

THE STRUCTURE OF RATHKE'S GLANDS IN THE SOFTSHELL TURTLES *APALONE MUTICA* AND *A. SPINIFERA*

MICHAEL V. PLUMMER^{1,2} AND STANLEY E. TRAUTH³

¹Department of Biology, Harding University, Searcy, Arkansas 72149, USA

²Corresponding author e-mail: plummer@harding.edu

³Department of Biological Sciences, Arkansas State University, P. O. Box 599, State University, Arkansas 72467-0599, USA

Abstract.—Although Rathke's glands are thought to be homologous among Testudines, we know little about the gland structure in many turtle lineages, including the trionychid softshells. We describe the macro, micro, and ultrastructural anatomy of Rathke's glands in the softshell turtles *Apalone mutica* and *A. spinifera*. Rathke's glands of both species are structurally similar and consist of two pair of anatomically similar glands, an axillary and an inguinal pair. Both are large exocrine glands derived from epidermal epithelium and appear to be specialized for the production and extrusion of a secretion onto the body surface. Glands consist of one or more apparent holocrine secretory lobules encased in a muscle and connective tissue capsule. Secretion droplets and cellular debris fill lobules that empty through secretory ducts leading to exterior pores. Unlike all non-trionychoid turtles, each axillary gland of *A. mutica* and *A. spinifera* has a lengthy secretory duct that empties through a pore on the leading edge of the carapace; otherwise, gland structure closely resembles that of Rathke's glands in other lineages of turtles. The overall similarity in the anatomy of Rathke's glands in softshells and other turtles supports the notion that Rathke's glands are ancient structures and homologous among all turtle lineages. We discuss possible functions of Rathke's glands in softshells and other turtles; however, the glands' function remains largely unknown and offers a challenging opportunity for behavioral and chemical ecologists.

Key Words.—*Apalone*, integumental glands, musk glands, Rathke's glands, scent glands, softshell turtles

INTRODUCTION

The presence of small osseous canals in the bridge of an early Jurassic *Kayentachelys* turtle shell provides evidence for the presence of externally-secreting Rathke's glands found widely among recent turtles and establishes Rathke's glands as the oldest known amniote integumental gland (Weldon and Gaffney 1998). Since their initial description (Rathke 1848), the literature variously referred to the glands as musk glands ("moschusdrüsen"), scent glands, axillary glands, or inguinal glands until Ehrenfeld and Ehrenfeld (1973) formally named them Rathke's glands. Despite structural similarities among the few descriptions of Rathke's glands in turtles, little is known about the gland morphology in most Chelonia, including the trionychoid lineage (families Trionychidae and Carretochelidae). Hatchlings have fully formed and functional Rathke's glands (Stromsten 1917; Zangerl 1941; Neill 1948; Rostal et al. 1991). One to five pairs of Rathke's glands are embedded in the ventrolateral aspect of the trunk and secrete a primarily carbohydrate-protein onto the body surface through slit-like pores located in the axillary, inguinal, or inframarginal regions (Waagen 1972; Ehrenfeld and Ehrenfeld 1973; Rainey 1981; Rostal et al. 1991; Weldon and Gaffney 1998). Rathke's glands appear to be specialized for the production and episodic forceful extrusion of a sometimes-malodorous secretion (Waagen 1972; Ehrenfeld and Ehrenfeld 1973);

however, their function is undetermined. The most comprehensive review of Rathke's glands available, gave nine patterns of external pore number and location among turtles (Waagen 1972). Fossil and recent evidence suggest the presence of Rathke's glands is the basal condition for all turtles (Peters 1848; Gadow 1923; von Eggeling 1931; Loveridge and Williams 1957; Waagen 1972; Weldon and Gaffney 1998; Hervet 2006). Their absence is presumably derived and occurs primarily in terrestrial turtles (testudinids and a few emydids; Ehrenfeld and Ehrenfeld 1973; Waagen 1972).

Despite knowledge of the presence of Rathke's glands in turtles for 160 years, descriptions of the glandular anatomy exist for only a few species in the families Chelydridae, Kinosternidae, Bataguridae, and Cheloniidae (*Chelydra serpentina*, Zangerl 1941; *Sternotherus odoratus*, Dahlgren and Kepner 1908; Ehrenfeld and Ehrenfeld 1973; *Mauremys caspica*, Müller 1961; *Chelonia mydas*, Ehrenfeld and Ehrenfeld 1973, Solomon 1984; *Lepidochelys olivacea*, Ehrenfeld and Ehrenfeld 1973; *Lepidochelys kempi*, Weldon and Cannon 1992; *Caretta caretta*, Stromsten 1917). The more comprehensive anatomical descriptions that contain illustrations include Ehrenfeld and Ehrenfeld (1973), Solomon (1984) and, to a lesser extent, Dahlgren and Kepner (1908).

Although the knowledge that softshell turtles possess Rathke's glands is not new (Rathke 1848; Smith 1931; Waagen 1972), there is no systematically described

gland morphology for any trionychid or carettocheliid species. Rathke's glands appear to be ancient structures in softshells because they are present in the primitive *Lissemys* (Deraniyagala 1939) and in the closest trionychid relative, *Carettochelys* (Waagen 1972).

Old World trionychids have three pair of Rathke's glands, whereas New World trionychids (*Apalone* spp.) have two pairs; an inguinal pair and an axillary pair, the pore occurring "far anterior" (Waagen 1972). Unlike other turtles, the axillary pores of trionychoids are uniquely located on the leading edge of the carapace just above the shoulders (Deraniyagala 1939; Waagen 1972) and connect via an elongated duct to the axillary gland located in the suprascapular region (Waagen 1972). Our objective in this paper is to systematically describe the structure of Rathke's glands in two New World softshell species, *Apalone mutica* and *A. spinifera*. We describe the macro, micro, and ultrastructural anatomy as revealed by a combination of gross dissection and light, scanning electron, and transmission electron microscopy. We also discuss the proposed functions of Rathke's glands in turtles and appeal to chemical and behavioral ecologists to study this interesting and enigmatic gland.

