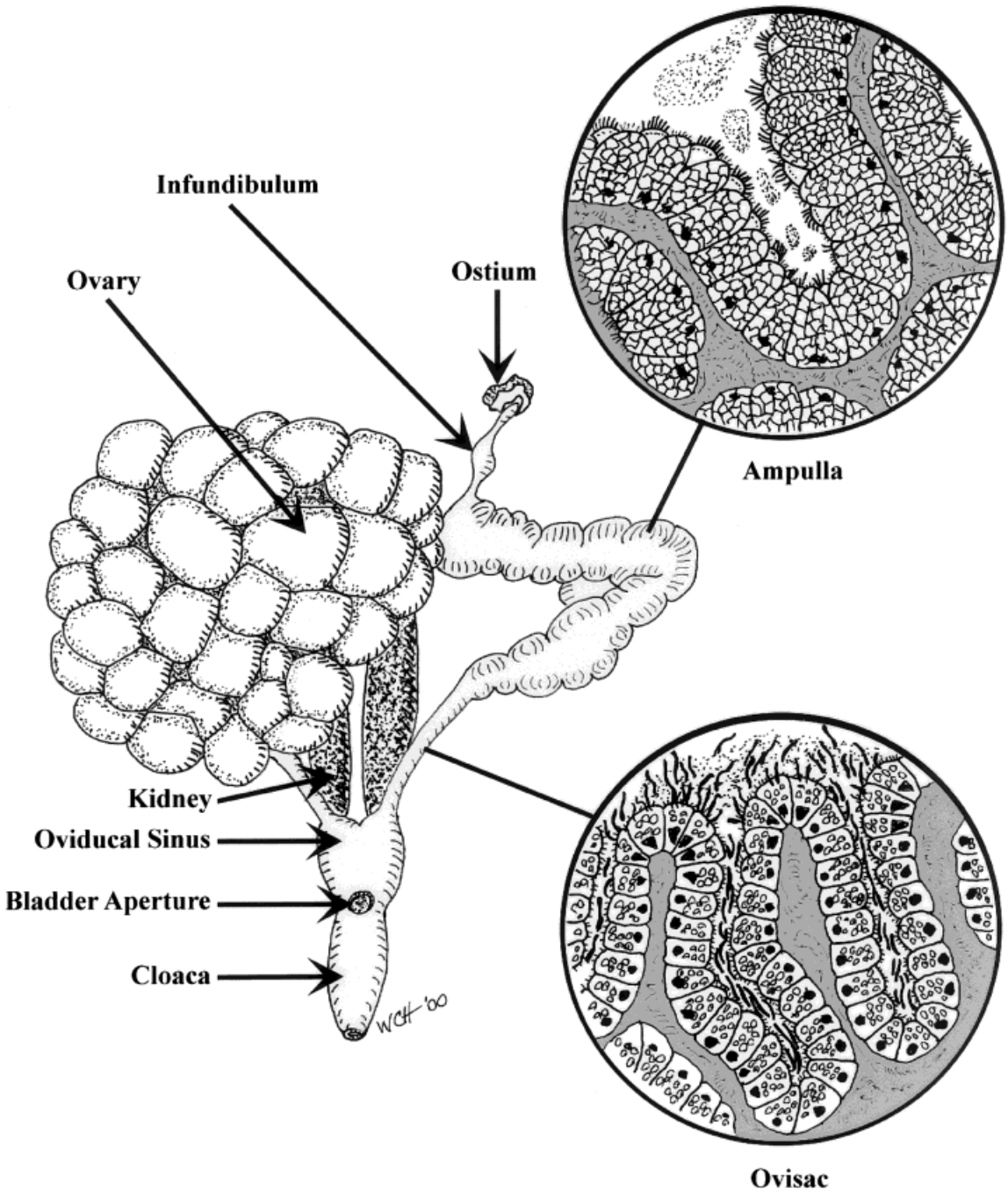

Fig. 2. Female *Ascaphus truei*. Drawing of the oviduct of the same specimen used in Figure 1, illustrating the histology of the ampulla and ovisac.

males was killed while in copulexus. These females both possess sperm in SSTs in the ovisac and are similar in other aspects of oviducal anatomy except for some features of the oviducal sinus. In the female in copulexus, sperm also are present in the oviducal sinus and epithelial sloughing occurs in the oviducal sinus (absent in the other female). Sections through the infundibulum are illustrated in Figure 3, the ampulla in Figure 4, the ovisac in Figures 5–6, and the oviducal sinus in Figures 7–8.

Infundibulum. The infundibulum is relatively thin-walled; the lining is rather smooth anteriorly but becomes rugose posteriorly toward the ampulla (Fig. 3A). The infundibulum possesses three basic layers found throughout the oviduct. The most superficial layer is the visceral pleuroperitoneum com-

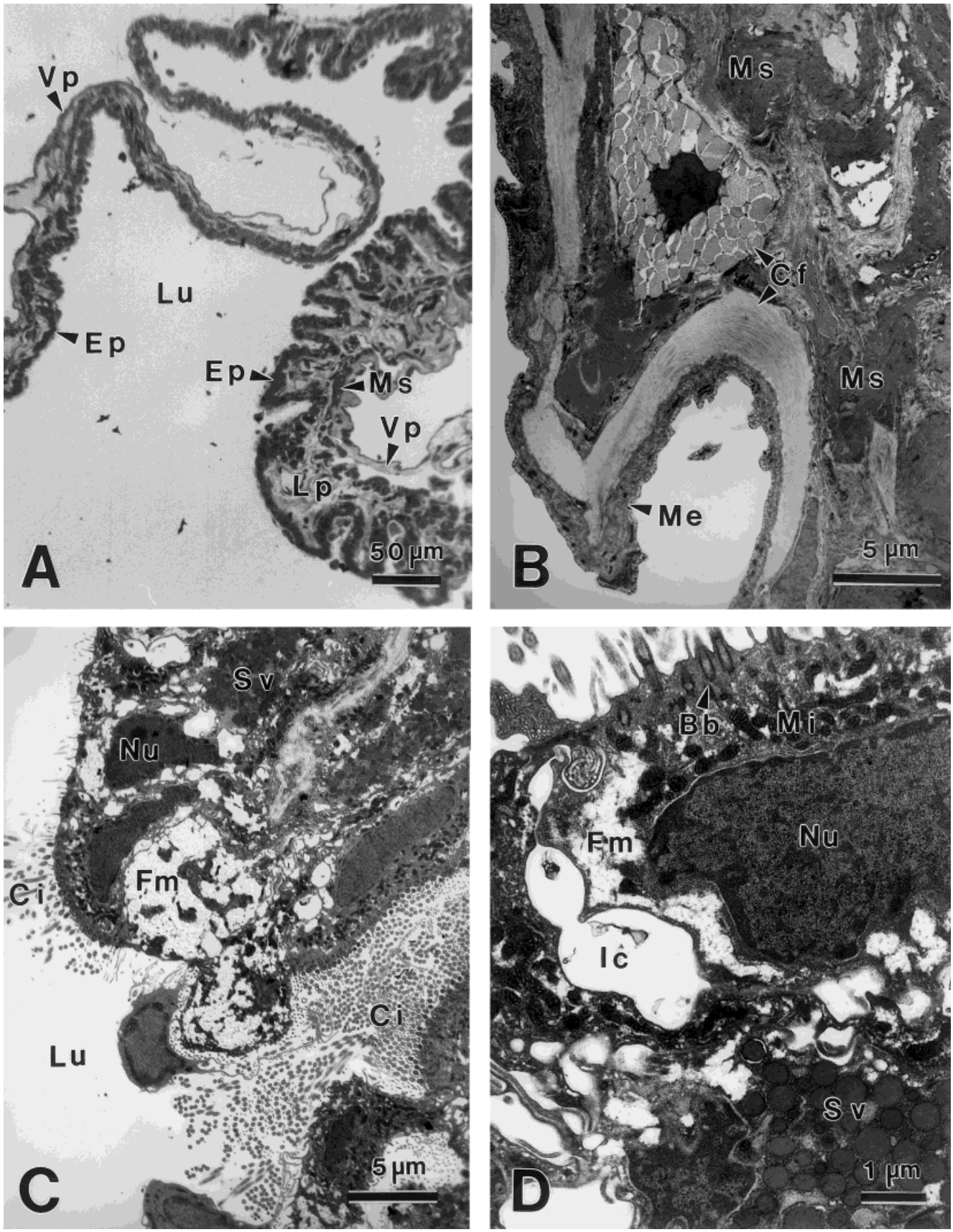

Fig. 3. Female *Ascaphus truei*. Light micrograph (**A**) and transmission electron micrographs of the infundibulum of a 47.2 mm SVL vitellogenic female sacrificed 12 July. **A:** Tissue layers of the infundibulum. **B:** Visceral pleuroperitoneum and muscularis. **C:** Luminal border. **D:** Adjacent ciliated and secretory cells. Bb, basal bodies; Cf, collagen fibers; Ci, cilia; Ep, epithelium; Fm, flocculent material; Ic, intercellular canaliculus; Lp, lamina propria; Lu, lumen; Me, mesothelium; Mi, mitochondria; Ms, muscularis; Nu, nuclei; Sv, secretory vacuoles; Vp, visceral pleuroperitoneum.

