ULTRASTRUCTURE OF THE MALE CLOACAL GLANDS OF THE EASTERN RED-BACKED SALAMANDER, PLETHODON CINEREUS

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ABSTRACT: This paper represents the first ultrastructural descriptions of the cloacal glands of a male salamander. All male salamanders in the suborder Salamandroidea possess these glands, which are required for internal fertilization. These glands are responsible for producing spermatophores and, in some species, certain glands produce female-attracting pheromones. Both scanning and transmission electron microscopy provided higher resolution and magnification of the cytology of cloacal glands than possible in previous studies limited to light microscopy. The glands are hypertrophied in October and April samples, and much reduced in size and secretory activity in June and August samples. Each of the cloacal glands, when active, has secretory vacuoles that are unique in appearance and that secrete carbohydrates and/or proteins. Kingsbury’s glands are modified mucous glands that have secretory vacuoles filled with a flocculent substance. Active pelvic glands have uniform electron-lucent secretory vacuoles that fill the cytoplasm and narrow the lumen. Ventral glands have squamous epithelium with moderately dense vacuoles scattered around the apical border, and a granular secretion that fills the wide lumen. In the breeding season, vent glands have biphasic secretory vacuoles characteristic of glycoproteins. All glands release their products by a merocrine process, and all have myoepithelial sheaths, which are best developed in ventral glands. Future research should focus on differences in cytology among the pheromone producing glands and comparisons with the ventral glands in salamander clades that do not produce spermatophores (e.g., Hynobiidae and Cryptobranchidae).

Key words: Glandular activity; Microanatomy; Pheromone glands; Reproductive histology; Spermaphore glands

Salamanders possess a variety of sexually dimorphic courtship and mating glands. The structure, function, and phylogeny of these glands were reviewed by Sever (2003), and hormonal control was described by Sever and Staub (2010). Among the most intensely studied glands are those associated with the cloaca, with the first histological studies occurring over a century ago (Siebold, 1858; Robin, 1874; Blanchard, 1881; Heidenhain, 1890).

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Salamanders in the suborder Salamandroidea (following the taxonomy of Duellman and Trueb [1986]) are unique among vertebrates in the possession of male cloacal glands that make spermatophores and female cloacal glands (spermathecae) that store sperm. The role of the various male cloacal glands in spermatophore formation was described by Sever and Houck (1985). Reviews of spermathecal anatomy and sperm storage, which have been studied more extensively than male cloacal glands, include those of Sever and Brizzi (1998) and Sever (2002).

Eggs are fertilized internally as they pass through the cloaca, except in viviparous salamandrids in which sperms migrate from spermathecae to the uterus to fertilize eggs.
As noted by Frost et al. (2006), the glands associated with internal fertilization are the only morphological characters that support a monophyletic Salamandroidea. In contrast, external fertilization occurs in the other three families of salamanders, Cryptobranchidae, Hynobiidae, and Sirenidae (Sever et al., 1996), all of which are considered basal to the Salamandroidea (Zhang and Wake, 2009; Pyron and Weins, 2011). Cryptobranchids and hynobiids generally possess just one type of cloacal gland, called the ventral gland, and sirenids lack cloacal glands (Sever, 1991b). Frost et al. (2006), however, consider Proteidae + Sirenidae as a sister clade to Ambystomatidae + Salamandridae, and the ancestor of these clades as a sister taxon to Plethodontidae. As far as cloacal glands are concerned, the most parsimonious explanation for the phylogeny of Frost et al. (2006) would be secondary loss of cloacal glands in sirenids.

Cloacal glands are also involved in the production of courtship pheromones (Malcarmne and Vellano, 1987; Kikuyama et al., 1995). In male salamanders, the pheromone-producing glands are called dorsal glands, and their elongate tubules can extend from orifices in the posterior end of the cloaca superior to other gland clusters to terminate in the posterior trunk (Sever, 2003). In other salamandrids, the homolog of the dorsal gland is called the vent gland, and it is typically limited to small tubules secreting onto the epidermal lining of the posterior end of the cloaca. Vent glands are most rudimentary in the Plethodontidae; these salamanders rely more on skin glands for pheromone delivery than a cloacal source (Houck and Reagan, 1990; Sever, 1991a).