MATERIALS AND METHODS

We examined the Rathke's gland axillary orifices and distal portions of the ducts in 15 museum specimens of *Apalone mutica* (5 males, 7 females, 3 juveniles) collected in 1972–73 in the Kansas River near Lawrence, Douglas County, Kansas, USA. We also examined freshly collected (2007) specimens of *A. mutica* (13 males, 1 female) and *A. spinifera* (1 male, 1 female) from the White River in the vicinity of Georgetown, White County, Arkansas, USA. We returned Arkansas turtles to the laboratory to be measured (carapace length, CL) and for sex determination. We used an intraperitoneal injection of sodium pentobarbital to euthanize the turtles prior to dissection.

For Arkansas specimens, we extracted and macroscopically photographed the axillary and inguinal Rathke's glands (with intact ducts, when available) and fixed them in either 10% neutral buffered formalin—NBF (for paraffin sectioning) for 48 h, or a 2% glutaraldehyde (GTA) solution buffered with 0.1 M sodium cacodylate at a pH of 7.2 (for plastic sectioning) for 2 h. For postfixation, we used 1% osmium tetroxide, buffered as above, for 2 h. We used routine histological techniques to prepare tissues for light microscopy (LM) and employed the paraffin embedding methods (Presnell and Schreiber 1997). We dehydrated glands and accompanying tissues in a graded series of increasing ethanol solutions (50–100%), cleared with xylene, and infiltrated and embedded in paraffin wax. We trimmed paraffin/tissue blocks of excess wax, serially sectioned them into ribbons 8 μ m thick using a rotary microtome,

and affixed sections to microscope slides using Haupt's adhesive while floating on a 2% NBF solution. Alternating sets of four slides received treatments of four histological stains: (1) hematoxylin and eosin (H & E) for general cytology; (2) Pollak trichrome stain for connective tissues and mucins; (3) alcian blue 8GX for carboxylated glycosaminoglycans; and (4) the periodic-acid Schiff (PAS) procedure for neutral carbohydrates, mucopolysaccharides and other carbohydrate-protein substances.

For LM of plastic-embedded tissues, we followed the methods of Bozzola and Russell (1992). Following fixation, we dehydrated tissues in a graded series of increasing ethanol solutions (70–100%), placed in a 50/50% acetone/plastic mixture for overnight infiltration, and embedded in Mollenhauer's Epon-Araldite #2 (Dawes 1988). For thick sectioning (approximately 1 μ m in thickness) and staining, we used glass knives on an LKB Ultratome (Type 4801A) with Ladd® multiple stain (LMS), respectively. We used a Nikon Eclipse 600 epifluorescent light microscope with a Nikon DXM 1200C digital camera (Nikon Instruments Inc, Melville, New York, USA) for photomicroscopy and selected macroscopic images. We also used a Konica Minolta Maxxum 7D digital single lens reflex camera fitted with a ProMaster AF Macro lens to photograph macroscopic images of turtles and glandular tissues.

For scanning electron microscopy (SEM), dehydration of glandular tissues occurred in a graded series of increasing ethanol solutions (70–100%), followed by fluid exchange to 100% amyl acetate. We used a Samdri-780 critical point drier (Tousimis Research Corporation, Rockville, Maryland, USA) to remove amyl acetate (31°C, 1072 psi, ventilation rate ~ 100 psi/min). We mounted tissue samples on 25.4 mm aluminum SEM specimen mounts and coated them with gold using a Cressington 108 sputter coater (Cressington Scientific Instruments Ltd, Watford, UK). Quantitative and qualitative analysis of tissues utilized a Vega TS 5136XM digital scanning electron microscope (Tescan USA Inc., Cranberry Township, Pennsylvania, USA) at 19.5 kV.

We also used the plastic-embedded samples prepared for LM for transmission electron microscopy (TEM). We sectioned tissue blocks with a glass or diamond knife. We picked up sections with 200 mesh copper grids, and then stained them with uranyl acetate (3% aqueous) and lead citrate for 30 min each. Examination of grids utilized a JEOL 100 CX-II transmission electron microscope (JEOL USA, Inc., St. Louis, Missouri, USA) at 60 kV (55 μ A). We generated positive digital images by scanning developed TEM negatives using an Epson Perfection 4990 scanner (Epson America, Inc., Long Beach, California, USA).

