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Regionalization of Eccrine and Spermiophagic Activity in Spermathecae of the Salamander *Eurycea cirrigera* (Amphibia: Plethodontidae)

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ABSTRACT The spermathecae of female *Eurycea cirrigera* are compound alveolar glands; narrow neck tubules connect the distal bulbs to a common tube that opens onto the roof of the cloaca. The common tube and neck tubules produce apical secretory vacuoles that contain a periodic acid-Schiff (PAS)+ substance for merocrine export into the lumen. This substance is produced throughout the year, although secretory vacuoles are less numerous during the period of reproductive inactivity in the summer. When sperm are present, the product from the secretory vacuoles bathes sperm in the lumen. Sperm are in orderly arrays and never are embedded in the cytoplasm of the common tube or neck tubules. The distal bulbs do not produce PAS+ secretory vacuoles, and are actively spermiophagic as long as sperm are present. Sperm become embedded in the epithelium of the distal bulbs where lysosomes degrade sperm.

Sperm storage glands, the spermathecae, occur in the dorsal cloacal wall of females of most species of salamanders (Sever, '91a). In oviparous species, ova are fertilized by sperm released from the spermathecae during oviposition (Boisseau and Joly, '75).

The spermathecae of *Eurycea cirrigera,* like those of other plethodontid salamanders, constitute a compound alveolar gland (Sever, '87). A common tube of stratified epithelium opens onto the middorsal wall of the cloaca. Twenty to twenty-four narrow neck tubules of simple epithelium arise from the common tube and pass posteriorly into the tunica propria. The distal bulbs possess simple columnar epithelium with a fluctuated luminal border (Sever, '87).

The spermathecae of *Eurycea cirrigera* contain a glycoprotein secretion that stains positively with periodic acid and Schiff's reagent (PAS) (Sever, '91b). This secretion is exported into the lumina of spermathecal tubules to bathe sperm during their storage (Sever, '91b); however, the secretion is absent in the distal bulbs of females during the 5 months after oviposition (May–September; Sever, '92). Many sperm remain in the distal bulbs after oviposition, and the epithelium of the distal bulbs is actively spermiophagic (Sever, '92).

In this report, we describe localization of secretory and spermiophagic activity in the spermathecae of *Eurycea cirrigera.* Samples from previous studies were reexamined, and observations were made on additional specimens to determine the effects of mating and oviposition on spermathecal cytology. We attempted to induce oviposition in laboratory-maintained animals with an agonist of luteinizing hormone releasing hormone (LHRH), which induces sexual activity and oviposition in females of other salamanders (Licht and Porter, '87; Moore et al., '87; Moore and Deviche, '88; Verrell, '89).

MATERIALS AND METHODS

The *Eurycea cirrigera* used in LHRH experiments were collected at Morgan-Monroe State Forest, Morgan County, Indiana. One group (Group I) of 12 female salamanders was collected on September 20, 1991. Two of these specimens were sacrificed within 4 days of capture, and 10 individuals were maintained in the laboratory through the end of the experimental period in March 1992. These individuals were housed in separate 17 × 31 × 9 cm vinyl containers in an incubator set at 16°C, on a 12:12 light:dark cycle, and provided with vials of *Drosophila* for food. A second group (Group II) of 10 females was

collected March 13, 1992 and maintained in the same manner as those collected in September.

On March 19, 1992, five females from each of the groups were anesthetized with 5% ether and injected intraperitoneally with 0.1 ml of an LHRH solution. The LHRH (des-Gly^{10} [im-Bzl-D-His^{6}]-LH-RH-ethylamide from Sigma Chemical Co., St. Louis, Missouri) was dissolved in amphibian saline (102 mM NaCl, 0.56 mM KCl, 0.23 mM $CaCl_2$; Verrell, '89). Five controls from each group were not injected with any substance. Three of the experimentals from Group II oviposited on 23–24 March and were sacrificed March 24 along with three controls. The remaining experimentals were reinjected on March 26, 1992; these latter individuals along with the remaining controls were sacrificed on March 30, 1992.

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

After fixation in NBF, the ovaries were removed from specimens containing follicles, and the number of large follicles of similar size was counted. The largest diameters of 10 randomly chosen follicles were measured. Mean follicular diameters of specimens sacrificed in March were 2.2–2.8 mm in diameter and considered mature (Sever, '88, '91b).

The spermathecae were rinsed in Millonig's buffer, postfixed in 2% osmium tetroxide, dehydrated in a graded ethanol series, cleared in propylene oxide, and embedded in an epoxy resin (EMBED-812, Electron Microscopy Science, Fort Washington, Pennsylvania). Semithin sections (0.5–1 μm) for light microscopy were 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 aqueous solutions of 2% uranyl acetate and 2.7% lead citrate. Sections were cut using RMC XL1000 and RMC MT-7 ultramicrotomes. Thin sections were viewed with a Hitachi H-300 transmission electron microscope.

Specimens were reexamined from previous studies (Sever, '88, '91, '92). Tissues for transmission electron microscopy were prepared as indicated above, and both semithin and ultrathin sections were cut and examined (Sever, '91b, '92). Tissues prepared for light microscopy were embedded in paraffin and 10 μm sections were cut (Sever, '88). These sections were stained with PAS, diagnostic for neutral carbohydrates. Spermathecae stained with PAS were examined from specimens collected before and after oviposition during the breeding season (April, May), from reproductively inactive specimens collected after the breeding season during the summer months (June, August), and from an unmated specimen collected prior to hibernation (October).