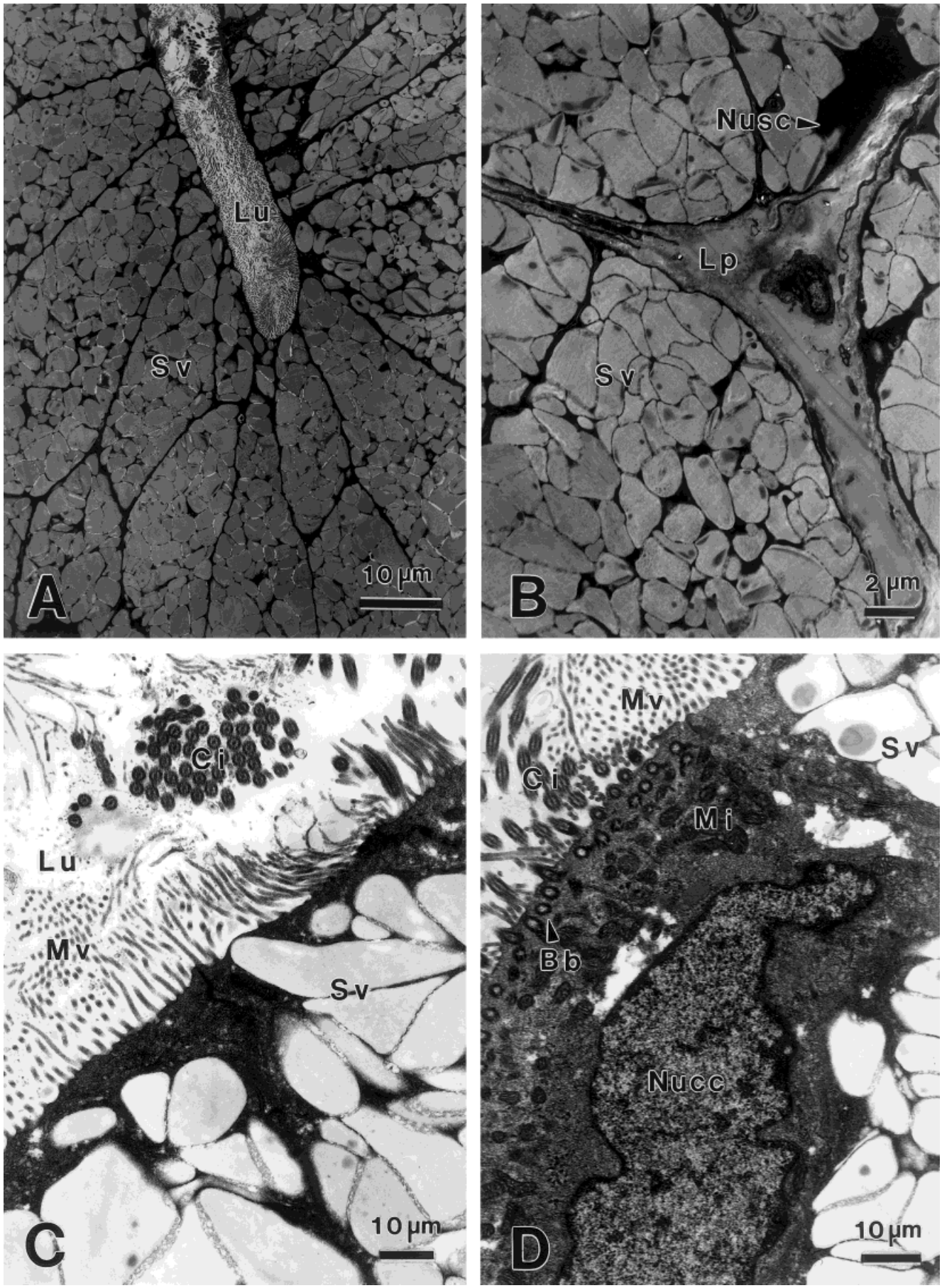
Lu
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Figure 4.

posed of simple squamous mesothelium and a thin, subsurface tunica propria of connective tissue proper that features a dense submesothelial layer of collagen fibers (Fig. 3B). The middle muscularis layer is thin in the infundibulum and is composed primarily of longitudinal smooth muscle fibers. Elsewhere in the oviduct both a superficial circular and a deep longitudinal layer are apparent, with these most prominent in the ovisac (Fig. 5A) and oviducal sinus (Fig. 12A). The deepest layer is the mucosa, composed of the epithelium lining the inner walls of the oviduct and the subsurface connective tissue of the lamina propria. The mucosa, especially the epithelium and intrinsic exocrine glands derived from the epithelium, shows the most variation throughout the oviduct and is the focus of the ultrastructural observations.

In the infundibulum, the mucosal epithelium is simple squamous to cuboidal, with irregular, densely staining nuclei (Fig. 3C). Cells with elongate cilia are numerous and interspersed among the ciliated cells are secretory cells characterized by apical microvilli. Ciliated cells often appear pyramidal in shape, with the broadest aspect in a luminal position and the truncated region projecting away from the lumen. The ciliated cells possess numerous supranuclear mitochondria and contain vacuoles, some of which appear empty, as well as others that contain a flocculent material (Fig. 3C,D). Intercellular canaliculi exhibit broad gaps between cells (Fig. 3D) that narrow to tight junctions apically and become labyrinthine basally. The secretory cells also contain the flocculent inclusions as well as dark, uniformly electron-dense secretory vacuoles (Fig. 3C,D) that stain intensely PAS+ and are AB− and BB− in paraffin sections.

Ampulla. The ampulla of the reproductively active but preovulatory females contain greatly hypertrophied oviducal glands (Fig. 4). These exocrine glands are so numerous, crowded, and enlarged that they obliterate any gaps between one another and compress the lamina propria (Fig. 4B) and muscularis into thin layers barely discernible with light microscopy.

Electron microscopy, however, reveals that two types of epithelial cells are still present: secretory and ciliated cells. The secretory cells are pyriform, with the base being the widest (Figs. 2, 4A). They have densely staining, irregular basal nuclei (Fig. 4B). The secretory vacuoles also are irregular in outline and their contents usually are uniform and moderately electron-dense, although occasionally a darker, eccentric circular area occurs in the vacuole (Fig. 4B,C). The vacuoles stain intensely PAS+ and are AB− and BB−. The apices of the secretory cells have microvilli and the unit membrane of the secretory vacuoles appears in places to be in contact with the plasmalemma of the apical epithelium (Fig. 4C), perhaps indicating the initial phase of merocrine secretion of the contents. No eggs, however, were in the ampulla and secretory products were not being elaborated from the vacuoles. The ciliated cells are small squamous cells squeezed between the apices of the secretory cells (Fig. 4D). The ciliated cells have irregular euchromatic nuclei that occupy much of the cytoplasm and numerous supranuclear mitochondria.

Ovisac. The upper portion of the ovisac is also characterized by the presence of intrinsic exocrine glands, but these glands are not packed with the irregular, moderately electron-dense secretory vacuoles found in the ampulla. The glands are simple tubular or simple branched tubular (Fig. 5A) and serve as SSTs. The lower portion of the ovisac lacks tubular glands. The lining of the SSTs and the luminal epithelium of the oviduct is simple columnar epithelium and consists of secretory cells with microvilli interspersed among ciliated cells (Fig. 5B,C). Intercellular canaliculi are wide, especially among groups of ciliated cells, and tortuous basally (Fig. 5B). Circular vacuoles of varying sizes and electron densities occur in secretory cells and are especially numerous and variable in size in the distal portions of the SSTs (Fig. 5D). These vacuoles are intensely PAS+ and are AB− and BB−. Both ciliated and secretory cells often possess vacuoles containing a flocculent material, especially in the epithelium of the oviducal lining (Figs. 5B, 6A).

Sperm are present in the lumen of the oviduct and the SSTs (Figs. 5, 6). Sperm in the lumen usually were loosely aggregated (Figs. 5B, 6A) and small clusters of sperm show similar orientations (i.e., similar sections through nuclei, tails aligned). Occasionally groups of sperm in the oviducal lumen are found embedded in an acellular matrix composed of a uniformly electron-dense substance and small vesicles (Fig. 6B). Sperm are also frequently found embedded in the secretory epithelial cells, especially in the SSTs (Fig. 6C,D). Sperm nuclei sometimes are found deep in these cells, in the infranuclear cytoplasm bordering the basal lamina (Fig. 6C). The nuclei are not vacuolated, appear normal in cytology, and often are in close proximity to secretory vacuoles (Fig. 6D).

Oviducal sinus. The epithelium of the oviducal sinus is rugose and consists of stratified cuboidal epithelium and lacks cilia and intrinsic tubular glands (Fig. 7A). A thick layer of collagen fibers occurs in the lamina propria superficial to the epithelium (Fig. 7A), and the muscularis is relatively wider than in other regions. In the female that was not in the act of mating when sacrificed, the apical

Fig. 4. Female *Ascaphus truei*. Electron micrographs of the ampulla of 47.2 mm SVL vitellogenic female sacrificed 12 July. **A:** Luminal border. **B:** Basal border. **C:** Secretory cell. **D:** Ciliated cell. Bb, basal bodies; Ci, cilia; Lp, lamina propria; Lu, lumen; Mi, mitochondria; Mv, microvilli; Nucc, nucleus of a ciliated cell; Nusc, nucleus of a secretory cell; Sv, secretory vacuoles.