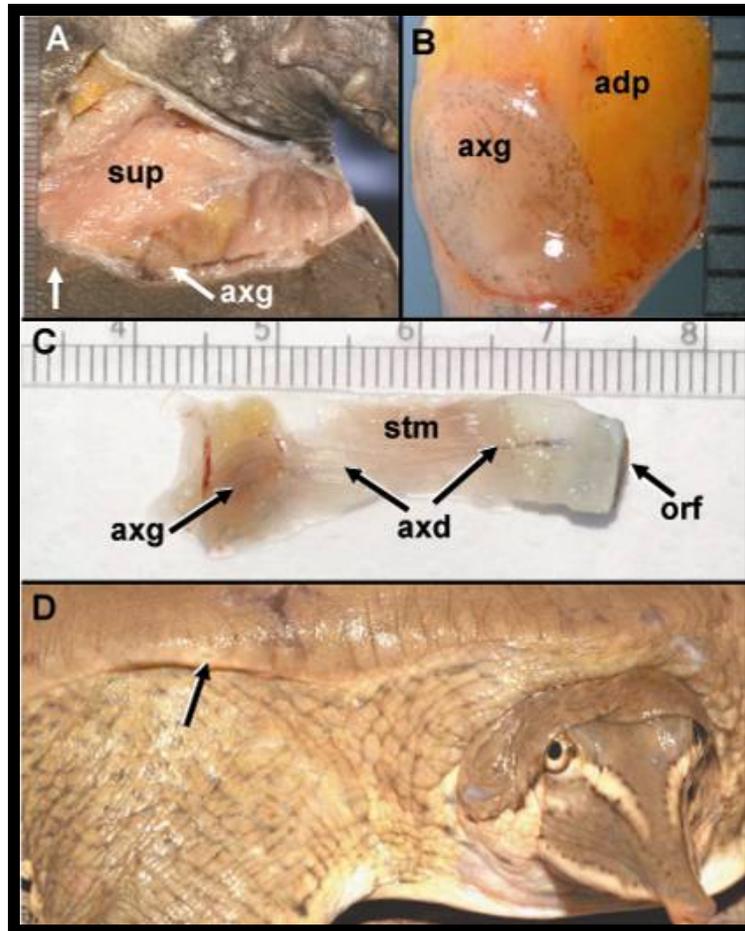


FIGURE 1. Axillary Rathke's glands in *Apalone mutica*. (A) Anatomical position (arrow at lower left points cranially) of the right gland (axg) of a male (ASUMZ 30733; CL = 174 mm; dorsal view) at postero-medial edge of suprascapular muscle (sup). Scale bar in mm. (B) Gland of a male (ASUMZ 30749; CL = 176 mm) embedded in adipose tissue (adp). Scale bar in mm. (C) Excised left gland (axg) and duct (axd) of a male (ASUMZ 30748; CL = 175 mm). Distal region of duct embedded in dense connective tissue of carapace before reaching orifice (orf). Duct appears as a thin faint black line (due to scattered melanocytes embedded in wall) within striated muscle (stm). Scale bar numbered in cm. (D) Male (ASUMZ 30748) exhibiting duct orifice (arrow) of right gland.

We generally followed Ehrenfeld and Ehrenfeld (1973) and Solomon (1984) for the descriptive terminology of Rathke's glands and ducts; however, we have modified some cellular features (e.g., we use secretory vacuoles rather than secretory granules) to better express the nature of these structures in softshell turtles.

RESULTS

Macroscopic Anatomy.—In male *Apalone mutica*, the axillary Rathke's glands lie midway beneath the anterior border of the third pleural bones of the carapace and are situated within an aggregate of loose connective tissue, striated muscle, blood vessels, nerves, and adipose tissue at a point near the postero-medial edge of the suprascapular muscle (Fig. 1A). Axillary Rathke's

glands range ~ 6.5 mm in length and ~ 4.5 mm in width (Fig. 1B). A tunic of striated muscle encapsulates each gland which possesses two lobules. The muscle is firmly attached to a dense connective tissue layer that immediately encircles the gland's secretory epithelium. A long, narrow axillary (secretory) duct extends cranially ~ 25 mm to open externally through a pore located on the anterior margin of the carapace (Fig. 1D). During gland dissection and removal, physical stimulation of nerves leading to the axillary Rathke's gland often resulted in spasmodic contraction of the muscular tunic.

A thin flat sheath of striated muscle envelopes the proximal portion of each axillary duct (Fig. 1C) tissue that firmly attaches to the carapace but gradually dissipates in an area ~ 18 mm from the gland proper. The distal half of the axillary duct becomes lightly-to-