Many species of female salamanders retain ventral glands like those found in hynobiids and cryptobranchids. Sever (1988) found that females of Eurycea cirrigera secrete a proteinaceous substance into the cloaca during the mating season, before oviposition. Because similar ventral glands are symplesiomorphic for salamanders, I proposed that the ancestral function of cloacal glands may involve a secretion during mating activity.

Thus, cloacal glands of salamanders are important to study because of their necessary role in transfer of sperm by spermatophores, and storage of sperm in spermathecae, in most species of salamanders. Furthermore, certain cloacal glands are required, at least in some species, for the attraction of mates.

My first publication on salamander cloacal glands (Sever, 1978b) concerned the histology of the cloaca and its glands in male P. cinereus and P. dorsalis. That paper was also only the third on the cloaca of members of the family Plethodontidae and the first in almost 50 yr. Kingsbury (1895) provided a brief description of the cloacal glands of male P. cinereus, P. glutinosus, and Desmognathus fuscus, and Noble and Pope (1929) briefly described the cloaca of male D. fuscus. The work by Sever (1978b) extended this knowledge by describing two new cloacal glands, named Kingsbury’s gland and the vent gland.

At the light microscopy level, male cloacal glands can be characterized by their staining reactions in hematoxylin and eosin. The basic dye hematoxylin stains acidic structures that have a high content of RNA and DNA (e.g., nuclei and endoplasmic reticulum) and also stains mucous glands (Young et al., 2006). The basophilic glands are (1) Kingsbury’s glands, a small group of glands around the lumen of the anterior cloacal tube; and (2) ventral glands, located around the cloacal orifice and divided into anterior and posterior groups (based upon location of their tubules and a lighter basophilic reaction in the posterior cluster). Eosin is an acidic dye that has affinity for basic proteins in the cytoplasm. The eosinophilic glands are (1) pelvic glands, located around the roof of the cloacal cavities (in plethodontids, these glands are usually divided into dorsal, lateral, and caudal groups based upon their relative locations); and (2) vent glands, a small group of glands arising from the epidermal lining of the caudal end of the cloacal orifice. The secretory products of cloacal glands generally contain complexes of carbohydrates and proteins, although lipids have been reported from some glands (Sever, 2003).

Ultrastructure examination using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) can reveal details about the production and release of secretory products, junctional complexes between cells,
and distribution of various organelles. Ultrastructural studies exist on sperm storage in the spermathecae in each of the families in the Salamandroidea (Sever, 2003), but no ultrastructural descriptions are available for male cloacal glands of salamanders. This situation is remedied in the current paper by descriptions of seasonal variation in ultrastructure of the male cloacal glands of *P. cinereus* using scanning and transmission electron microscopy.

**Materials and Methods**

Specimens of *P. cinereus* were collected in 2002 at the Swamp Rose Area, Potato Creek State Park, Saint Joseph County, IN, USA. Four adult males were collected for each sample on 16 April, 16 June, 14 August, and 16 October. Courtship and mating have not been observed in this population, but sperm have been reported in the vasa deferentia of males collected in October and March and are absent in May and August (Sever, 1978b). Snout–vent length (SVL) ranged from 37.5 to 48.1 mm. Specimens were euthanized in 10% tricaine methanesulfonate (MS-222) in 50% ethanol. After death, the cloacal region was excised from each specimen and fixed in Trump's fixative (2.5% glutaraldehyde and 2.5% formaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4; Electron Microscopy Sciences, Hatfield, PA, USA) for electron microscopy. Two cloacae from each month were prepared for TEM and two were prepared for SEM.