RESULTS

Effects of LHRH injections

Injections of LHRH did not induce oviposition in the five treated females collected prior to the breeding season. These individuals were determined to be unmated by absence of sperm in the spermathecae. The five treated females collected during the breeding season have abundant sperm in their spermathecae. Three of the mated females oviposited 10–11 days after a single injection and the other two mated females oviposited 4 days after a second injection. In addition, dissection revealed that unmated females injected with LHRH had not ovulated (i.e., transferred follicles from the ovaries to the oviducts).

None of the control females ovulated or oviposited eggs. All of the Group I control females lack sperm in their spermathecae except for one individual, which has abundant sperm, apparently from an earlier September mating. Group II controls have abundant sperm in their spermathecae. Specimens injected with LHRH did not differ from controls in spermathecal cytology.

Staining with PAS

In all spermathecae stained with PAS, the apical border of the common tube and neck tubules is PAS+, and PAS+ substances are absent in the epithelium of the distal bulbs (Fig. 1). The PAS+ reaction is weakest in a specimen sacrificed June 18, 1.5–2 months after oviposition (Fig. 1C), but strong in a reproductively inactive specimen sacrificed August 13 (Fig. 1D).

Occurrence of secretory vacuoles

Secretory vacuoles occur in the apical cytoplasm of the common tube and the neck

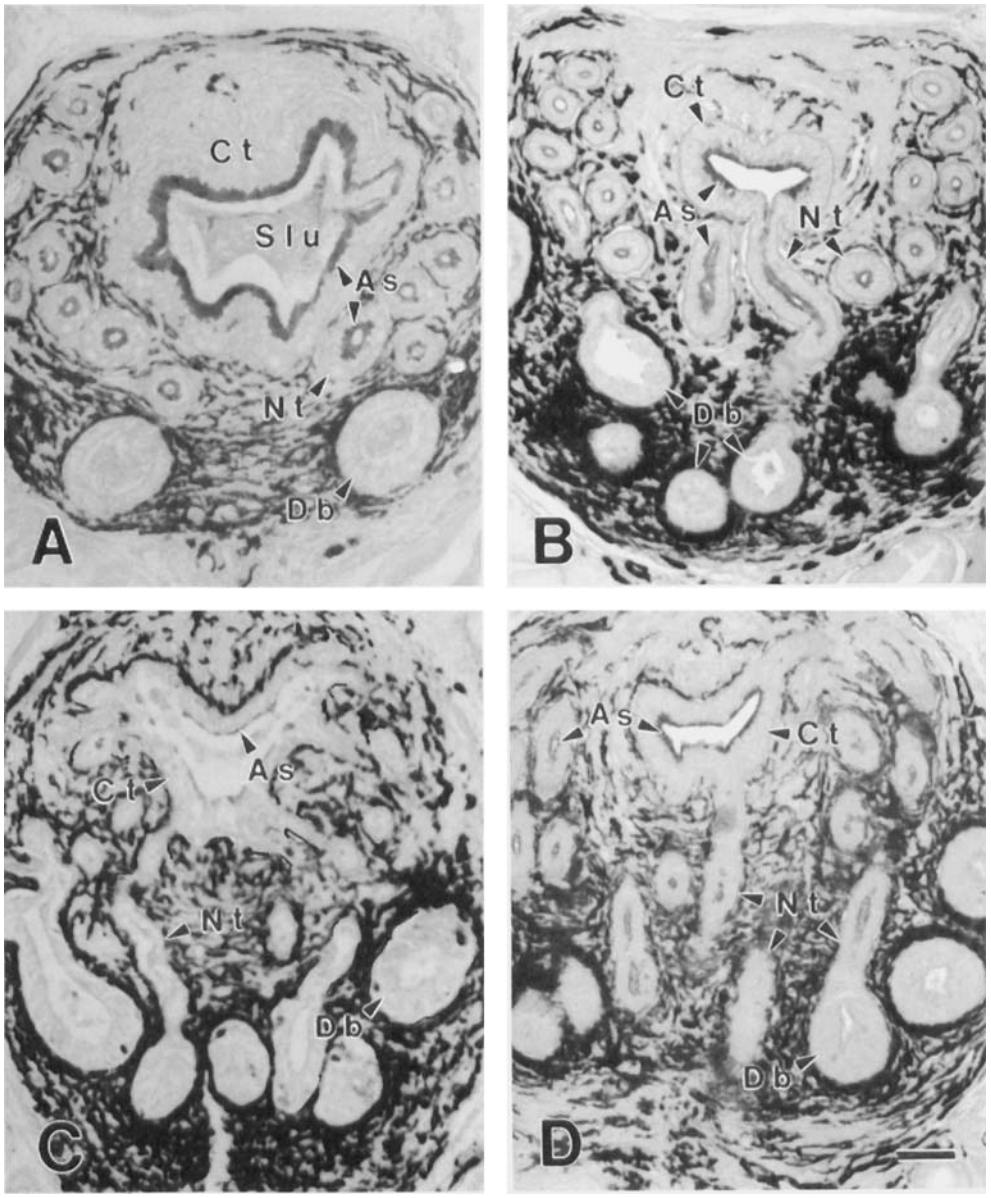

Fig. 1. *Eurycea cirrigera*. Reaction of thick sections of paraffin-embedded spermathecal tubules with PAS. The dark border around the lumen indicates the presence of PAS+ apical secretory vacuoles (As). Note the presence of PAS+ vacuoles in the common tube (Ct) and neck tubules (Nt) and their absence in the distal bulb (Db). Additional data on these specimens are given in Table 2. **A:** Specimen (43.2 mm SVL) in breeding condition collected April 10 and sacrificed April 12, 1976 prior to oviposition. **B:** Specimen (47.4 mm SVL) in breeding condition collected April 10 and sacrificed April 12, 1976 after oviposition. **C:** Specimen (42.5 mm SVL) in nonreproductive condition collected June 17 and sacrificed June 18, 1976. **D:** Specimen (44.1 mm SVL) in nonreproductive condition collected August 12 and sacrificed August 13, 1976. As, apical secretory vacuoles; Ct, common tube; Db, distal bulbs; Nt, neck tubules; Slu, sperm in the lumen. Scale bar in lower right corner for A–D = 90 μm.

tubules but are absent in the epithelium of the distal bulbs (Figs. 2–5). These vacuoles are 0.7–1 μm in diameter and are responsible for the PAS+ staining reactions noted above. In the following sections, the ultrastructure of the spermathecae is described for specimens in five different stages of reproductive activity: Stage I, specimens collected in September while unmated and in an inactive reproductive condition; Stage II, specimens sacrificed in reproductive condition during the breeding season but prior to mating and oviposition; Stage III, specimens sacrificed during the breeding season after mating and prior to oviposition; Stage IV, specimens sacrificed within 24 hours of oviposition; and Stage V, specimens sacrificed between May 15 and September 2 after having been removed from their nests of recently oviposited eggs on April 26.