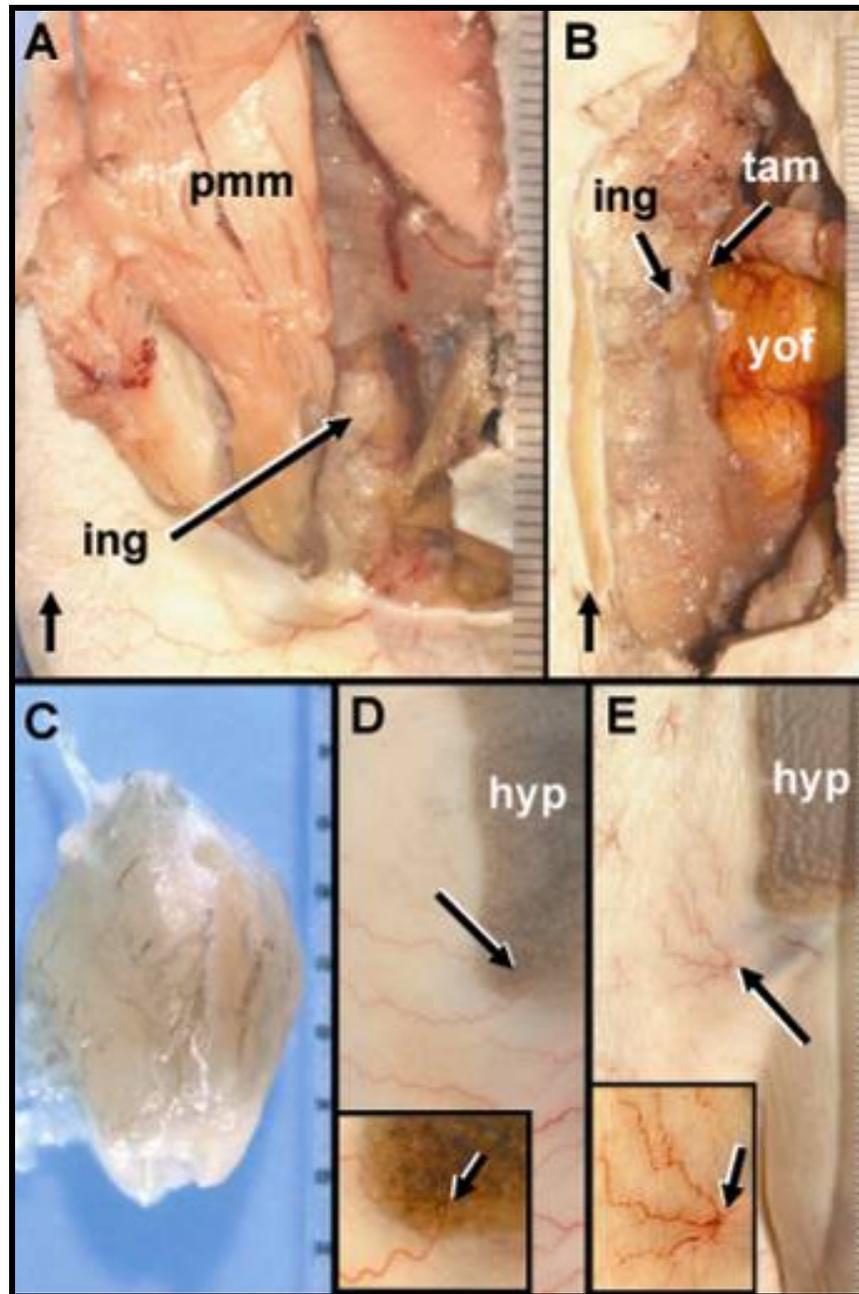


FIGURE 2. Inguinal Rathke's glands in *Apalone mutica*. (A) Anatomical position (arrow at lower left points cranially) of the right gland (ing) of a male (ASUMZ 30733; ventral view). Scale bar in mm; ppm = pectoralis major muscle. (B) Anatomical position of the right gland (ing) of a female (ASUMZ 30738; CL = 268 mm). Scale bar in mm; yof = yolked ovarian follicle; tam = transversus abdominis muscle. (C) Gland of specimen in B. Scale in mm. (D) External orifice (not clearly visible at tip of arrow) of gland duct of a male (ASUMZ 30807; CL = 197 mm); inset box reveals position of orifice (at tip of arrow at the confluence of two blood vessels) on the hypoplastron bone (hyp). (E) Orifice (arrow) of gland duct of female specimen in B (inset box reveals position of orifice at the confluence of several blood vessels) away from the hypoplastron. Scale in mm for both D and E.

darkly pigmented (Fig. 1C) as the duct proceeds distally; the pigmentation is especially evident as the duct enters the carapace proper. The most distal 5 mm segment of the duct extends through the dense connective tissue of the anterior carapacial margin (Figs. 1C; 4A). A pale region is found circumscribing the external orifice of the

duct in both sexes (Fig. 1D) rendering the minute pore visually conspicuous dorsal to each forelimb. The pore is especially noticeable when the duct is sufficiently full to force the orifice open and reveal the pale greenish secretory material contained within.

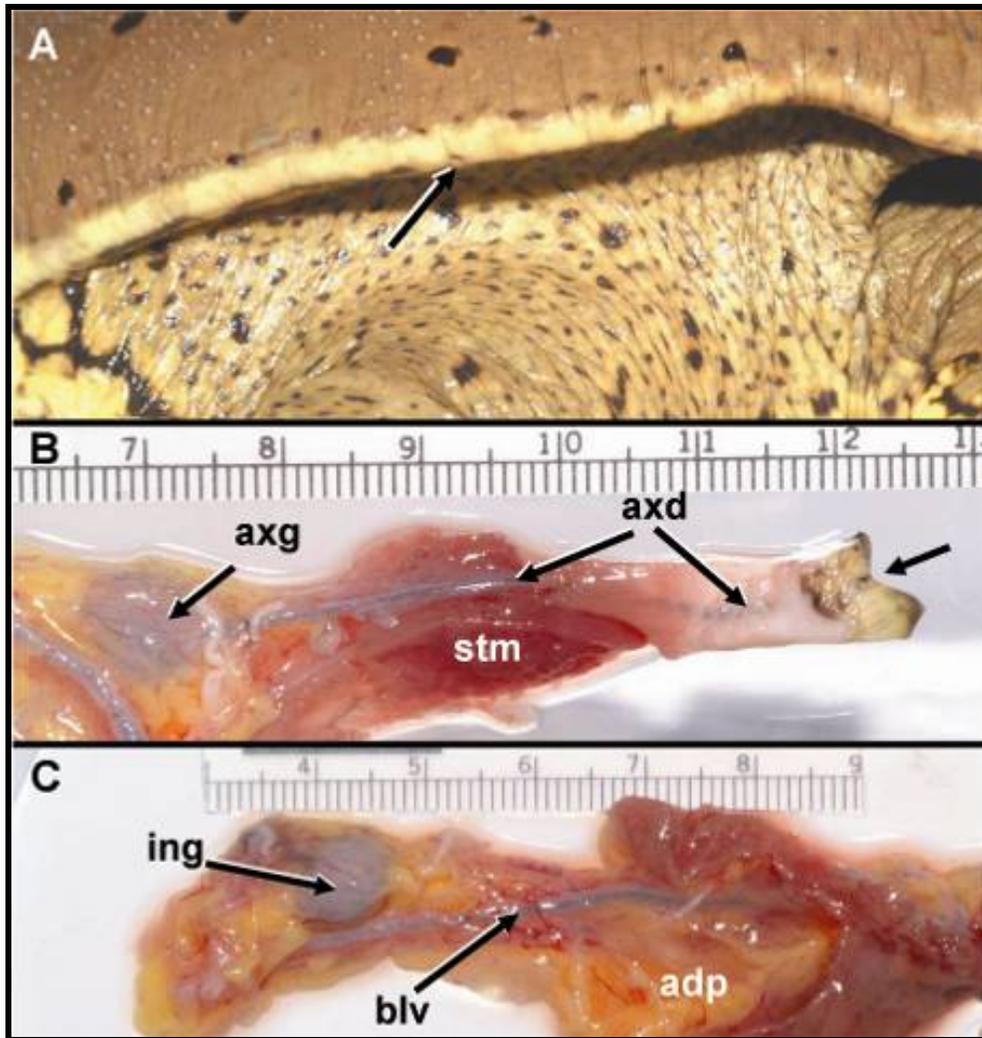


FIGURE 3. Axillary and inguinal Rathke's glands in *Apalone spinifera*. (A) Orifice (arrow) of axillary gland duct of male (ASUMZ 30773; CL = 212 mm). (B) Excised left axillary gland (axg) and duct (axd) of a female (ASUMZ 30772; CL = 343 mm); stm = striated muscle. Distal region of darkly pigmented duct embedded in dense connective tissue of carapace before reaching orifice (arrow). Scale bar numbered in cm. (C) Excised left inguinal gland (ing) embedded in adipose tissue of female in B. Scale bar numbered in cm; adp = adipose tissue; blv = blood vessel.