Tissues for TEM were rinsed in deionized water, postfixed in 2% osmium tetroxide, dehydrated through a graded series of ethanol solutions, critical point dried with a Denton DCP-1, and sputter coated with gold palladium (Denton Vacuum, Moorestown, NJ, USA). Specimens were viewed and photographed using a Philips XL20 scanning electron microscope (Philips Electronics, Eindhoven, The Netherlands).

**Results**

**April.—**The cloacal glands are in their most hypertrophied condition in the April sample (Figs. 1–4). Sperm are present in the anterior cloaca in all specimens (Figs. 1A, 2A). Kingsbury's glands have an abundance of secretory material in the cytoplasm, but the lumen is empty (Fig. 1B). Plasma membranes between epithelial cells of Kingsbury's glands are not apparent, and the cytoplasm appears syncytial (Fig. 1B–D). Microvilli are absent along the apical border, and the secretory vacuoles abut upon the luminal plasma membrane (Fig. 1C). The secretory vacuoles have a flocculent appearance characteristic of a mucoid secretion. Nuclei are irregular, heterochromatic, and generally basal. The occurrence of Golgi bodies and condensing vacuoles in the supranuclear region indicates synthetic activity (Fig. 1D).

All regions of the pelvic gland are similar in cytology. The cytoplasm of pelvic glands is packed with secretory material, generally obscuring the lumina (Fig. 2A,B). When a lumen is observed, the apical border of the epithelial cells has a fringe of microvilli (Fig. 2C). The secretory vacuoles have a uniform, electron-lucid appearance (Fig. 2C,D). Scant cytoplasm exists around the secretory vacuoles, and heterochromatic nuclei are restricted to the basal border of the cells (Fig. 2D). Intercellular canaliculi are narrow and appear to lack junctional complexes.
Ventral glands, like pelvic glands, show little variation (Fig. 3). The cytoplasm is dense and squamous, and a granular secretory product fills the lumen (Fig. 3A). Superficial to the epithelial cells is a myoepithelial layer with a relatively thick sheath of actin fibers (Fig. 3B,C). Myoepithelium also occurs around the other glands, but it is not obvious in active glands and only apparent when the epithelial cells are reduced in size. The cytoplasm of the epithelial cells of the ventral glands contains two kinds of vacuoles: vacuoles with fine granules, interpreted as condensing vacuoles; and vacuoles with a homogeneous substance, interpreted as mature secretory vacuoles (Fig. 3B). Condensing vacuoles often abut upon nuclei, and many secretory vacuoles lie against the apical

FIG. 1.—Anterior cloacal tube of a 45.4-mm snout–vent length male *Plethodon cinereus* collected 16 April 2002. (A) Light micrograph of a semithin transverse section stained with toluidine blue. (B) Transmission electron micrograph showing overview of a Kingsbury’s gland (Kg). (C) Transmission electron micrograph of luminal border of a Kg. (D) Basal border of a Kg. Avg = anterior ventral glands, Bl = basal lamina, Cf = collagen fibers, Cs = cloacal sheath, Cv = condensing vacuoles, Dpg = dorsal pelvic glands, Go = Golgi bodies, Lu = lumen, Nu = epithelial cell nucleus, Sp = sperm in the lumen, and Sv = secretory vacuoles.
plasma membrane. A prominent nucleolus occurs in many sections through the heterochromatic nuclei of epithelial cells (Fig. 3B). Microvilli are scattered along the luminal border (Fig. 3D). The only organelles obvious in the dense cytoplasm are occasional clusters of mitochondria with tubular cristae.

Vent glands, much like Kingbury’s glands, lack conspicuous amounts of secretory material in their lumina (Fig. 4A,B). Secretory vacuoles fill the cells, and other cytoplasm is scant. The secretory vacuoles are biphasic, with a central, moderately dense core surrounded by an electron-lucid ring (Fig. 4B–D). Secretory vacuoles with this appearance are generally interpreted as have a protein core surrounded by carbohydrate. Occasional globules of secretory product found in the lumen have the same appearance as the central core. Apparently, membrane fusion occurs between the secretory vacuoles and the luminal border causing release of the secretion by a merocrine process. Epithelial nuclei are basal and euchromatic (Fig. 4B,D). Microvilli are scattered along the luminal border (Fig. 4C). Intercellular canaliculi are narrow and lack junctional complexes (Fig. 4C,D).