Common tube

Specimens from Stages I–IV possess abundant apical secretory vacuoles in the stratified epithelium of the common tube (Fig. 2). Active production of the secretory vacuoles and export of the contained substance into the lumen occur in each stage. Release of the product into the lumen occurs by fusion of the vacuole membrane with the luminal border and, therefore, is considered merocrine (Fig. 2A). Golgi complexes in the perinuclear cytoplasm produce condensing vacuoles that are the precursors of the secretory vacuoles (Fig. 2B). Rough endoplasmic reticulum (Rer) and free ribosomes are abundant in the cytoplasm, indicating peptide synthesis (Fig. 2A,B).

The product in the secretory vacuoles probably is a combination of neutral polysaccharides (accounting for the PAS+ reaction) and proteins. The linking of carbohydrates to protein molecules ("glycosylation") usually begins in the rough endoplasmic reticulum and is completed in or near the Golgi apparatus (Holtzman and Novikoff, '84). Depending on the concentration of carbohydrate (Spicer, '65), the secretion may be a glycoprotein ($< 4\%$ carbohydrate) or a mucoprotein ($> 4\%$ carbohydrate). Multivesicular bodies, of uncertain origin and function, also occur in the lumen (Fig. 2C).

Sperm are present in the common tube in mated specimens sacrificed before (Stage III) and after (Stage IV) oviposition (Fig. 2C,D). Although some sperm are adjacent to the cytoplasmic border, none is embedded in the cytoplasm; the sperm appear normal in cytology. Sperm sections in clusters usually represent the same regions of different sperm cells, indicating that the sperm are oriented in the same direction (Fig. 2C).

Neck tubules

The secretory activity of neck tubules is similar to that of the common tube (Fig. 3). Golgi complexes that actively produce condensing vacuoles occur in the perinuclear cytoplasm, and the resulting secretory vacuoles are abundant along the luminal border in Stages I–IV (Fig. 3). The product is exported into the lumen to bathe the sperm after mating. The neck tubules are narrower in diameter than the common tube, and sperm are in dense clusters in mated specimens (Fig. 3C). As in the common tube, sperm usually are in the same orientation (Fig. 3C); although sperm may lie adjacent to the cytoplasmic border, none is embedded in the spermathecal epithelium (Fig. 3C,D).

Distal bulbs

Secretory vacuoles such as those observed in the common tube and neck tubules are absent in the distal bulbs (Fig. 4). Instead, the cytoplasm of the distal bulbs bears pervasive Golgi-Rer-lysosome (GERL) complexes in all stages. In unmated, reproductively inactive specimens (Stage I), small membrane-bound dense bodies (200–250 nm in diameter) that may represent primary lysosomes are abundant (Fig. 4A), and larger (0.65–1 μm), more heterogenous particles occur during the breeding season (Fig. 4B).

Sperm found in the distal bulbs of the spermathecae after mating are randomly oriented (Fig. 4C; Sever, '91b). As described elsewhere (Sever, '91b, '92), sperm in contact with the apical cytoplasm of the distal bulbs are embedded within phagosomes (Fig. 4D), where they are degraded through the action of lysosomes (Sever, '92).