The inguinal Rathke's glands in male *A. mutica* lie dorsal to the pectoralis major muscle in a region ~ 8 mm cranial to the inguinal notch and beneath the posteriolateral edge of the hypoplastron bones. Each inguinal Rathke's gland has at least two lobules, and is embedded in adipose tissue and encapsulated by a thin muscular tunic (Fig. 2A–C). Inguinal Rathke's glands occur just outside the pleuroperitoneal cavity, immediately adjacent to the transversus abdominis muscle (Fig. 2B). Inguinal Rathke's glands are slightly smaller than axillary glands ranging approximately ~ 5 mm in length and ~ 3.5 mm in width (Fig. 2C). The inguinal duct passes through a foramen in the hypoplastron bone of males or extends deep and caudolaterally away from the surface of the hypoplastron ~ 10 mm in females (Fig. 2D, E). In either case, the

orifice is an inconspicuous, minute pore. In both sexes, the pore site features an array of surface blood vessels that radiate mostly laterally away from the orifice (Fig. 2D, E).

The axillary and inguinal Rathke's glands of female *A. mutica* are generally similar to those of males, except that the glands and ducts are slightly longer and larger in diameter (compare Figs. 1C and 3B–C). Females also exhibit more adipose tissue in areas surrounding the glands.

The axillary and inguinal Rathke's glands and duct systems of *A. spinifera* differ slightly from those of *A. mutica* in part due to its larger body size. For example, the axillary duct pore may be obscured in *A. spinifera* (Fig. 3A) by the yellowish marginal edge among the small-scattered pigmented patches and surface

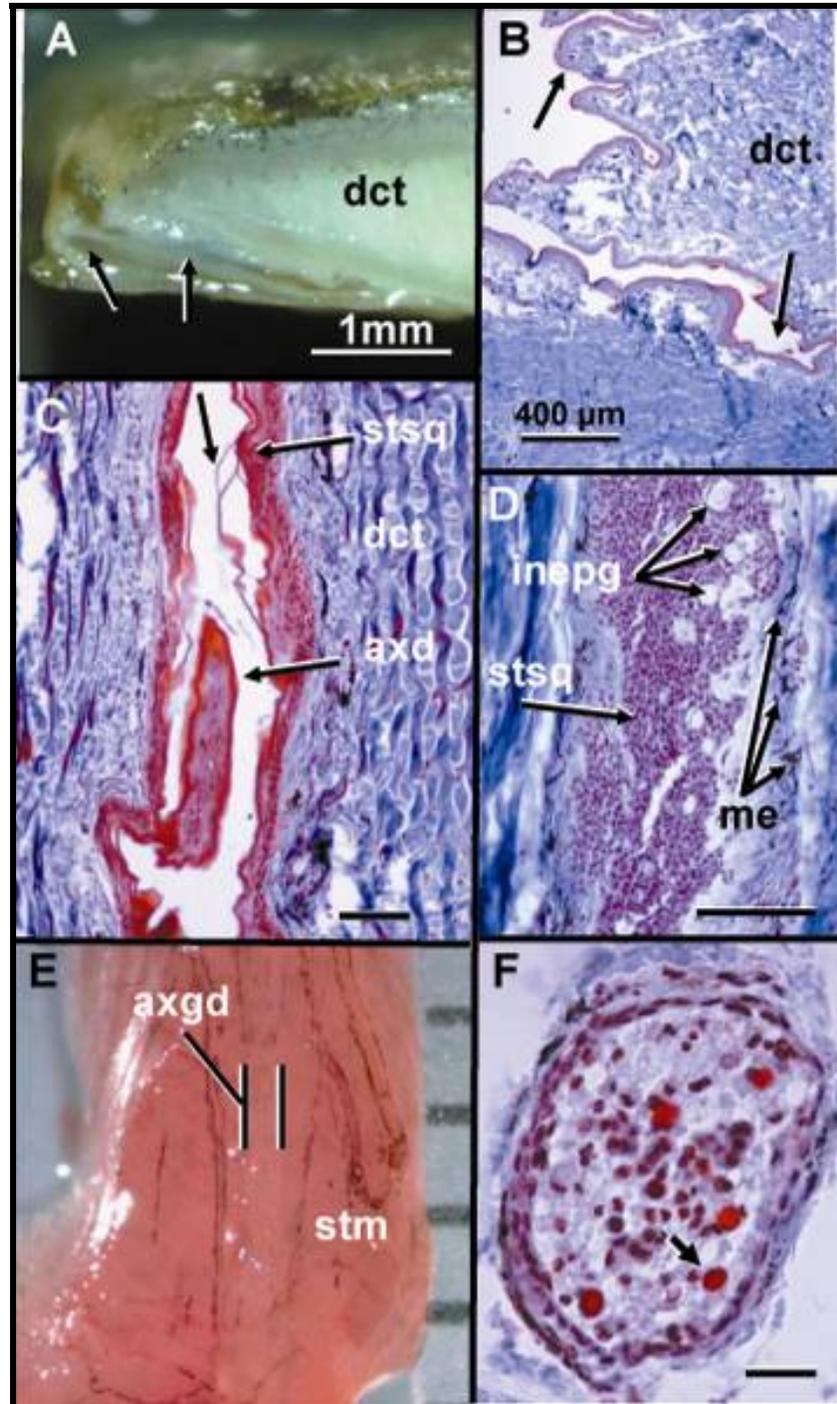


Figure 4. Duct morphology of Rathke's glands in *Apalone mutica* (A-D; F) and *A. spinifera* (E). (A) Mid-sagittal macroscopic view of distal portion of right duct in *A. mutica*. Left arrow points to region near duct orifice; right arrow points to duct passageway obliquely transversing through ventral surface of the anterior margin of carapace. dct = dense connective tissue. (B) Light micrograph of distal portion of duct (arrow) stained with Pollak trichrome stain in male specimen (ASUMZ 30748). Stratified squamous epithelium of skin (upper arrow) extends internally to line the duct wall into a region beyond duct orifice; lower arrow points to lumen of duct. Abbreviation as in A. (C) Light micrograph of segment of right axillary duct (axd) revealing a stratified squamous epithelium (stsq) and a lamellar layering of the dct of carapace. Some sloughing of cornified stsq (arrow within duct) is apparent. Pollak trichrome stain; line = 100 µm. (D) Light micrograph of longitudinal section through the wall of the right duct (ASUMZ 30748) revealing numerous intraepithelial glands (inepg) embedded within the stratified squamous epithelium (stsq) of duct wall; melanocytes (me) lie along the exterior of the duct wall; Pollak trichrome stain; line = 100 µm. (E) Macroscopic view of mid-region of axillary duct (axgd) surrounded by deep red striated muscle (stm) in a male (ASUMZ 30773). Scale bar in mm. (F) Light micrograph of cross section of mid-regional area of axillary duct in a male (ASUMZ 30746; CL = 160 mm) revealing a lumen filled with large acidophilic secretory vacuoles (arrow), smaller dark staining vacuoles, and cellular debris. Pollak trichrome stain; line = 20 µm.