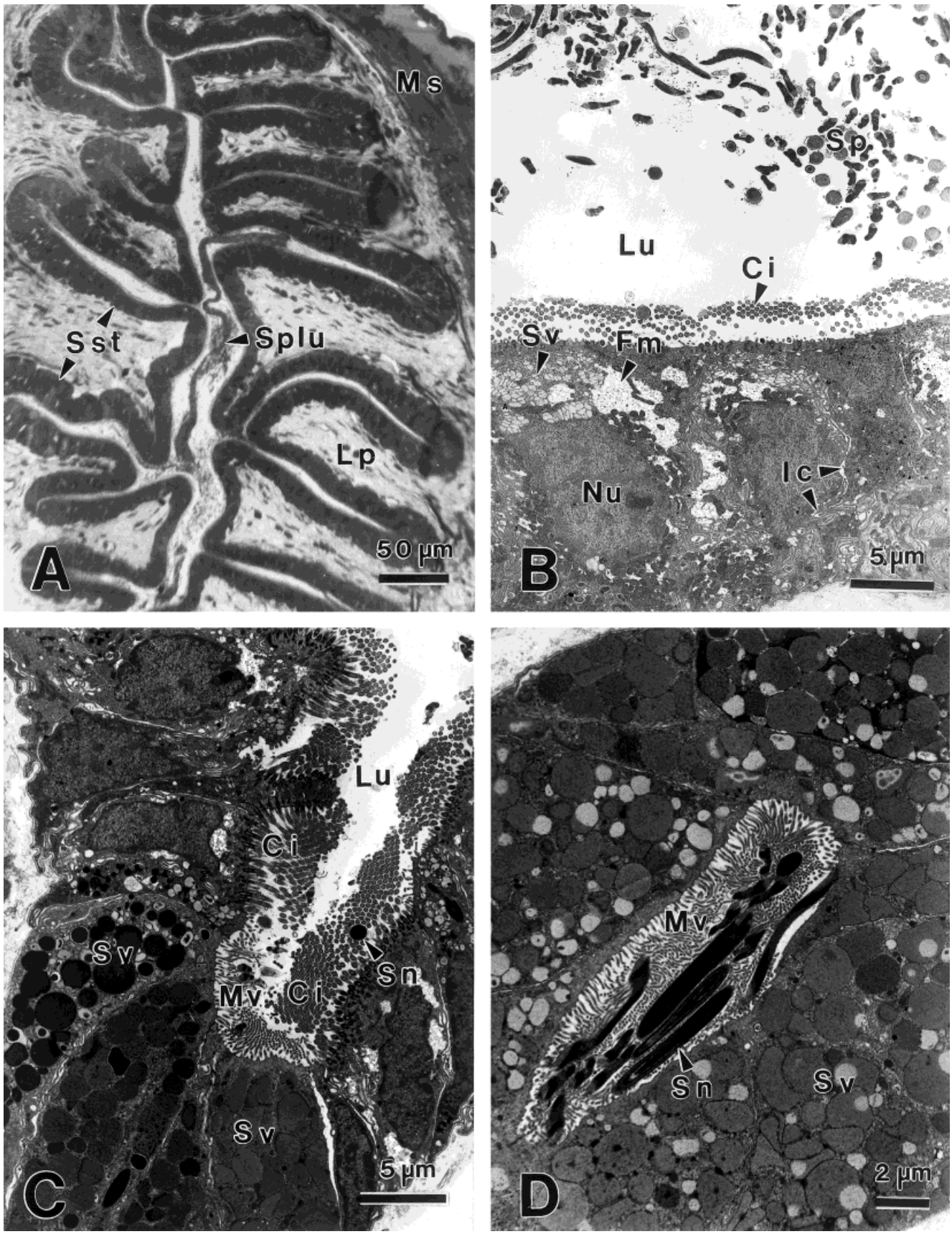

Fig. 5. Female *Ascaphus truei*. Light micrograph (**A**) and transmission electron micrographs of the ovisac of a 47.2 mm SVL vitellogenic female sacrificed 12 July. **A:** Sperm storage tubules (Sst) in the anterior ovisac. **B:** Epithelial border of the ovisac. **C:** Orifice of an Sst. **D:** Distal portion of an Sst. Ci, cilia; Fm, flocculent material; Ic, intercellular cannalculi; Lp, lamina propria; Lu, lumen; Ms, muscularis; Nu, nucleus; Sn, sperm nucleus; Sp, sperm; Splu, sperm in the lumen; Sst, sperm storage tubule; Sv, secretory vacuole.

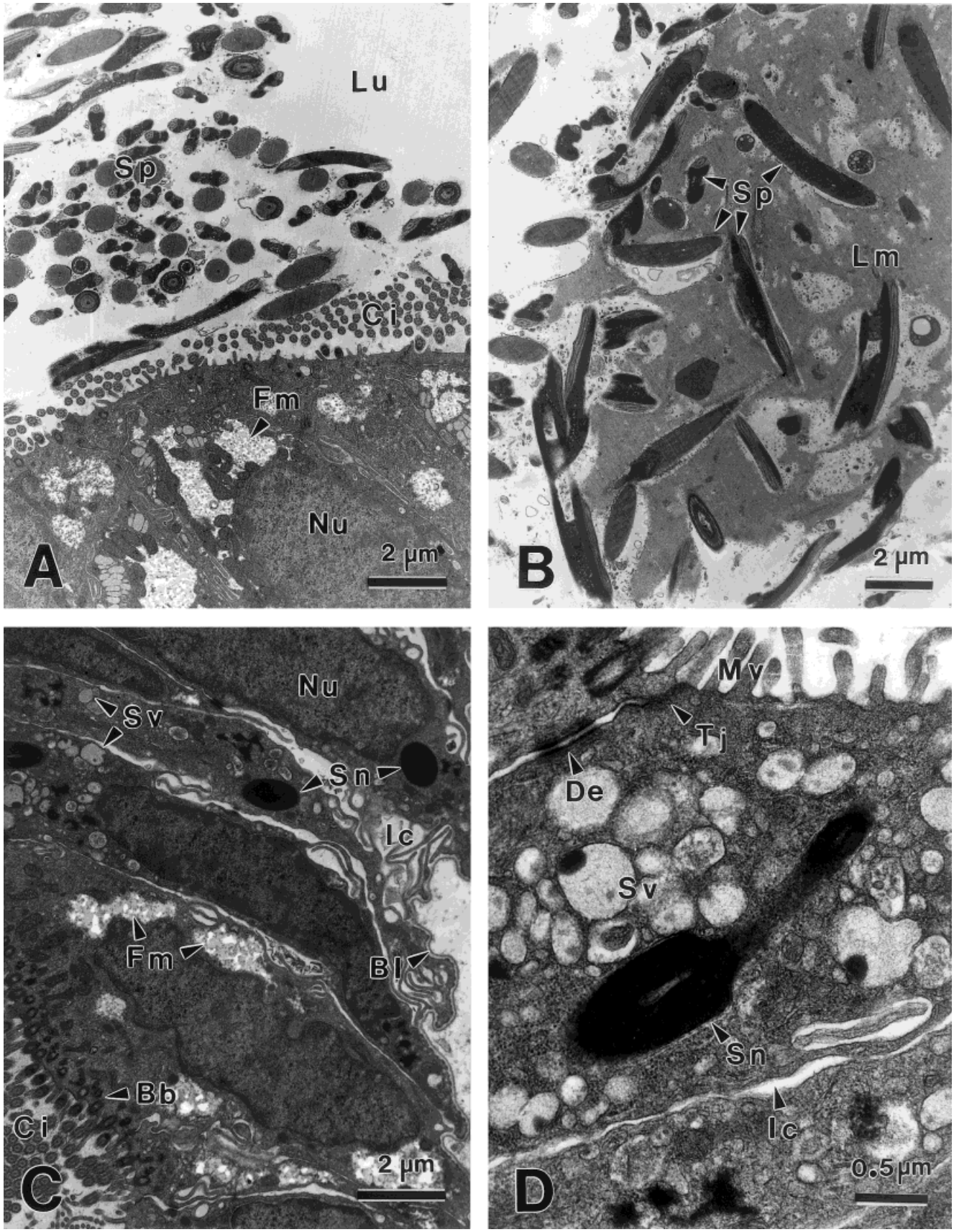

Fig. 6. Female *Ascaphus truei*. Transmission electron micrographs of the ovisac of a 47.2 mm SVL vitellogenic female sacrificed 12 July. **A:** Luminal border. **B:** Sperm in luminal matrix (Lm). **C:** Basal border of an Sst. **D.** Sperm nucleus embedded in an Sst. Bl, basal lamina; Ci, cilia; De, desmosome; Fm, flocculent material; Ic, intercellular canaliculus; Lm, luminal matrix; Mv, microvilli; Sn, sperm nucleus; Sp, sperm; Sv, secretroy vacuole; Tj, tight junction.