Common tube and neck tubules in stage V

As noted above, the apical cytoplasm of the common tube and neck tubules is PAS+ in specimens collected in June and August (Fig. 1C,D). Cytological examination of spent individuals removed from tended nests of eggs on April 26 and sacrificed at various intervals between March 15 and September 2 confirms that secretory activity in these regions persists throughout the summer period of reproductive inactivity (Fig. 5). Secretory vacuoles, however, are less numerous than during the height of the breeding season. Some

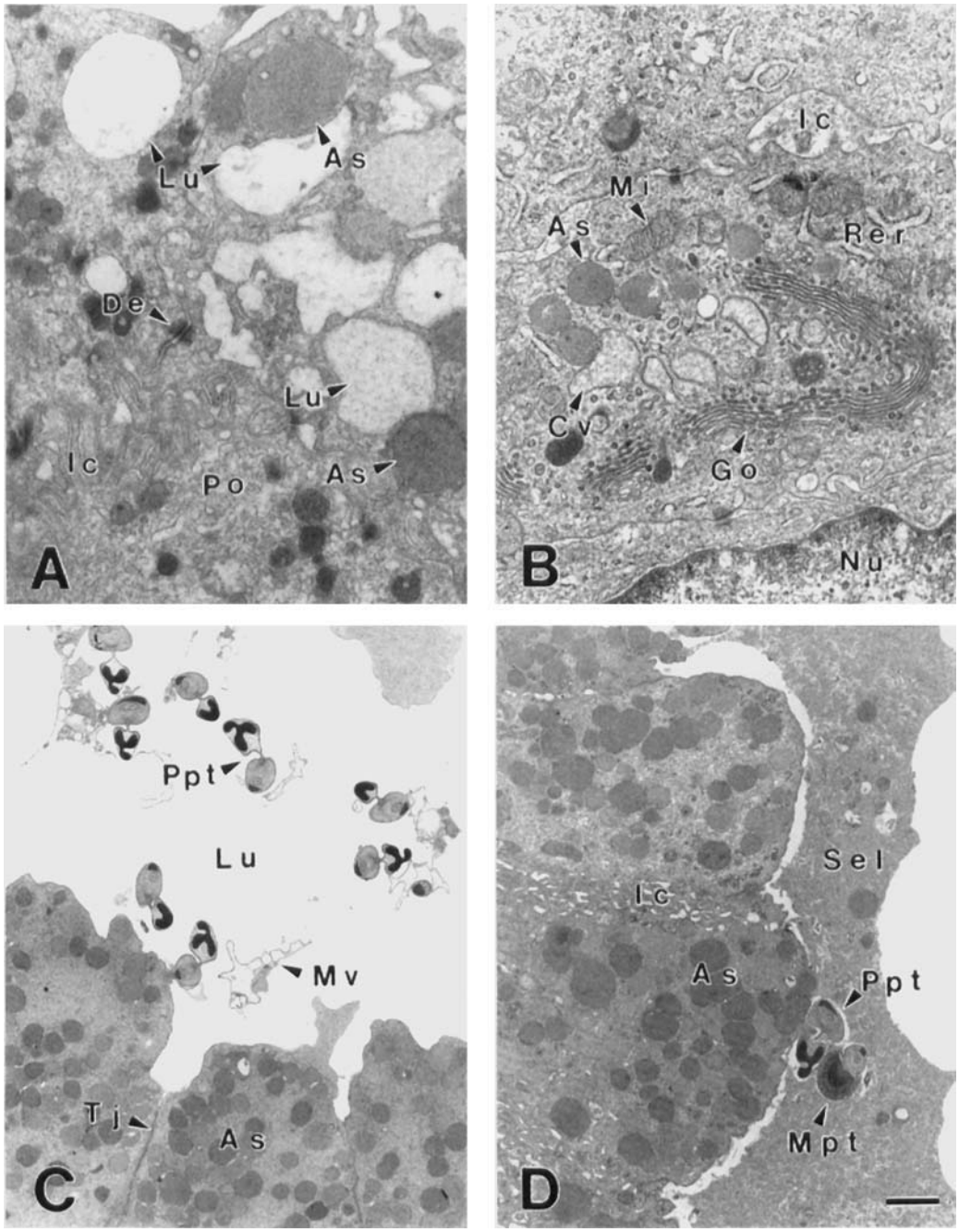

Fig. 2. *Eurycea cirrigera.* Ultrastructure of the luminal border of the spermathecal common tube of females in various reproductive stages. **A:** Specimen (40.9 mm SVL) collected September 20, 1991 and sacrificed September 24, 1991 prior to reproductive activity, showing active export of product into the lumen. **B:** Specimen (41.6 mm SVL) collected September 20, 1991 and sacrificed March 30, 1992 in reproductive condition, but prior to mating and oviposition, showing organelles in active production of the apical secretory vacuoles (As). **C:** Specimen (44.0 mm SVL) collected September 20, 1991 and sacrificed March 30, 1992 after mating and prior to oviposition. **D:** Specimen (41.1 mm SVL) collected March 13 and sacrificed March 30 after mating and oviposition. As, apical secretory vacuoles; Cv, condensing vacuole; De, desmosome; Go, Golgi apparatus; Ic, intercellular canaliculi; Lu, lumen; Mi, mitochondria; Mpt, middle piece of a sperm tail; Mv, multivesicular body; Nu, nucleus of an epithelial cell; Po, polyribosomes; Ppt, principle piece of a sperm tail; Rer, rough endoplasmic reticulum; Sel, secretory product in the lumen; Tj, tight junction. Scale bar in the lower right corner = 513 nm for A, 545 nm for B, and 1.7 μm for C and D.