Fig. 2.—Anterior cloacal tube of male *Plethodon cinereus* collected 16 April 2002, continued. (A) Scanning electron microscopy overview of a 43.1-mm snout–vent length (SVL) specimen. (B) Same specimen as in (A), showing higher magnification of dorsal pelvic glands (Dpg). (C) Transmission electron microscopy overview of a Dpg from a 45.4-mm SVL specimen. (D) Same specimen as in (C), showing detail of basal border. Kg = Kingsbury’s glands, Ic = Intercellular canaliculus, Mv = microvilli, Nu = epithelial cell nucleus, Sm = secretory material, Sp = sperm in lumen, Sv = secretory vacuoles, and Tp = tunica propria.
Fig. 3.—Ventral glands of male *Plethodon cinereus* collected 16 April 2002. (A) Light micrograph of a semithin transverse section of an anterior ventral gland from a 45.4-mm snout–vent length (SVL) specimen stained with toluidine blue. (B) Transmission electron micrograph of portions of two anterior ventral glands from the same specimen as in (A). (C) Transmission electron micrograph of the posterior ventral gland of a 45.5-mm SVL specimen. Af = actin fibers, Cv = condensing vacuoles, Gr = granules in the lumen, Mi = mitochondria, Mv = microvilli, My = myoepithelial cell nucleus, No = nucleolus, Nu = epithelial cell nucleus, Smlu = secretory material in the lumen, and Tp = tunica propria.
June.—In specimens from the June sample, the cloacal glands are much reduced in size and secretory activity (Figs. 5 and 6). Basically, the different glands can be recognized only because of their location (Fig. 5A). Anteriorly, most pelvic glands are devoid of secretory product but possess dense, irregular apocrine blebs that extend into the lumen (Fig. 5B). The myoepithelial layer that was difficult to discern in active glands is now apparent (Fig. 5B). Some pelvic glands, although much reduced, still contain residual clusters of secretory vacuoles (Fig. 5C). Ventral glands have narrow lumina and no indications of secretory product (Fig. 5D).

Posteriorly, cloacal glands of the caudal pelvic gland group show some additional variation (Fig. 6A,B). Some caudal pelvic

Fig. 4.—Posterior cloacal chamber of a 45.4-mm snout–vent length (SVL) male *Plethodon cinereus* collected 16 April 2002. (A) Light micrograph of a semithin transverse section stained with toluidine blue. (B) Transmission electron micrograph showing overview of a vent gland (Vg). (C) Transmission electron micrograph of luminal border of a Vg. (D) Basal border of a Vg. Cf = collagen fibers, Cpg = caudal pelvic glands, Ic = intercellular canaliculus, Lu = lumen, Mv = microvilli, Nu = epithelial cell nucleus, Pvg = posterior ventral gland, and Sv = secretory vacuoles.
glands have supranuclear intracytoplasmic spaces and a granular substance in the lumen (Fig. 6A). Others, like some pelvic glands more anteriorly, possess residual secretory vacuoles (Fig. 6B). Again, the actin fibers of myoepithelial cells are much more apparent than in active glands (Fig. 6B).

Vent glands, unlike those in April, often possess many dense secretory vacuoles in the lumen, surrounded by a granular matrix (Fig. 6C). Indications of synthetic activity occur in the supranuclear cytoplasm, including the presence of rough endoplasmic reticulum and condensing vacuoles (Fig. 6D).