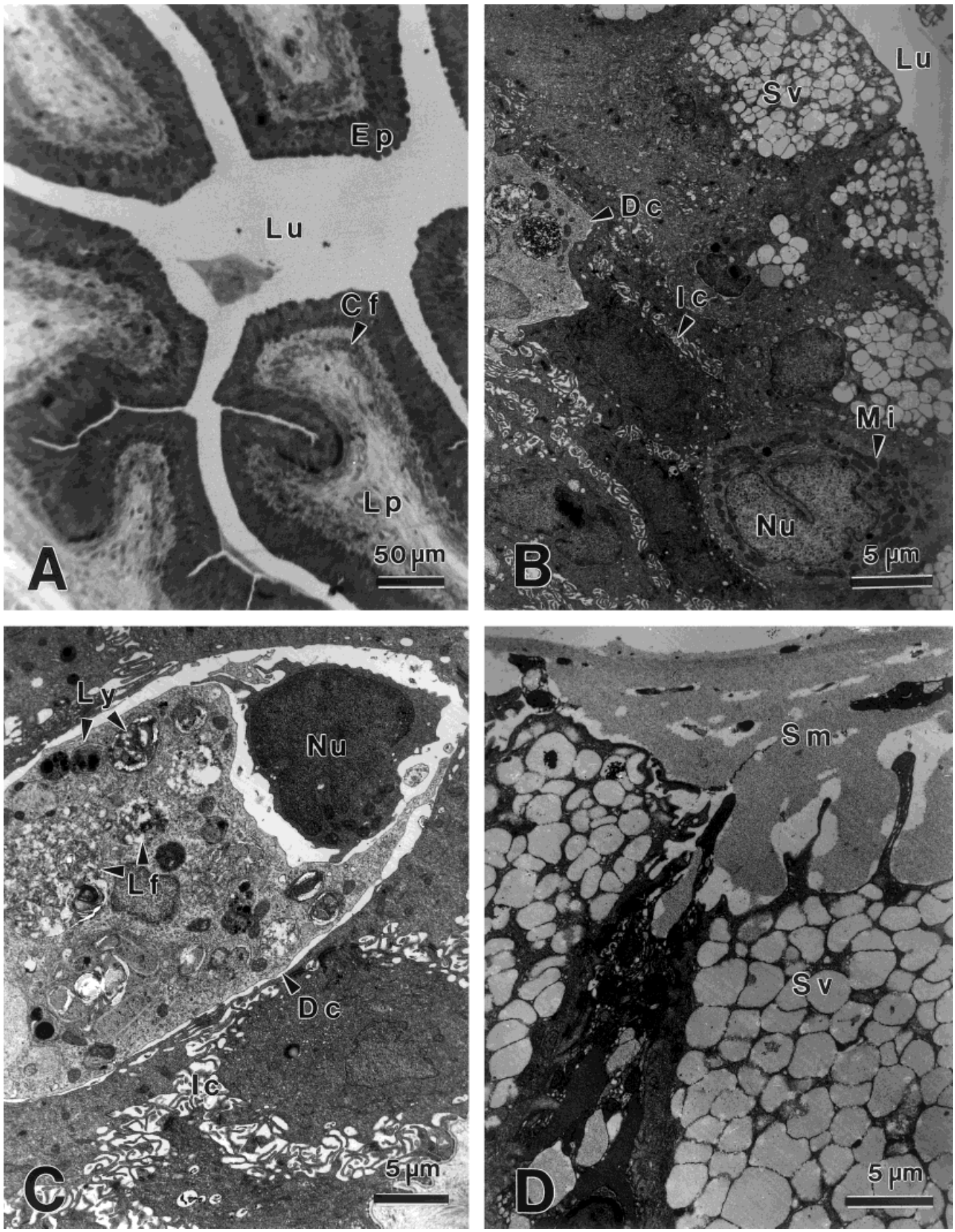

Fig. 7. Female *Ascaphus truei*. Light micrograph (**A**) and transmission electron micrographs of the oviducal sinus of a 47.2 mm SVL vitellogenic female sacrificed 12 July. **A:** Mucosa and submucosal collagen layer. **B:** Apical cytoplasm. **C:** Degenerating epithelial cell. **D:** Merocrine secretory process. Cf, collagen fibers; Ep, epithelium; Ic, intercellular canaliculus; Lf, lipofuscin particles; Lp, lamina propria; Lu, lumen; Ly, lysosomes; Mi, mitochondria; Nu, nucleus; Sm, secretory material; Sv, secretory vacuoles.

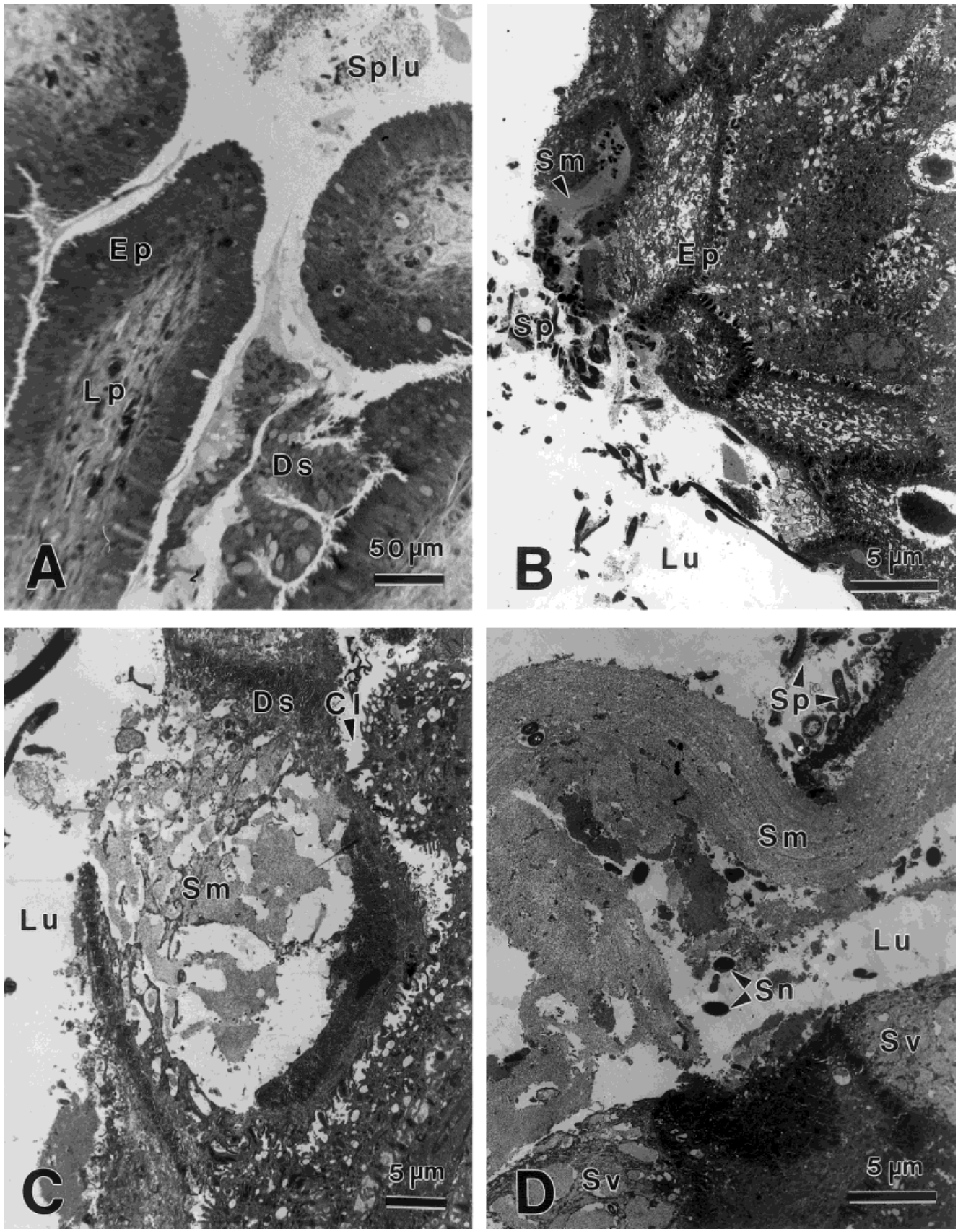

Fig. 8. Female *Ascaphus truei*. Light micrograph (**A**) and transmission electron micrographs of the oviducal sinus of a 52.5 mm SVL vitellogenic female sacrificed in copulexus on 18 June. **A:** Mucosa. **B:** Luminal border. **C:** Desquamating cell. **D:** Secretory material and sperm in the lumen. Cl, cleavage line; Ds, desquamating cells; Ep, epithelium; Lp, lamina propria; Lu, lumen; Sm, secretory material; Sn, sperm nucleus; Sp, sperm; Splu, sperm in the lumen; Sv, secretory vacuoles.

cytoplasm is packed with electron-lucent secretory vacuoles (Fig. 7B) that are PAS+ and AB− and BB− in paraffin sections. A few cells bordering the lumen lack secretory vacuoles and have numerous mitochondria in the apical cytoplasm (Fig. 7B). Some sloughing of the epithelium is apparent, as cells with apparently deteriorating cytoplasm are occasionally found among normal cells (Fig. 7B,C). Complex interdigitations occur in the intercellular spaces between adjacent epithelial cells (Fig. 7B,C). In some areas, a product is observed during release from these vacuoles into the oviducal lumen by the merocrine process (Fig. 7D).

In the female sacrificed while in copulexus, sperm occur in the oviducal lumen (Fig. 8A) including the folds of the rugae. Some sloughing of the apical epithelium is apparent (Fig. 8A–C), and this process results in an abundance of secretory material that is associated with sperm in the lumen (Fig. 8D).

Nonvitellogenic Females Sacrificed in Summer

Two females examined from June–July contain small ovarian follicles (1.2–1.4 mm mean dia) and narrow, relatively unconvoluted ampullae of the oviduct. These individuals obviously are not in the same stage of reproductive readiness as those females with larger follicles and hypertrophied oviducts. Both of the females with small follicles, however, contain sperm in their SSTs.

Infundibulum. The infundibulum (Fig. 9A) did not appear significantly different from that of females with larger ovarian follicles (Fig. 3A). Ciliated and secretory cells occur in the epithelium and the secretory cells contain dark, uniformly electron-dense vacuoles that stain PAS+ in paraffin sections. A minor difference is that the secretory vacuoles are more irregular in outline than those in the infundibulum of reproductively active females (compare Figs. 3D, 9A). The epithelium contains numerous clear vacuoles as noted in reproductively active females.

Ampulla. The oviducal glands in the ampulla in females with small ovarian follicles (Fig. 9B–D) are much reduced relative to the condition found in females with large ovarian follicles (Fig. 4). Ciliated cells are not prominent. The secretory cells of the glands lack the large, moderately electron-dense secretory vacuoles and the pyramidal shape of active glands. Instead, the secretory cells are columnar and contain numerous elongate secretory vacuoles that vary in electron density (Fig. 9B–D) but are generally darker than those in active glands. The secretory vacuoles observed in this condition may be precursors to those in glands ready to form egg capsules. Debris is frequently found in the lumina of the glands (Fig. 9C). Like ampullary glands in reproductively active females, the secretory cells of the oviducal glands in nonreproductive females are characterized by elongate microvilli (compare Figs. 4A,C, 9C,D).