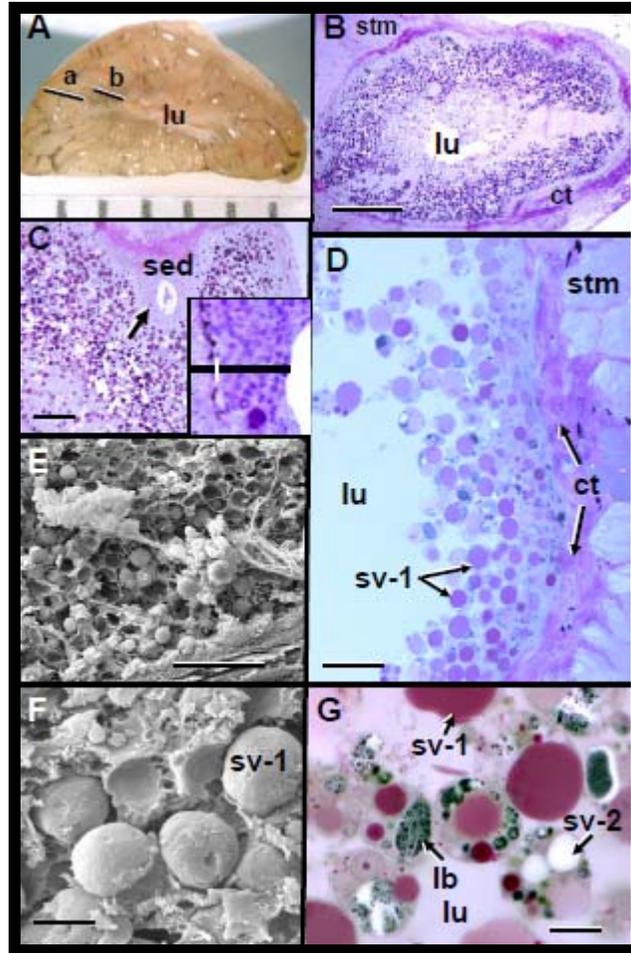


FIGURE 5. Secretory epithelium of axillary and inguinal Rathke's glands in *Apalone mutica* (B, C, E, and F) and *A. spinifer* (A, D, and G). (A) Macroscopic view of section through a freshly necropsied axillary gland (ASUMZ 30773). Notice muscular tunic (a), thick epithelial lining (b), and open lumen (lu); b may vary in thickness due to detachment or sloughing of glandular secretions. Scale bar in mm. (B) Light micrograph (thick section) of cross section of portion of axillary gland (ASUMZ 30748) encapsulated by a layer of striated muscle (stm) attached to a dark-staining layer of connective tissue (ct), which immediately surrounds secretory epithelium. The lumen (lu) is surrounded by numerous spherical secretory vacuoles and cellular debris. Ladd multiple stain; line = 400 μ m. (C) Light micrograph (thick section) of anterior margin of axillary gland revealing a prominent, thick-walled secretory duct (sed). Inset shows magnification of duct wall in region of arrow tip. Horizontal black bar within inset spans duct epithelium and adjacent secretory epithelium (vertical white bar separates the epithelia); Ladd multiple stain; line = 100 μ m. (D) Portion of axillary gland secretory epithelium (ASUMZ 30773) exhibiting cells containing large, PAS positive, Type 1 secretory vacuoles (sv-1). Ladd multiple stain; abbreviations as in other figures; line = 50 μ m. (E) Scanning electron micrograph of an inguinal gland (ASUMZ 30773) in region near epithelium exposing Type 1 secretory vacuoles; line = 50 μ m. (F) Scanning electron micrograph of several Type 1 secretory vacuoles of an axillary gland (ASUMZ 30733) exposed in secretory vacuole-apoptotic cell complex. Line = 20 μ m. (G) Light micrograph of intact secretory cells within lumen of axillary gland (ASUMZ 30773) exhibiting Type 1 and Type 2 secretory vacuoles (sv-2) along with multiple vacuoles containing lamellar bodies (lb). Color digitally enhanced; line = 10 μ m.

indentations instead of being outlined by a pale region. In addition to being a longer duct (~ 45 mm in an adult female; Fig. 3B) stemming from overall greater body size in females, pigment occurs along the entire length of the axillary duct. Finally, the gland is proportionally larger in *A. spinifer* and is darker in coloration than in *A. mutica*.

Microscopic Anatomy.—The duct systems of axillary Rathke's glands of both *Apalone* species are similar (Fig.

4). In *A. mutica*, the distal 8 mm segment of the duct lies along the ventral surface of the anterior margin of the carapace (Fig. 4A). A relatively thick stratified squamous epidermal epithelium (~ 40 μ m in height) penetrates the distal portion of the duct near its orifice (Fig. 4B). The epithelium rests upon a dermis of dense connective tissue organized into alternating dark and light layers of collagenous fiber bundles (Fig. 4C) that become less apparent near the duct orifice (Fig. 4B). The duct passageway is mostly irregular, exhibiting