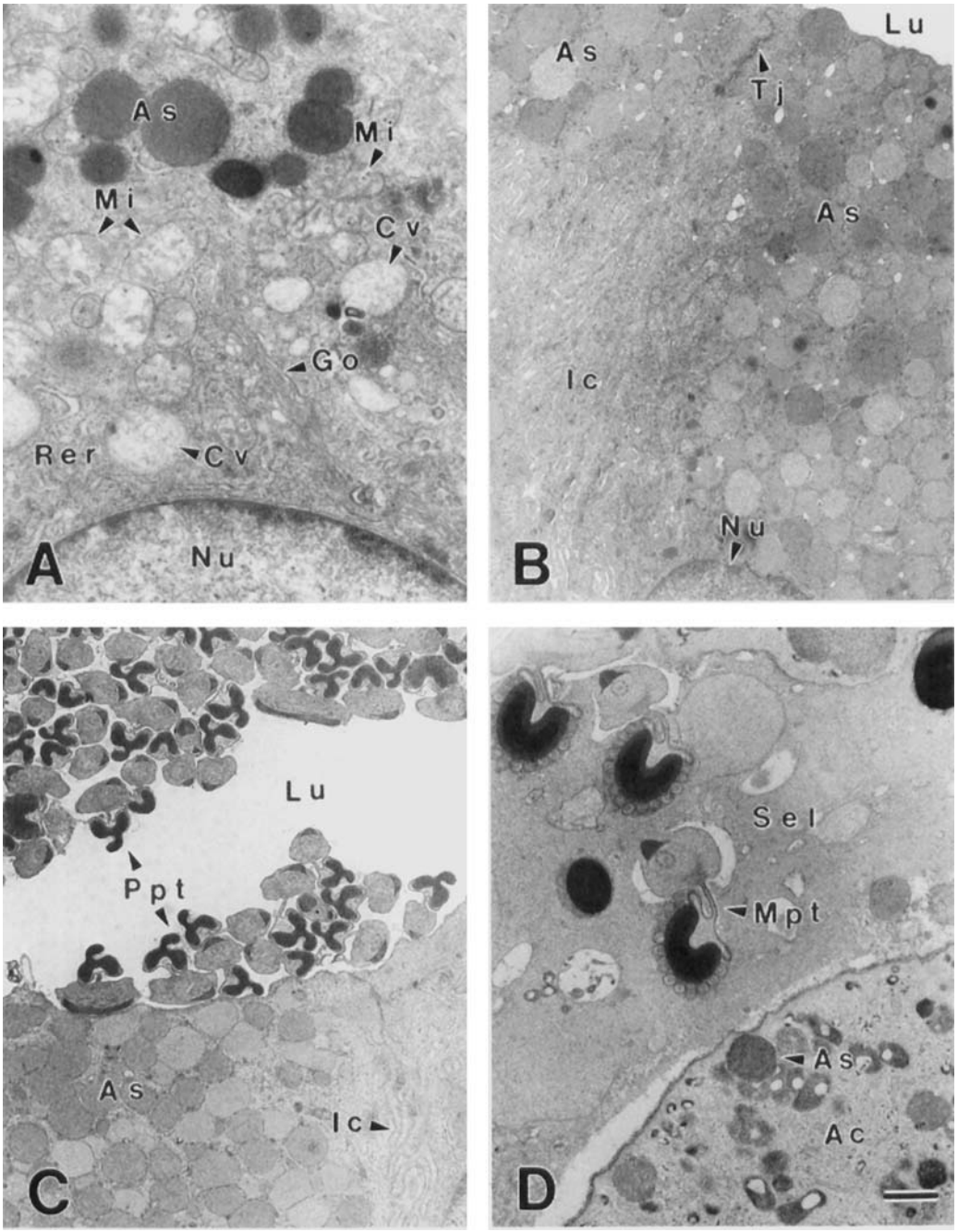

Fig. 3. *Eurycea cirrigera.* Ultrastructure of the spermathecal neck tubules of females in various reproductive stages. **A:** Specimen (40.9 mm SVL) collected September 20, 1991 and sacrificed September 24, 1991 prior to reproductive activity, showing organelles in active production of the apical secretory vacuoles (As). **B:** Luminal border of a specimen (44.0 mm SVL) collected September 30, 1991 and sacrificed March 30, 1992 in reproductive condition but prior to mating and oviposition. **C:** Luminal border of a specimen (39.2 mm SVL) collected March 13 and sacrificed March 30, 1992 after mating and prior to oviposition. **D:** Luminal border of a specimen (41.1 mm SVL) collected March 13 and sacrificed March 30, 1992 after mating and oviposition. Ac, apical cytoplasm; As, apical secretory vacuoles; Cv, condensing vacuole; Go, Golgi apparatus; Ic, intercellular canaliculi; Lu, lumen; Mi, mitochondria; Mpt, middle piece of a sperm tail; Nu, nucleus of an epithelial cell; Ppt, principle piece of a sperm tail; Rer, rough endoplasmic reticulum; Sel, secretion in the lumen; Tj, tight junction. Scale bar in the lower right corner = 565 nm for A, 1.25 μm for B, 1 μm for C, and 700 nm for D.