Ovisac. Sperm occur in the oviducal lumen and the SSTs of the anterior ovisac (Figs. 10, 11) of the nonvitellogenic females examined from June and July (Table 1). Sperm are especially numerous and closely packed in the SSTs and clusters of sperm often exhibit the same orientation of their axes (Fig. 10B–D, 11A). Occasional sperm nuclei are embedded in the apical cytoplasm of SSTs and these sperm, like those in the lumen, appear normal in cytology (Fig. 11B). No evidence for spermiophagy was observed. Ciliated cells were numerous. Many secretory cells lack extensive clusters of secretory vacuoles, but other cells contain numerous, electron-dense, PAS+ secretory vacuoles (Fig. 11A,C). Vacuoles with a lighter density as noted in females with large ovarian follicles (Figs. 5D, 6D) are lacking. Occasional lipid droplets (Fig. 11B,C) occur in the cytoplasm of secretory cells and these lipids stain Sudan Black+ in frozen sections. Capillaries closely abut the basal lamina (Figs. 10B, 11A). In some secretory cells that lack large vacuoles, mitochondria and Golgi complexes are prominent in the perinuclear cytoplasm (Fig. 11D).

Oviducal sinus. The epithelial lining of the oviducal sinus in females with small ovarian follicles (Fig. 12) is similar to that of the vitellogenic female that was not sacrificed while in copulexus (Fig. 7). Most cells bordering the oviducal lumen are filled with large secretory vacuoles. In females with small ovarian follicles, however, these vacuoles are of varying densities rather than the more uniform densities observed in females with large ovarian follicles. Interdigitations and junctions between adjacent epithelial cells are complex (Fig. 12B,C). Some apical cells lack secretory vacuoles and contain numerous mitochondria with tubular cristae (Fig. 12D).

Females Sacrificed in November and April

None of the females sacrificed in November or April possess sperm in their oviducts and observations were limited to light microscopy. Both of the females sacrificed in November and one of the three sacrificed in April contain only small ovarian follicles (0.6–1.3 mm mean dia.) and have relatively undeveloped oviducts. Oviducal histology is similar to that of nonvitellogenic females examined from June and July. Two females from the April sample, however, have large, vitellogenic ovarian follicles (2.6 mm mean dia.) and hypertrophied, sinuous oviducts. In these two females, oviducal histology resembles that of gravid females from June and July, with the oviducal glands in the ampulla filled with large secretory vacuoles. Whether the oviduct is hypertrophied or not, the lining is PAS+, AB−, and BB−.

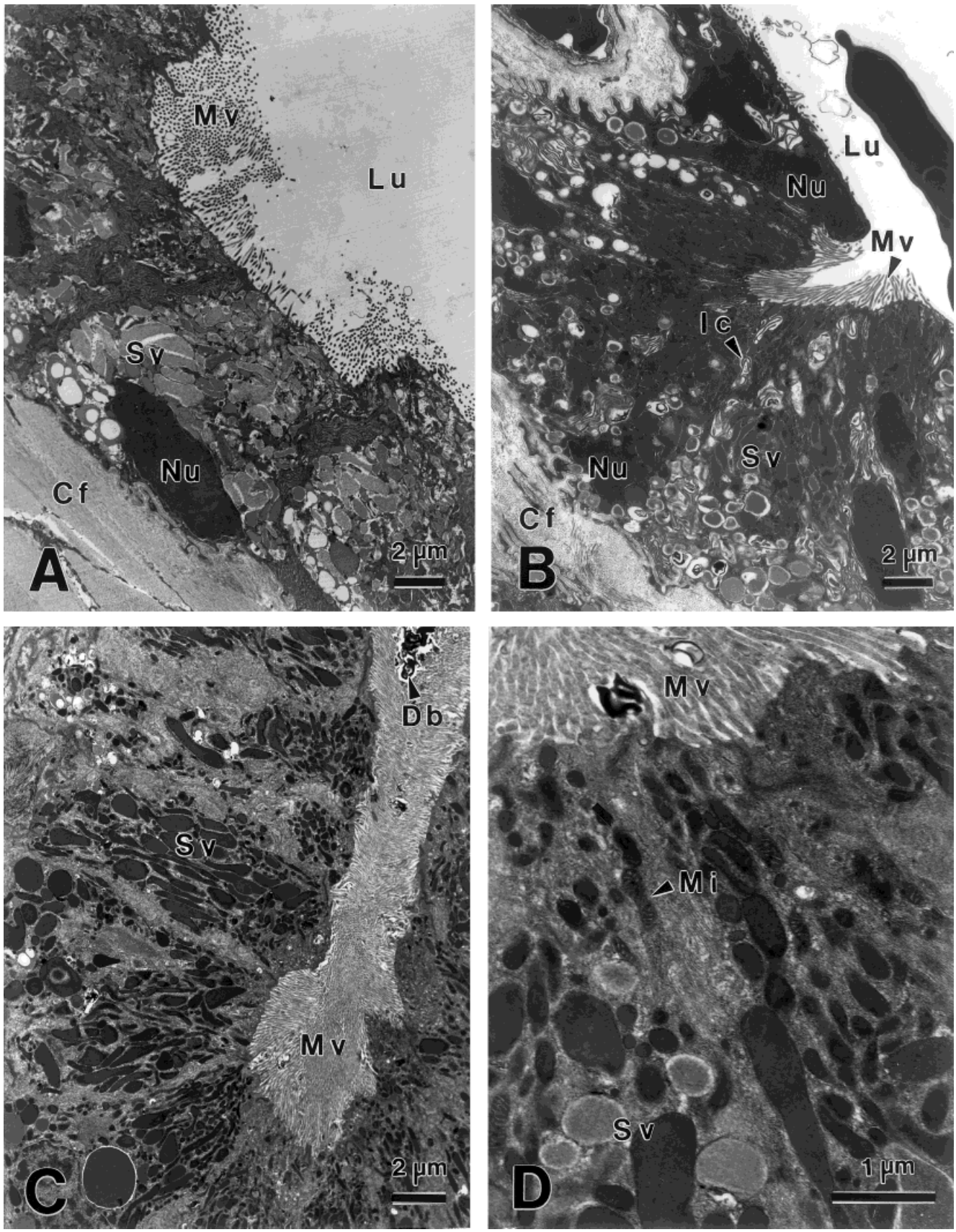

Fig. 9. Female *Ascaphus truei*. Transmission electron micrographs of the infundibulum (**A**) and ampulla (**B–D**) of a 48.1 mm SVL nonvitellogenic female sacrificed 12 July. **A:** Mucosa of the infundibulum. **B:** Muscoa and oviducal gland in the ampulla. **C:** Oviducal gland in the ampulla. **D:** Luminal border of an oviducal gland in the ampulla. Cf, collagen fibers; Db, debris; Ic, intercellular canaliculus; Lu, lumen; Mi, mitochondria; Mv, microvilli; Nu, nucleus; Sv, secretory vacuoles.