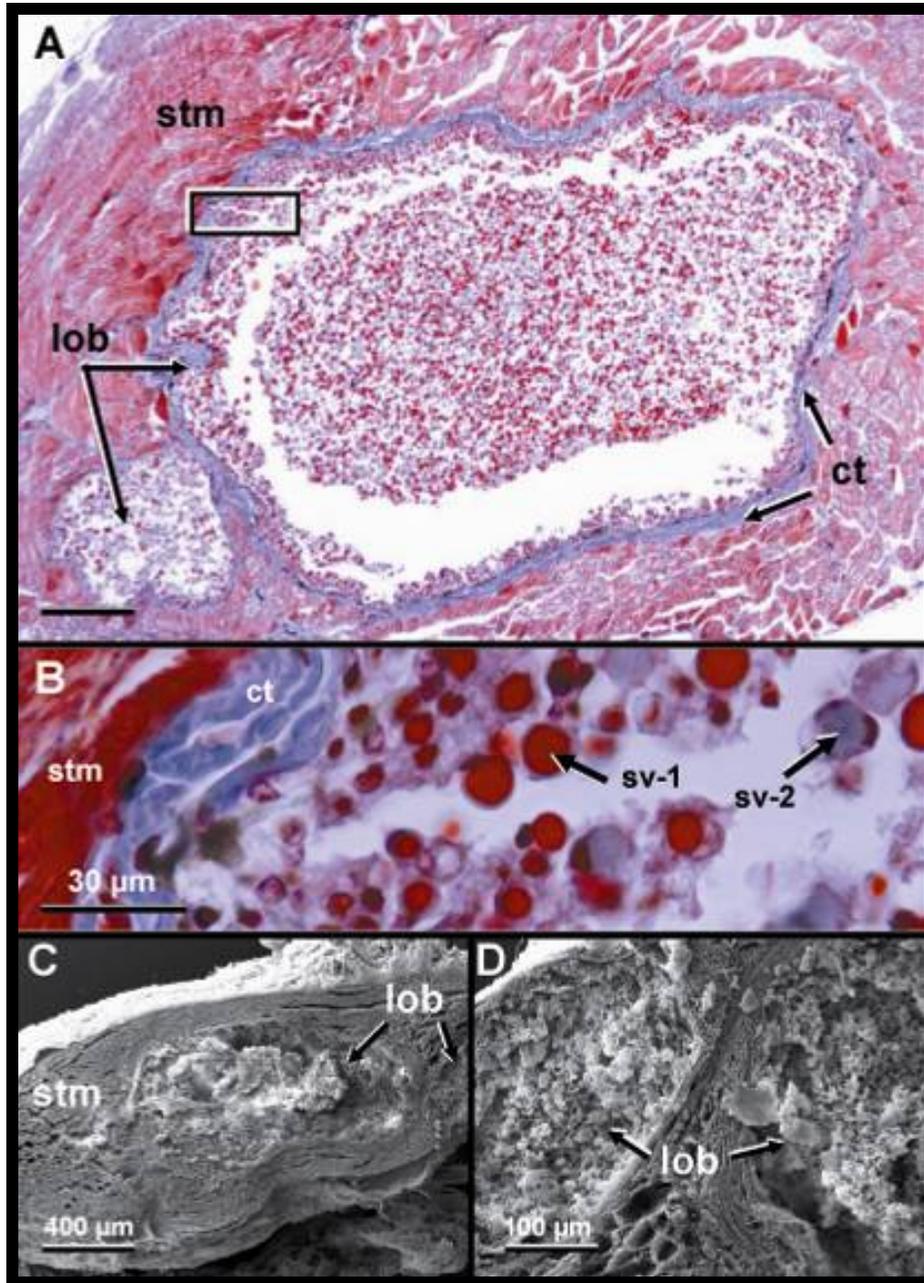


FIGURE 6. Secretory lobules of axillary and inguinal Rathke's glands in *Apalone mutica*. (A) Light micrograph of cross section through right axillary gland (ASUMZ 30746) exhibiting portions of two lobules (lob), each surrounded by a layer of connective tissue. Pollak trichrome stain; line = 200 μ m. See B for inset box. (B) Inset from A of gland wall illustrating Types 1 (red) and 2 (blue) secretory vacuoles, thick connective tissue, and striated muscle of gland tunic. (C) Scanning electron micrograph of section through an inguinal gland (ASUMZ 30733) revealing two secretory lobules. Note thick muscle-connective tissue tunic. (D) Scanning electron micrograph of cross section of middle region of same gland in C showing separating of secretory lobules by thick layers of striated muscle. See previous figures for abbreviations

small outpocketings and depressions (Fig. 4C); a thin stratum corneum loosely adheres to the epidermal surface. Numerous intraepithelial glands are embedded within the stratified squamous epithelium near its basal lamina in the distal region (Fig. 4D). This region of the duct is especially evident due to melanocytes bordering

the basal lamina (Fig. 4D). Striated muscle envelopes the middle and proximal regions of the duct (Figs. 1C). The muscle tissue of freshly necropsied ducts of *A. mutica* is less vascularized compared to its counterpart in *A. spinifera* (Fig. 4E). The middle segment of the duct is less conspicuous than the distal region due to the near