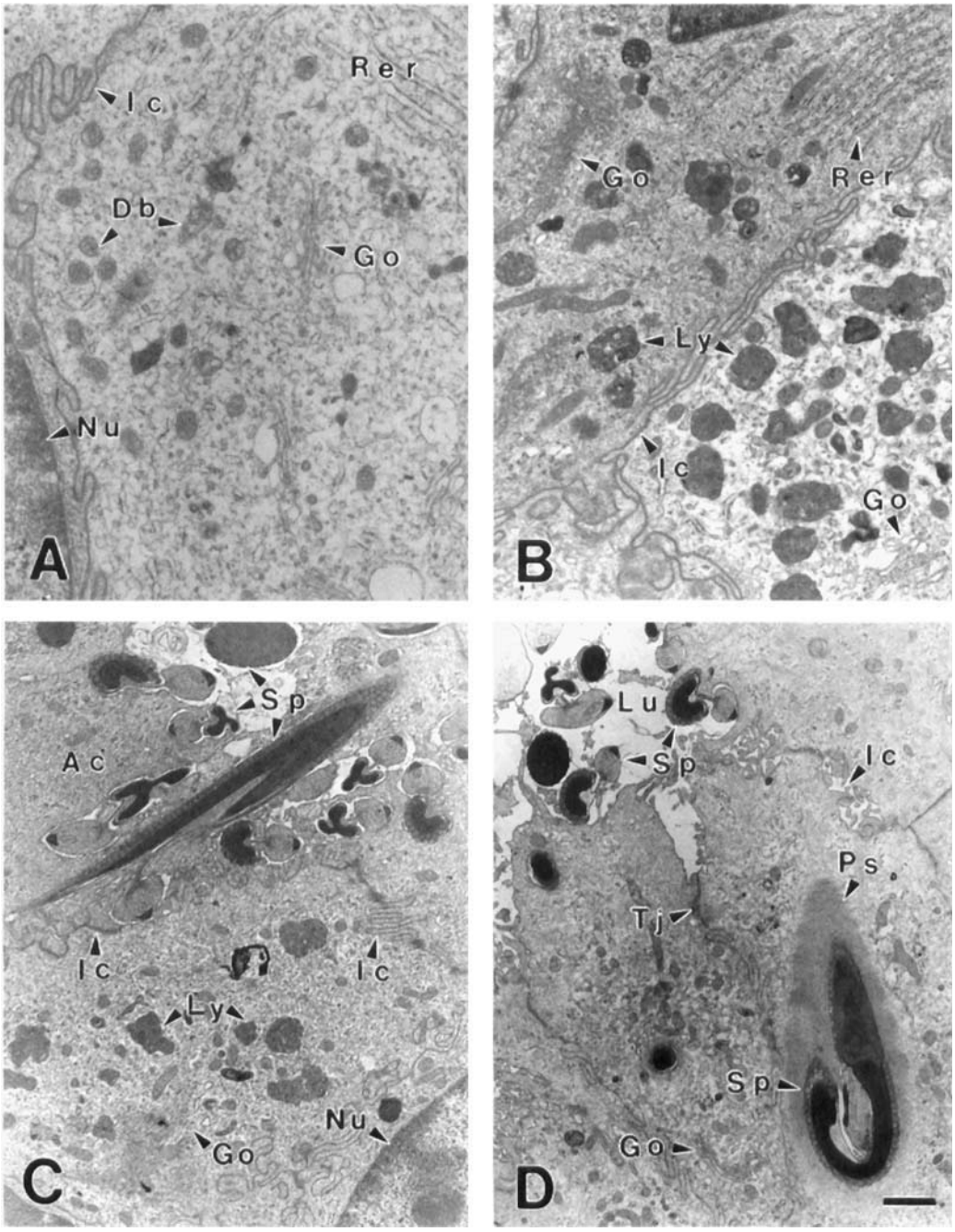

Fig. 4. *Eurycea cirrigera.* Ultrastructure of the spermathecal distal bulbs of females in various reproductive stages. **A:** Specimen (40.9 mm SVL) collected September 20, 1991 and sacrificed September 24, 1991 prior to reproductive activity, showing characteristic appearance of the perinuclear cytoplasm. **B:** Specimen (44.0 mm SVL) collected September 30, 1991 and sacrificed March 30, 1992 in reproductive condition but prior to mating and oviposition, showing a typical GERL complex. **C:** Luminal border of a specimen (39.2 mm SVL) collected March 13 and sacrificed March 30, 1992 after mating and prior to oviposition. **D:** Luminal border of a specimen (44.1 mm SVL) collected March 13 and sacrificed March 30 after mating and oviposition. Ac, apical cytoplasm; Db, dense bodies, probably primary lysosomes; Go, Golgi apparatus; Ic, intercellular canaliculi; Lu, lumen; Ly, lysosomes; Nu, nucleus of an epithelial cell; Ps, phagosome; Rer, rough endoplasmic reticulum; Sp, portions of sperm cells; Tj, tight junction. Scale bar in the lower right corner = 500 nm for A, 844 nm for B, and 1.4 μm for C and D.