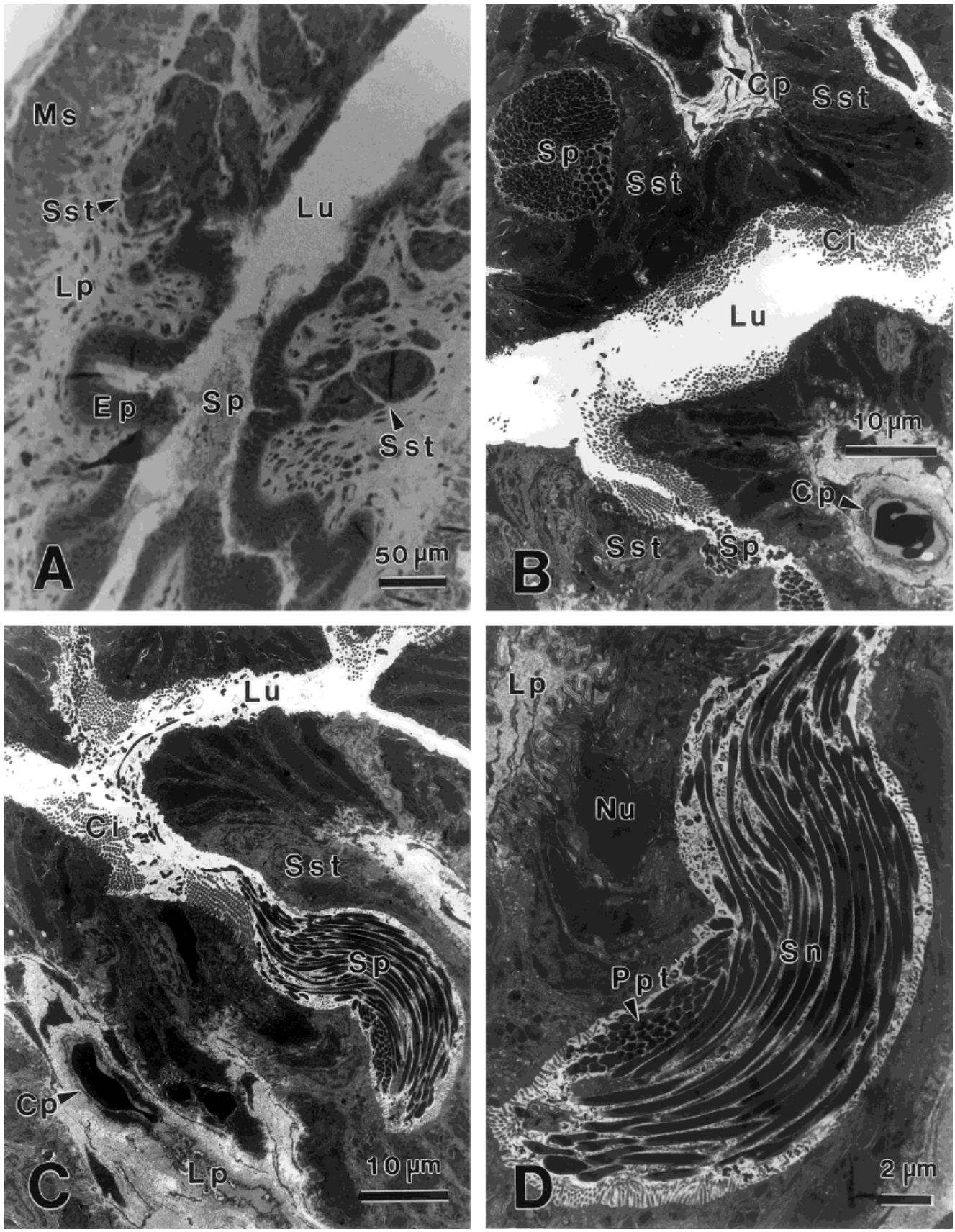

Fig. 10. Female *Ascaphus truei*. Light micrograph (**A**) and transmission electron micrographs of the ovisac of a 48.1 mm SVL nonvitellogenic female sacrificed 12 July. **A:** Tissue layers in the Sst region. **B,C:** Mucosa and Ssts. **D:** Distal portion of an Sst. Ci, cilia; Cp, capillary; Ep, epithelium; Lp, lamina propria; Lu, lumen; Ms, muscularis; Nu, nucleus; Ppt, principal piece of the tail; Sn, sperm nucleus; Sp, sperm; Sst, sperm storage tubule.

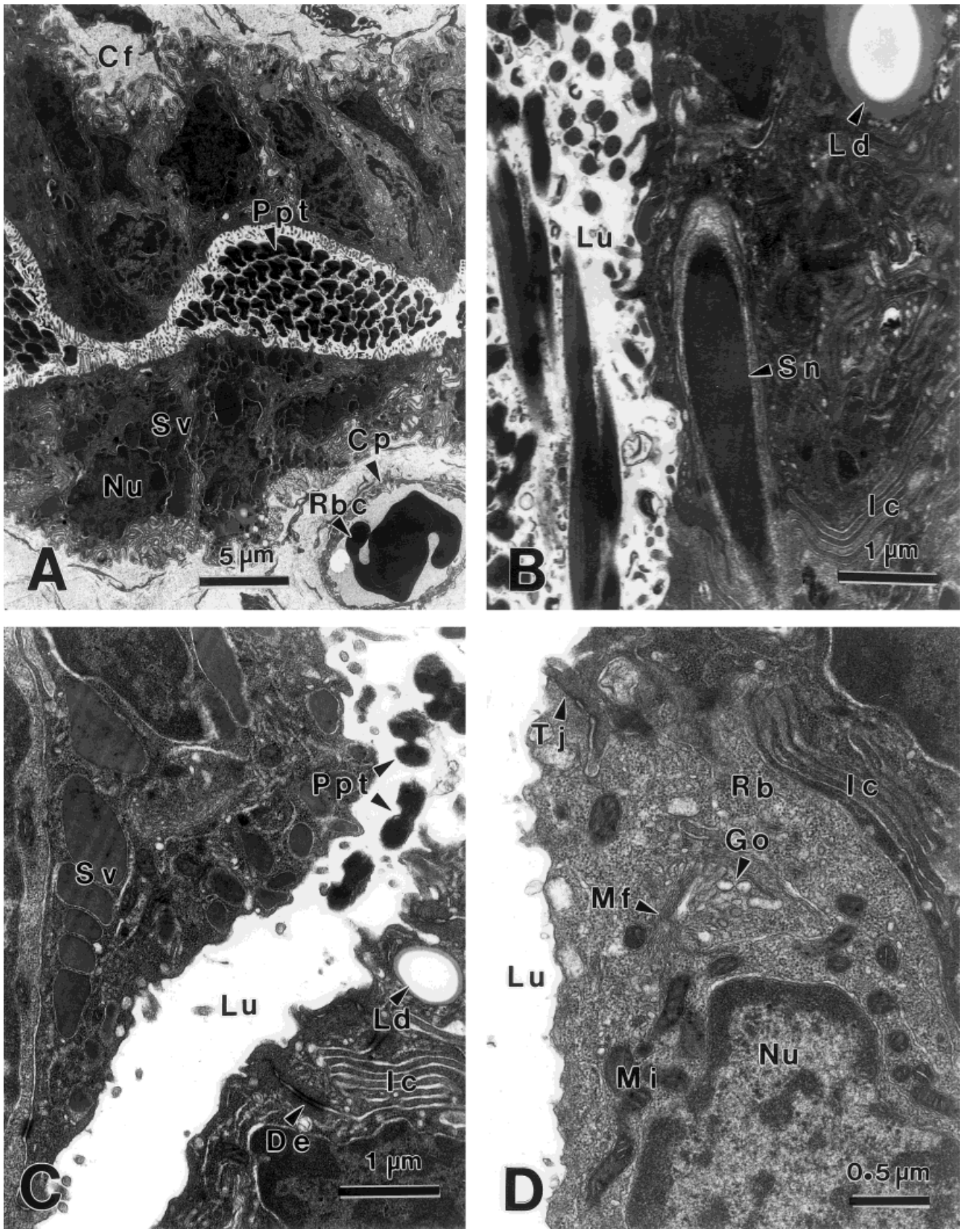

Fig. 11. Female *Ascaphus truei*. Transmission electron micrographs of the ovisac of a 48.1 mm SVL nonvitellogenic female sacrificed 12 July. **A:** Sperm storage tubule (Sst). **B:** Sperm nucleus embedded in the epithelium. **C:** Apical cyctoplasm of an Sst, showing secretory vacuoles. **D:** Apical cytoplasm of an Sst, showing synthetic organelles. Cp, capillary; De, desmosome; Go, Golgi complex; Ic, intercellular canaliculus; Ld, lipid droplet; Lu, lumen; Mf, microfilaments; Mi, mitochondria; Nu, nucleus; Ppt, principal piece of the tail; Rb, ribosomes; Rbc, red blood cell; Sn, sperm nucleus; Sv, secretory vacuoles; Tj, tight junction.

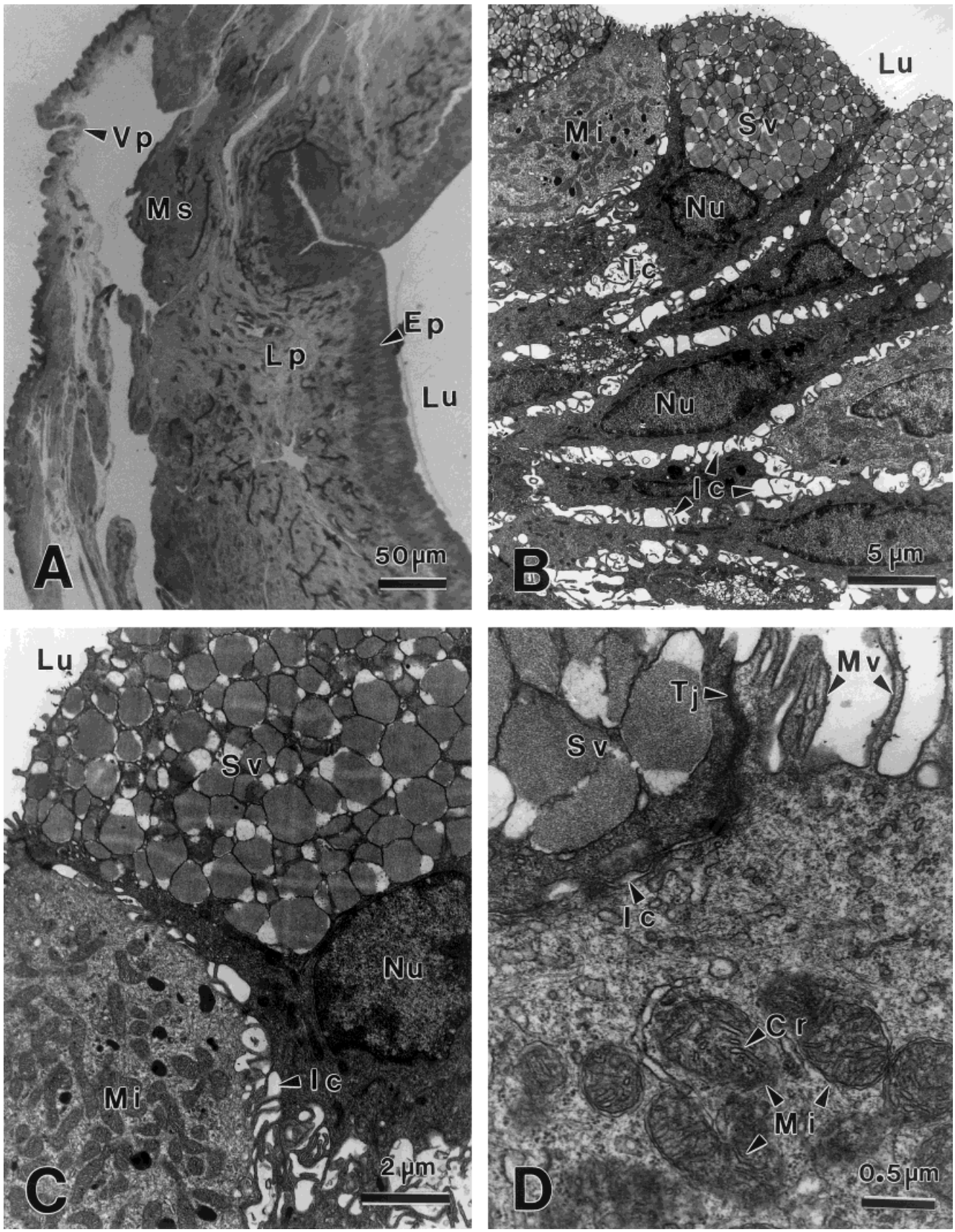

Fig. 12. Female *Ascaphus truei*. Light micrograph (**A**) and transmission electron micrographs of the oviducal sinus of a 48.1 mm SVL nonvitellogenic female sacrificed 12 July. **A:** Tissue layers. **B:** Epithelium. **C:** Luminal border. **D:** Adjacent secretory and nonsecretory cells. Cr, cristae; Ep, epithelium; Ic, intercellular canaliculus; Lp, lamina propria; Lu, lumen; Mi, mitochondria; Ms, muscularis; Mv, microvilli; Nu, nucleus; Sv, secretory vacuoles; Tj, tight junction; Vp, visceral pleuroperitoneum.