**August.**—The cloacal glands in the August sample are even more reduced than those in June, but at least pelvic glands and ventral glands are recognizable (Fig. 7). The cyto-
plasm of the pelvic glands is dense, and the lumen is empty except for a few globules and some granular material (Fig. 7A). In the apical cytoplasm, however, are clusters of what appear to be condensing vacuoles, although adequate resolution of these was not achieved (Fig. 7B). Likewise, the ventral glands appear inactive (Fig. 7C), but examination of the supranuclear cytoplasm indicates the presence of rough endoplasmic reticulum (Fig. 7D). Thus, these glands appear to be initiating synthetic activity. The apical cytoplasm of the ventral glands also contains numerous small electron-dense bodies (Fig. 7D). These resemble primary lysosomes, but their identity and function remain unknown.

October.—Specimens from the October sample have cloacal glands nearly as hyper-
trophied as those from April (Fig. 8). Two of the specimens had sperm in their anterior cloacal tubes (Fig. 8A). Some dorsal pelvic gland and anterior ventral glands did not seem quite as “full” as in April (Fig. 8A,B,D), but others were packed tightly with secretory material (Fig. 8C). Vent glands, like those in April, have little indication of secretory material in the lumen (Fig. 8D).

**DISCUSSION**

Sever (1978b) reported that males of *P. cinereus* contain sperm in their vasa deferentia (=Wolffian ducts) in October and March and that sperm are absent in May and August. Sperm occurs in most gravid female *P. cinereus* collected from March to May, and some residual sperm may remain for a short period after oviposition in early summer.
Females collected in August and October did not contain sperm, even those collected in the latter month with developing ovarian follicles. Caldwell (1975) found spermatophores in the cloacae of 17 of 29 females with developing ova collected in December, January, and February in Indiana. Thus, although males in October have hypertrophied cloacal glands and sperm in their vasa deferentia, and they could therefore potentially make spermatophores and mate, mating is restricted to winter and spring.

Sever (1978b) also found that cloacal glands of *P. cinereus* are most hypertrophied during the breeding season, except for the vent gland.
which showed little variation. In the present study, the vent gland was the only gland that showed much secretory activity in June. The vent gland is not involved in spermatophore formation and is considered a pheromone-producing gland (Sever and Houck, 1985; Sever, 2003).

All of the cloacal glands have secretory vacuoles that abut upon the apical plasma membrane, and apparently fusion of the membranes occurs, causing release of the secretion by a merocrine process. Each of the cloacal glands has secretory vacuoles that are unique in appearance, but all are consistent with secretion of carbohydrates, proteins, or both. Kingsbury’s glands, which secrete upon ciliated epithelium in the anterior cloacal tube, are modified mucous glands. The secretions of pelvic and ventral glands appear more complex, and the biphasic secretory vacuoles of the vent gland indicate a glycoprotein.

This study provides the first ultrastructural description of male cloacal glands in salamanders. Although all males in Salamandroidea examined thus far possess the same basic suite of glands, there is considerable variation among them. For example, the vent glands of plethodontids are relatively rudimentary compared with those of other salamandroids. This is especially true for the Salamandridae, in which the vent gland homolog, the dorsal gland, can be the largest of any of the gland clusters (Sever, 1992). Two female-attracting pheromones, the decapeptides sodefrin and silefrin, have been isolated from the dorsal glands of *Cynops pyrrhogaster* (Kikuyama et al., 1995, 1997) and *C. ensicauda* (Yamamoto et al., 2000), respectively. The cellular details of the production of these substances would be interesting to reveal and compare among taxa. Pheromone-producing glands might show more variation in cytology in salamanders than spermatophore-producing glands.

Also of interest would be an examination of the ultrastructure of cloacal glands in two of the nonspermatophore-producing families, Cryptobranchidae and Hynobiidae. Both sexes in these families possess only one type of gland, the ventral gland (except for the hynobiid *Onychodactylus japonicas* that has a unique suite of glands). The ventral gland in cryptobranchids and hynobiids is hypothesized to secrete pheromones (Sever, 1991b). The comparison of the cytology of these ventral glands to ventral glands and pheromone-producing glands in salamandroids could reveal insight into the phylogeny of mating glands in salamanders.

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**LITERATURE CITED**


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