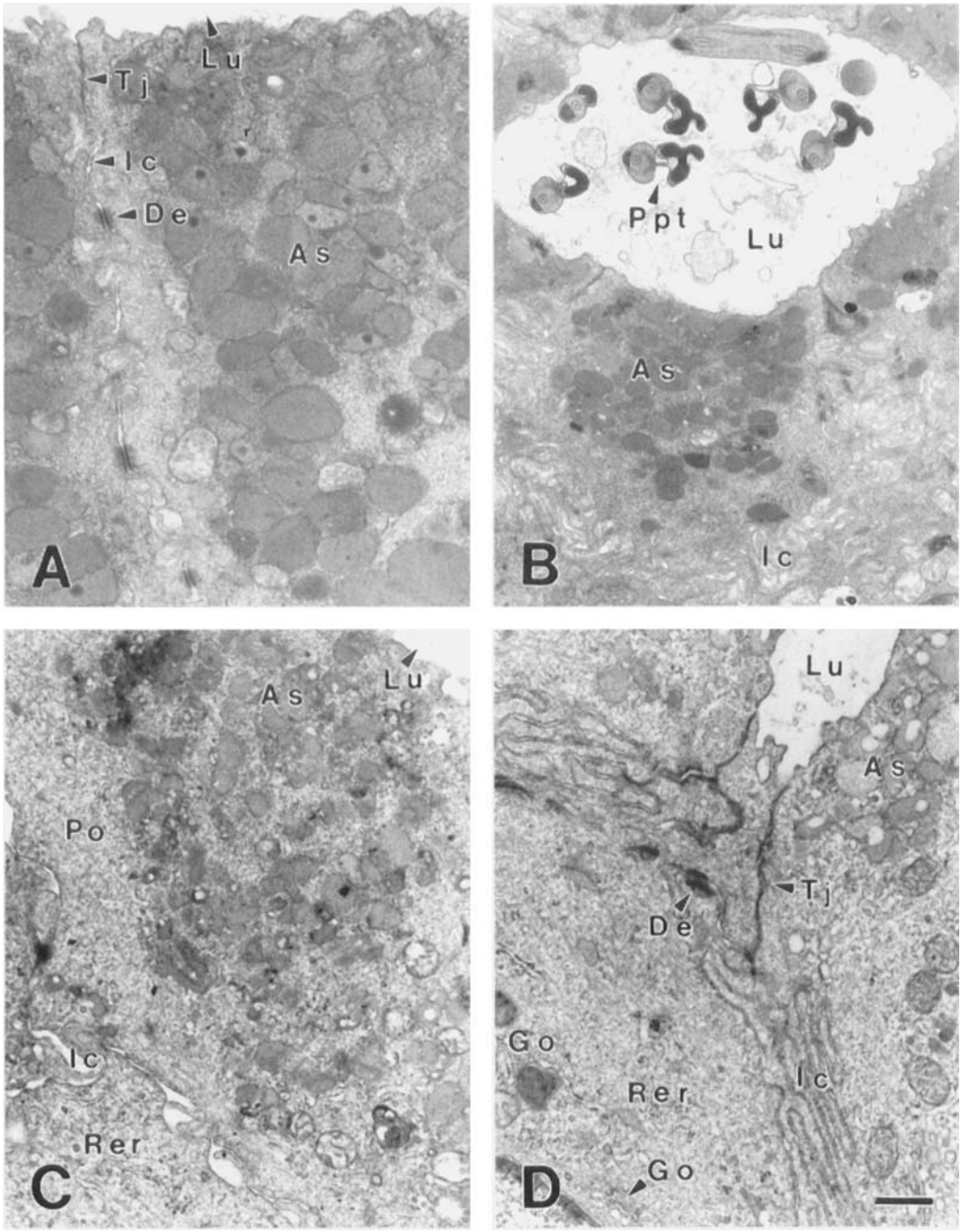

Fig. 5. *Eurycea cirrigera.* Ultrastructure of the luminal border of the common tubes and neck tubules of spent females sacrificed 20–67 days after removal from nests of tended eggs. **A:** Common tube of a specimen collected April 26, 1991 and sacrificed May 15, 1991. **B:** Same specimen as A, showing a neck tubule with sperm in the lumen. **C:** Common tube of a specimen collected April 26, 1991 and sacrificed July 1, 1991. **D:** Neck tubule from the same specimen as C. As, apical secretory vacuoles; De, desmosome; Go, Golgi apparatus; Ic, intercellular canaliculi; Lu, lumen; Po, polyribosomes; Ppt, principle piece of a sperm tail; Rer, rough endoplasmic reticulum; Tj, tight junction. Scale bar in the lower right corner = 667 nm for A, 1.6 μm for B, 625 nm for C, and 500 nm for D.

sperm occur in the neck tubules of a specimen sacrificed May 15 (Fig. 5B) and one sacrificed on September 2. These sperm appear normal and are not embedded in the apical cytoplasm (Fig. 5B).