DISCUSSION

Reproduction and Reproductive Cycle

The original description of *Ascaphus truei* by Stejneger (1897) was based on a single specimen that evidently was a female (Gaige, 1920:259), and the first observations of the male tail were not reported until Van Denburgh (1912:261), who suggested the tail may be a sexual organ. Gaige (1920) presented the first illustration of the tail and reported on the breeding habits of *A. truei* near Lake Cushman at the base of Mount Rose, Washington. She noted that many females collected 27 July – 24 August had large eggs, whereas others were "normal," which she believed indicated an extended breeding season (Gaige, 1920:5). Further support of this notion was provided by: variation in male breeding condition, by finding larvae of all stages of development on the same date, and Slater's (1931) observation of a pair copulating on 17 May in the Carbon River Valley of Mount Rainier.

Noble (1925:17) suggested that the tail is pressed against the cloaca of the female in copulation and was the first to report sperm in the oviducts females. Noble stated that his sections of the urinogenital organs of breeding females revealed great masses of spermatozoa in the lumen of the oviducts and particularly in the glands along the posterior part of the oviducts. Sections of the oviducts of females taken after their eggs had been laid show many of the glands of the posterior oviduct still filled with spermatozoa.

The actual intromission of the tail in copulation was first described by Noble and Putnam (1931), using specimens from the Lake Cushman locale frequented by Gaige (1920). They also reported finding pairs in the field in copulo from 12 June – 6 July, although a longer breeding season was apparent as females possessed large follicles throughout July, and a male collected 4 September still exhibited amplectic behavior. In the latter regard, however, it is interesting to note that later Metter (1967) reported that males, regardless of development of their secondary sexual characters, will clasp other individuals (male or female) at any time of year.

Van Dijk (1955:65) reported that the turgid tail can only be applied to the cloacal orifice of the female and not inserted into it. This statement is contrary to our observations of the entire turgid member completely inserted into the female. The structure of the flaccid and turgid intromittant organ is the subject of an article in preparation by Hamlett and Sever. We introduce the term "copulexus" to describe the unique form of mating that results in internal fertilization in this species. Amplexus is the term that describes the act of the male grasping the female to effect external fertilization. Copulation is the act of sexual intercourse and implies internal fertilization. Copulexus is characterized by both mechanisms occurring simultaneously to effect internal fertilization with a unique copulatory organ.

In a subsequent article, Van Dijk (1959:222) illustrated a transverse section through the oviduct of *Ascaphus truei*, "showing spermia." However, no explanation or description of the plate was presented.

Metter (1964a) studied the reproductive biology of two geographically close but isolated populations of *Ascaphus truei* in northern Idaho and the Blue Mountains of western Washington. Females apparently have a biennial breeding season (see also Metter, 1967). Secondary sexual characters reach their peak in early fall and copulation was observed between 4 September – 3 October. Oviposition, however, occurs from late June – early August. Thus, sperm for fertilization are retained from breeding the previous fall.

Metter (1964b) subsequently studied the occurrence of sperm in 19 females sacrificed in November. Ten of these females were in a group with large follicles (2.9–3.3 mm dia.) and would have oviposited the following summer while the others had small follicles (0.8–1.3 mm dia.) and presumably would have delayed fertilization and ovulation for almost 2 years. Metter (1964b) reported that 15 of the females contained sperm, although he was unclear how this number was divided among vitellogenic and nonvitellogenic females except to note (p. 711), "Three females collected in September had eggs in the smaller size class. . . [and] contained sperm. This would indicate the sperm can remain viable for 2 years." He also mentioned that one female with large vitellogenic eggs contained no sperm despite repeated copulations with males over 2 weeks.

Our observations indicate that the height of the mating season is June and July in northern California. Our limited sample of two vitellogenic and two nonvitellogenic females from this period all contained sperm in their SSTs, even though the oviducts of the nonvitellogenic females were not as hypertrophied and actively secretory as those of vitellogenic females. Among salamanders (the other amphibians known to store sperm), Sever et al. (1996b) found sperm storage in biennially breeding female *Amphiuma tridactylum* that were not expected to oviposit in the forthcoming year. Whether these findings indicate that these females can store viable sperm through successive breeding seasons requires experimental verification.

We must consider, however, the possibility that sperm storage could occur for approximately a year in the population we studied. However, storage for 2 years, as suggested by Metter (1964a,b) for Idaho and Washington populations would appear unlikely. Metter found mating in the fall so that even vitellogenic females must wait nearly a year until the subsequent summer to oviposit, while nonvitellogenic females mating in the fall were hypothesized to wait almost 2 years. In our population, sperm

were not found in any females sacrificed in fall or spring, even though two of the females sacrificed in April contained vitellogenic follicles nearly as large as those found in gravid females during June and July. Although our sample is small, we suggest that oviposition generally follows summer mating in vitellogenic females. Thus, nonvitellogenic females mating at the same time would presumably yolk follicles and oviposit the following year, resulting in a maximum of 1 year of sperm storage. The California coastal populations also differ from inland populations in having a 1–2 year larval period (Wallace and Diller, 1998) rather than 2–4 years as reported by Metter (1964a) in Idaho and southwest Washington. Thus, considerable geographic variation occurs in the reproductive biology of this species.

Metter (1964b) reported that sperm are stored in the lower, straight portion of the oviduct with none in the upper coiled portion. We found SSTs limited to the anterior portion of the ovisac, the "straight portion" of the oviduct. The coiled portion, the ampulla, is the area where the gelatinous coats are applied to the eggs after they enter the oviduct. Fertilization probably occurs as the jelly-coated eggs pass through the ovisac. It is well known in anurans that application of the jelly coat is necessary for successful fertilization of the eggs (Wake and Dickie, 1998).

Metter (1964b) also noted that sperm taken from the oviducts of *Ascaphus truei* were highly motile when placed in saline and that secretions from the oviduct may provide "nutrients" for the sperm. This supposition has also been made for sperm storage in spermathecae of salamanders, but no evidence exists that nourishment of stored sperm occurs (Sever and Kloepfer, 1993). In contrast, Hardy and Dent (1986) found that sperm stored in salamander spermathecae are quiescent during storage, and spermathecal secretions may therefore provide the chemical/osmotic environment for sperm quiescence (Sever and Kloepfer, 1993). In our sample, sperm in both vitellogenic and nonvitellogenic females of *A. truei* appear normal and are similar in abundance and distribution. Occasionally, sperm are embedded in the SST epithelium, but we found no evidence of involvement of the epithelium in either nourishment of sperm or spermiophagy.

Seasonal Variation of the Oviduct in *Ascaphus truei*

We find it noteworthy that all regions of the oviduct have a PAS+ secretory product, although the vacuoles differ somewhat in cytology. The lack of an AB+ or BB+ reaction indicates that the product contains neutral carbohydrates. The reaction is most intense when glands are most hypertrophied, but a PAS+ reaction can be found throughout the year. The only lipid droplets that were observed were in the oviducal epithelium of the ovisac of nonvitellogenic females.

The infundibulum shows little seasonal variation. In the oviducal sinus, the variation is limited to increased uniform density of the secretory vacuoles in reproductively active females and the sloughing of epithelium in the female in copulexus. We believe this sloughing of the epithelium may be associated with insertion of the male intromittent organ. Variation in the ampulla is associated with hypertrophy of the oviducal glands that provide gelatinous envelopes as eggs pass through this region. Possibly some differences in secretion occur in different regions of the ampulla that may correlate with differences in the jelly layers around the eggs, as known in other amphibians (McLaughlin and Humphries, 1978). However, whether different layers exist in the jelly coats of the eggs of *Ascaphus truei* is unknown, and our observations indicate that the ampullary region is homogeneous in structure and secretory activity.

The condition of the SSTs in the specimens containing sperm is most interesting, because several females contain sperm despite having regressed oviducts and small follicles, i.e., they are not in a condition where oviposition seemed likely in the current breeding season. The SSTs of the nonvitellogenic females exhibit less secretory activity than those of the vitellogenic females. The nonvitellogenic females, however, have lipid droplets in the oviducal epithelium, and lipid was not noted in other regions or other seasons. Despite these differences in secretory activity of the SSTs, sperm appear normal in both vitellogenic and nonvitellogenic females. Thus, secretions of SSTs may not be important in sperm maintenance. On the contrary, sperm in the nonvitellogenic females could be the result of a recent mating, and lack of sustenance from the secretions was not yet a factor in sperm maintenance.