As described by Sever ('92), the distal bulbs are actively spermiophagic throughout the summer months, and sperm retained in the bulbs from the previous breeding season are degraded prior to the start of the next cycle.

DISCUSSION

Effects of mating and LHRH injections on oviposition

LHRH has been used to induce oviposition in other salamanders (Verrell, '89), but whether mating must occur for the injections to succeed has not been addressed previously. Although the sample sizes used here are small, the five unmated females of *Eurycea cirrigera* injected with LHRH did not ovulate or oviposit, whereas the five mated females that were injected did oviposit. Moore et al. ('79) reported that gravid females of the salamandrid *Taricha granulosa* exposed to courtship and insemination ovulated, whereas gravid females isolated from males exhibited no signs of ovulation; this indicates that the stimulus of mating is necessary for ovulation in this species. However, females of *Triturus vulgaris* (Salamandridae) begin egg deposition in the spring after hibernation without having been inseminated (Pecio, '92). Although the spermathecae of *Triturus vulgaris* were not examined for presence of sperm, all the eggs were unfertilized; this fact contraindicates mating or between-season storage of viable sperm in the spermathecae (Pecio, '92). Clearly, more research is needed on the interrelationships of mating, sperm storage, and the hormones involved in follicle maturation, ovulation, and oviposition in salamanders.

Regionalization of secretory activity

Spermathecae occur in seven families of salamanders that constitute the suborder Salamandroidea (Sever, '91a). In all these families except the Plethodontidae, the spermathecae consist of numerous simple alveolar glands opening individually into the roof of the cloaca (Sever, '87); these types of glands are referred to here as "simple spermathecae." The only ultrastructural studies on simple spermathecae concern several species in the Salamandridae (Dent, '70; Boisseau and Joly, '75; Brizzi et al., '89) and Ambystomatidae (Sever and Kloepfer, '93).

Although the distal ends of simple spermathecae are alveolar, no cytological differences have been reported between proximal and distal regions (Sever and Kloepfer, '93). All regions seem to produce a secretion composed of carbohydrates and proteins for export into the lumen (Boisseau and Joly, '75). Sperm are immobile during storage in the spermathecae (Hardy and Dent, '86), and the apical secretion may provide the chemical/osmotic environment necessary for sperm quiescence (Sever and Kloepfer, '93).

Phagocytosis of stored sperm by the spermathecal epithelium has been reported in several species with simple spermathecae (Dent, '70; Davitt and Larsen, '88b; Sever and Kloepfer, '93). Other studies have proposed that sperm embedded in the spermathecal cytoplasm actually are receiving nourishment (Benson, '68; Marynick, '71; Boisseau and Joly, '75). Spermiophagy has not been reported to the degree that it occurs in the distal bulbs of *Eurycea cirrigera*. Perhaps most sperm remaining in simple spermathecae after oviposition "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).

Eurycea cirrigera possesses "complex spermathecae" composed of compound alveolar glands, with regional differences in secretory activity and function. The exclusively spermiophagic function of the distal bulbs of *Eurycea cirrigera* may be correlated with the complexity of the spermathecae. For sperm to leak out or be expelled from the distal bulbs in this species, sperm must pass down narrow neck tubules and a common tube. Perhaps spermiophagy by the distal bulbs is energetically a more advantageous way for disposal of residual sperm. As noted previously, sperm are quiescent while in the spermathecae, and some energy expenditure would be necessary for myoepithelial contractions to expel sperm. The ambystomatid *Ambystoma opacum*, with simple spermathecae, exports a lipid material into the connective tissue surrounding the spermathecae, and this substance may be involved in myoepithelial contraction (Sever and Kloepfer, '93). *Eurycea cirrigera* lacks a similar lipid product in its spermathecae.

The regionalization of spermathecal secretory activity in *Eurycea cirrigera* was neither noted by Sever ('91b) nor reported in other plethodontids that have been examined ultrastructurally (Pool and Hoage, '73; Davitt and Larsen, '88a, '90), although Davitt and

Larsen ('90) reported that different types of secretory cells occur in the spermathecae of an unidentified plethodontid salamander. Apparently, all plethodontids possess similar complex spermathecae, and the presence of a common tube is considered an autapomorphy for the family (Sever, '87, '91a).

Failure of Sever ('91b) to recognize regional cytological differences in the spermathecae of *Eurycea cirrigera* led to some misinterpretations. Although secretory vacuoles are less numerous in the common tube and neck tubules in the summer than in the breeding season, we have demonstrated a prolonged period of secretory stasis, as described by Sever ('91b), is absent in these regions. Figure 3 of Sever ('91b) shows a neck tubule containing secretory vacuoles, and the other illustrations in that paper depict various sections through distal bulbs, all of which lack secretory vacuoles. Spermiophagic activity by the distal bulbs of *Eurycea cirrigera* was first described by Sever ('91b), but a more detailed account is presented by Sever ('92).

Additional research is needed to document whether regionalization of spermathecal secretory activity occurs in other plethodontids and salamanders of other families. Formulation of hypotheses concerning the significance of sperm storage in female salamanders requires a clear understanding of the morphological interaction between the "foreign" sperm cells and "host" spermathecal cells that may variously serve to attract, nurture, deactivate, activate, or destroy sperm.

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