Comparative Biology

To date, *Ascaphus truei* is the only amphibian in which oviducal sperm storage has been reported. Indeed, the only other anamniotes in which oviducal sperm storage is known are elasmobranchs (Pratt, 1993; Hamlett et al., 1998, 1999; Hamlett and Koob, 1999) in the class Chondrichthyes, which is not considered the sister taxon of Amphibia. Females of some teleosts in the Osteichthyes store sperm (Howarth, 1974), but they lack homologs to the oviduct (Kardong, 1995). Instead, sperm are stored in the ovary or a gonaduct (ovarian duct) formed from ovarian tissue (Howarth, 1974; Constanz, 1989).

The extant representatives of Actinistia and Dipnoi, descendant taxa of sarcopterygiian sister groups of amphibians (Schultze, 1994), possess oviducts (Millot and Anthony, 1960; Wake, 1987) and *Latimeria* is viviparous (Smith et al., 1975), indicating that fertilization is internal (Fig. 13). Sperm stor-

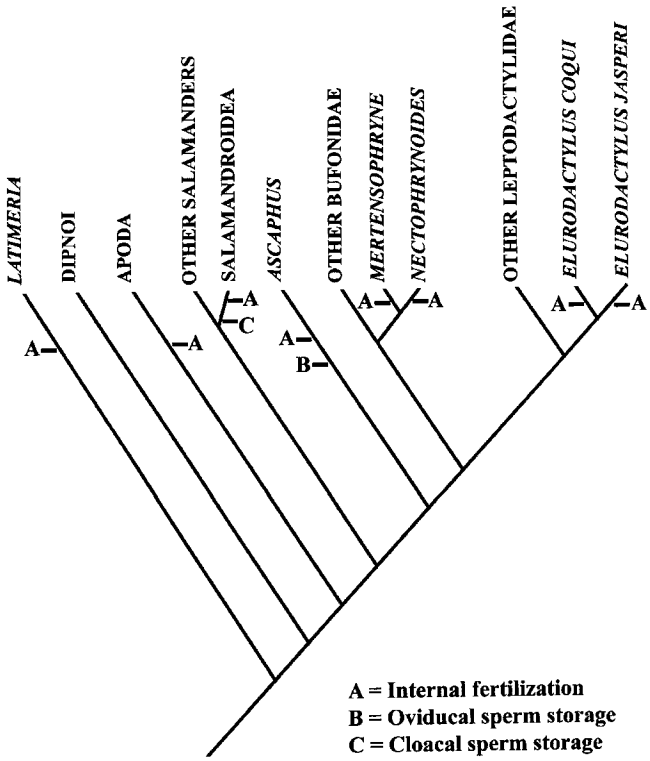

Fig. 13. "Scenariogram" (see Wake and Larson, 1987) showing distribution of internal fertilization and sperm storage in the Lissamphibia and extant sarcopterygiians (descendant taxa of piscine ancestors to amphibians). Only relevant and otherwise most inclusive taxa are shown. Two of the four species of *Nectophrynoides* reported by Wake (1980) to have internal fertilization are now designated *Altiphrynoides malcolmi* and *Nimbaphrynoides occidentalis* by some workers (Dubois, 1986; Graybeal and Cannatella, 1995). Independent origin of internal fertilization, as illustrated here, would not be most parsimonious in this tree. Oviducal sperm storage is an autapomorphy for *Ascaphus truei* within the Amphibia and sister taxa, but evidence for sperm storage should be sought in other taxa with internal fertilization.

age, however, has not been reported in *Latimeria* or any of the extant lungfish.

The Lissamphibia is generally considered monophyletic and consists of three groups, the Anura, Caudata, and Apoda. Most evidence supports a frog + salamander clade (Pough et al., 1998) (Fig. 13). Sperm storage is unknown in female apodans, even though internal fertilization apparently occurs in all taxa, and many caecilians are viviparous (Wilkinson and Nussbaum, 1998). Aside from *Ascaphus*, only a few anurans have internal fertilization, with sperm transfer accomplished by cloacal apposition. These species include *Mertensophryne micranotis* (Grandison and Ashe, 1983) and four species of *Nectophrynoides* (Wake, 1980) within the Bufonidae from Africa, and *Eleurodactylus jasperi* (Wake, 1978) and *E. coqui* (Townsend et al., 1981) within the Leptodactylidae from Puerto Rica. Recent work, however, has indicated that *Nectophrynoides* may not be monophyletic (Dubois, 1986; Graybeal and Cannatella, 1995). Two internally fertilizing species remain in the genus, *N. tornieri* and *N. viviparus*, while the other such taxa have been designated *Altiphrynoides malcolmi* and *Nimbaphrynoides occidentalis* (Dubois, 1986). *Nectophrynoides, Altiphrynoides*, and *Nimbaphrynoides* may not be closely related (Graybeal and Cannatella, 1995).

Obviously, research needs to be done to determine whether oviducal sperm storage occurs in caecilians and internal-fertilizing bufonids and leptodactylids. *Ascaphus truei*, however, is not the sister taxon of any caecilian or of the other internal-fertilizing frogs (Fig. 13), so oviducal sperm storage must be considered independently derived in *A. truei*.

Thus, oviducal sperm storage in *Ascaphus truei* is a classic example of homoplasy through convergence (Sanderson and Hufford, 1996). Structural and functional similarities in sperm storage between *A. truei* and other vertebrates with oviducal sperm storage therefore are not based on direct descent but related either to similar functional adaptations and/or to internal design restraints (Wake, 1991). In the latter case, structural and physiological constraints on the basic vertebrate oviduct and sperm morphologies (the "bauplans") may limit the options for expression of oviducal sperm storage.

The group of anamniotes phyletically closest to frogs and with which frogs share the most developmental similarities (the closest generative system; Wake, 1996) is the Caudata. Sperm storage occurs in females of all seven families of salamanders that compose the suborder Salamandroidea (Sever, 1991, 1994). Instead of oviducal sperm storage, however, sperm are stored in cloacal glands (spermathecae) which consist of a single compound tubulo-alveolar gland (Plethodontidae) or numerous simple tubular glands (other families). The ancestral condition for salamanders is lack of sperm storage glands, a condition found in three families (Sever, 1994). The ultrastructure of sperm storage in salamanders has been studied extensively and was recently reviewed by Sever and Brizzi (1998).

Numerous differences occur between the spermathecae of salamanders and the SSTs of *Ascaphus truei*. The distal portions of the spermathecae of salamanders are typically alveolar, lack cilia, and possess basal myoepithelium (Sever and Brizzi, 1998). Secretory activity in salamander spermathecae is sometimes regionalized and seasonal, depending on the taxa (Sever, 1994). A great deal of variation also occurs in reaction to carbohydrate stains with, however, most species showing AB+ reactions for carboxylated glycosaminoglycans (Sever, 1994).

In some forms the sperm are in orderly arrays in the spermathecae (Sever and Hamlett, 1998), whereas in others sperm are in tangled masses (Sever et al., 1999). Alignment of sperm may depend to some degree on the anatomy of the spermatheca (more orderly in compound glands than simple tu-

bular). Spermiophagy by the spermathecal epithelium has been described in various taxa of salamanders (Sever and Brizzi, 1998).

The SSTs of *Ascaphus truei* more closely resemble those of squamate reptiles (Fox, 1956; Girling et al., 1997; Sever and Ryan, 1999). Oviducal sperm storage glands are known from all groups in the reptile–bird clade except Crocodilia, Amphisbaenia (in which they likely occur) and Rhynocephalia (Gist and Jones, 1987). Like reptiles, the SSTs of *A. truei* are simply continuations of the oviducal lining and contain ciliated nonsecretory cells and nonciliated secretory cells. Myoepithelium is absent, but the oviduct possesses layers of smooth muscle (tunica muscularis) superficial to the mucosa. The linings and glands of reptilian oviducts are generally described as PAS+, like those of *A. truei*, with little reaction to acidic mucosubstances. Sperm in the SSTs of *A. truei* are generally in close alignment, although in squamates this condition varies (Fox, 1956; Sever and Ryan, 1999). Although sperm nuclei are sometimes found embedded in SSTs of reptiles (Sever and Ryan, 1999) and of *A. truei*, no evidence exists for spermiophagy in these taxa.

Thus, oviducal SSTs in distantly related taxa show more similarities than SSTs in *Ascaphus truei* and spermathecae in salamanders, members of sister taxa. The basic structure of the vertebrate oviduct, therefore, may limit the range of features associated with oviducal sperm storage.

Limits to Further Studies

The fact that we describe individual specimens rather than summarize observations on large samples is a result of our concern for conservation of this unique species. We realize, however, that we did not address some critical aspects of the reproductive anatomy of *Ascaphus truei*, and that we have perhaps engendered more questions than we have answered. For example, we would like to sample females with eggs passing through the ampulla, ovisacs, and oviducal sinuses in order to study the processes of formation of egg envelopes and fertilization. We need to dissect or examine sections of a male and female fixed in copulexus to confirm our suspicion that the engorged tail extends through the cloaca into the oviducal sinus. We would like to determine the fate of sperm remaining in the oviduct after oviposition (a condition noted by Noble, 1925). Observations of these and various other internal reproductive phenomena obviously result in the killing of gravid females, but we will continue to limit the number of animals killed to the minimum necessary for such studies.

ACKNOWLEDGMENTS

We thank Lowell V. Diller of the Simpson Timber Company for overseeing the collection and shipment of specimens, critically reading the manuscript, and offering many insights into the biology of *Ascaphus truei*. We thank Laura Burkholder, Elizabeth Ryder, and Joel Thompson for aid in the collections and Chris Hysell for help in the laboratory. This is publication number 17 from the Saint Mary's College Electron Microscopy Facility.

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