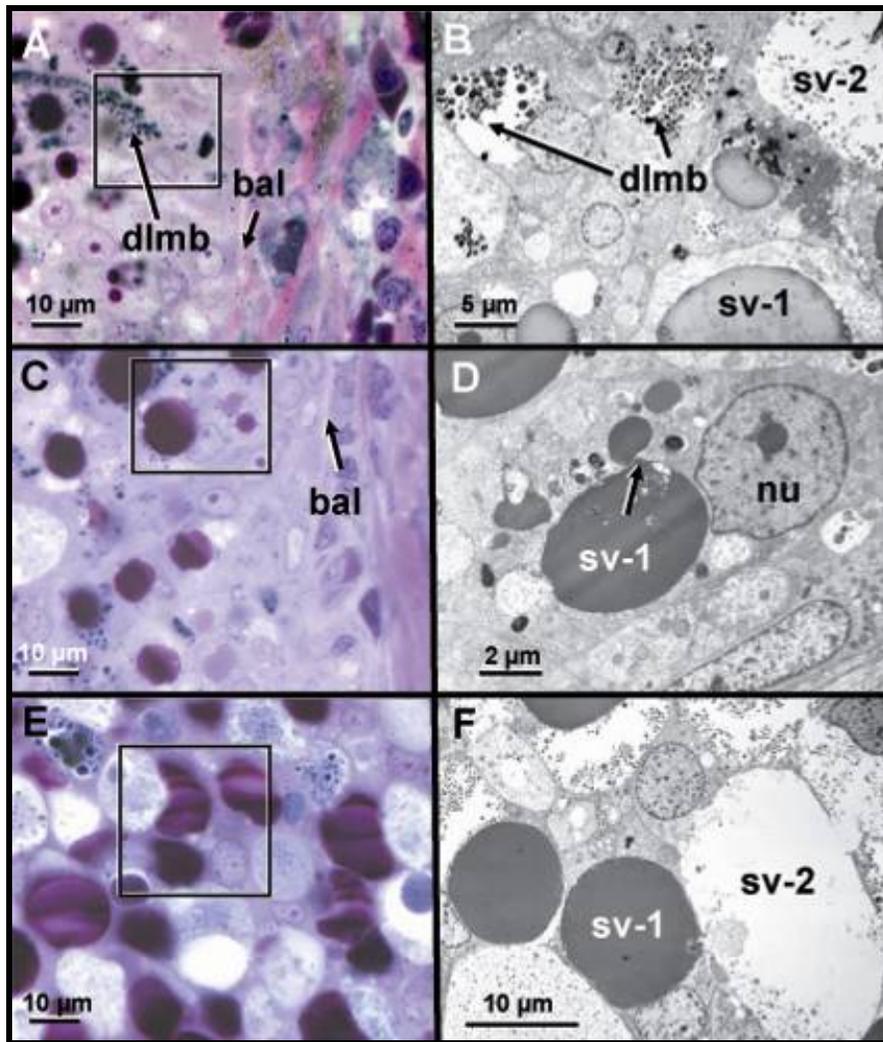


FIGURE 7. Secretory epithelium of axillary Rathke's gland in *Apalone mutica* (ASUMZ 30748). (A) Light micrograph (thick section) of epithelial cells near basal lamina (bal) exhibiting dark-staining, granular vesicles (presumably dense lamellar bodies = dlmb). Ladd multiple stain; see B for inset box. (B) Transmission electron micrograph (representative of inset box in A) exhibiting epithelial cells containing Types 1 and 2 secretory vacuoles; several Type 2 vacuoles exhibit dense lamellar bodies. (C) Light micrograph (thick section) of epithelium near basal lamina revealing developing Type 1 secretory vacuole. Ladd multiple stain; see D for inset box. (D) Transmission electron micrograph (representative of inset box in C) exhibiting epithelial cells containing Type 1 secretory vacuoles. Arrow points to cytoplasmic portal into enlarging Type 1 vacuole; nu = nucleus of epithelial cell. (E) Light micrograph (thick section) of secretory cells near lumen revealing maximum development of secretory vacuoles. Ladd multiple stain; see F for inset box. (F) Transmission electron micrograph (representative of inset box in E) exhibiting intact epithelial cells dominated by Types 1 and 2 secretory vacuoles.

absence of melanocytes. A cross-section of the duct in this region reveals a thin 2–3 layer epithelium and evident secretory material (Fig. 4F).

Internally, a secretory epithelium bound closely to a thick (~ 30 µm) layer of connective tissue lines the glandular medulla of the axillary and inguinal Rathke's glands of both *A. mutica* and *A. spinifera* (Fig. 5A; B). The secretory duct of the axillary gland enters the medulla by projecting a prominent ridge along an anterior wall (Fig. 5C). Here, the duct is ~ 150 µm in diameter, and the epithelium is 6–8 cell layers thick (Fig. 5C inset). The glandular epithelium also resides along the external surface of the duct's exposed tubular eminence within the medulla.

The glandular lining of Rathke's glands is a complex stratified holocrine epithelium composed of two cell types: basal epithelial cells, which reside along a thin basal lamina, and secretory cells, which apparently progressively enlarge as a wall of proliferating cells push them toward the open lumen of the medulla (Figs. 5–7). The largest secretory cells vary from ~ 28 µm in diameter in *A. spinifera* to ~ 25 µm in *A. mutica*. Secretory cells contain two kinds of primary secretory vacuoles based upon their staining properties and ultrastructural characteristics. The most conspicuous type, the large Type 1 secretory vacuole (sv-1; Fig. 5D–G), appear mildly acidophilic with H & E, highly acidophilic using Pollak trichrome, mostly basophilic

using LMS, strongly PAS+, and show no affinity for Alcian blue. Type 1 secretory vacuoles appear as mostly homogenous, spherical-to-somewhat oblong organelles that dominate the cytoplasm of most secretory cells. They maintain their cylindrical integrity within the glandular medulla and within the secretory duct (Fig. 4F) following elaboration from disintegrating secretory cells. Their individual epithelial cells loosely encase the Type 1 vacuoles which can be freely liberated from the apoptotic cell complex (Fig. 5E–F). The largest sv-1 in *A. mutica* was ~ 10.0–16.4 μm (\bar{x} = 13.7 \pm 1.65 μm ; n = 20) and (\bar{x} = 11.7 \pm 1.62 μm ; n = 20) in axillary and inguinal glands, respectively; the sv-1 of axillary glands of *A. spinifera* are larger (\bar{x} = 20.9 \pm 2.68 μm ; range = 17.8–25.0 μm ; n = 10). The deposition of secretory material into sv-1 is visible with TEM (Fig. 7D and 8C).

A second category of secretory vacuole, Type 2 (sv-2), appeared as mostly spherical, translucent-to-opaque organelles. We observed sv-2 (Fig. 5G) in all LM tissue preps as partially-to-mostly evacuated spheres (Fig. 5B–D; G). They appeared mildly blue (basophilic) when stained with Pollak trichrome (Fig. 6A and B) and a light blue (basophilic) when stained with LMS, revealing a flocculent intravacuolar material (Fig. 7).

Ultrastructural Anatomy.—We examined the glandular ultrastructure of secretory cells in axillary Rathke's glands of *A. mutica* progressing from the basement membrane (Fig. 7A–D) toward the lumen (Fig. 7E–F). Micrographs of secretory cells taken near the basal epithelium (Fig. 7A) revealed not only developing vacuoles (sv-1 and sv-2), but also diffuse clusters of osmophilic granules (left upper quadrant in Fig. 7A

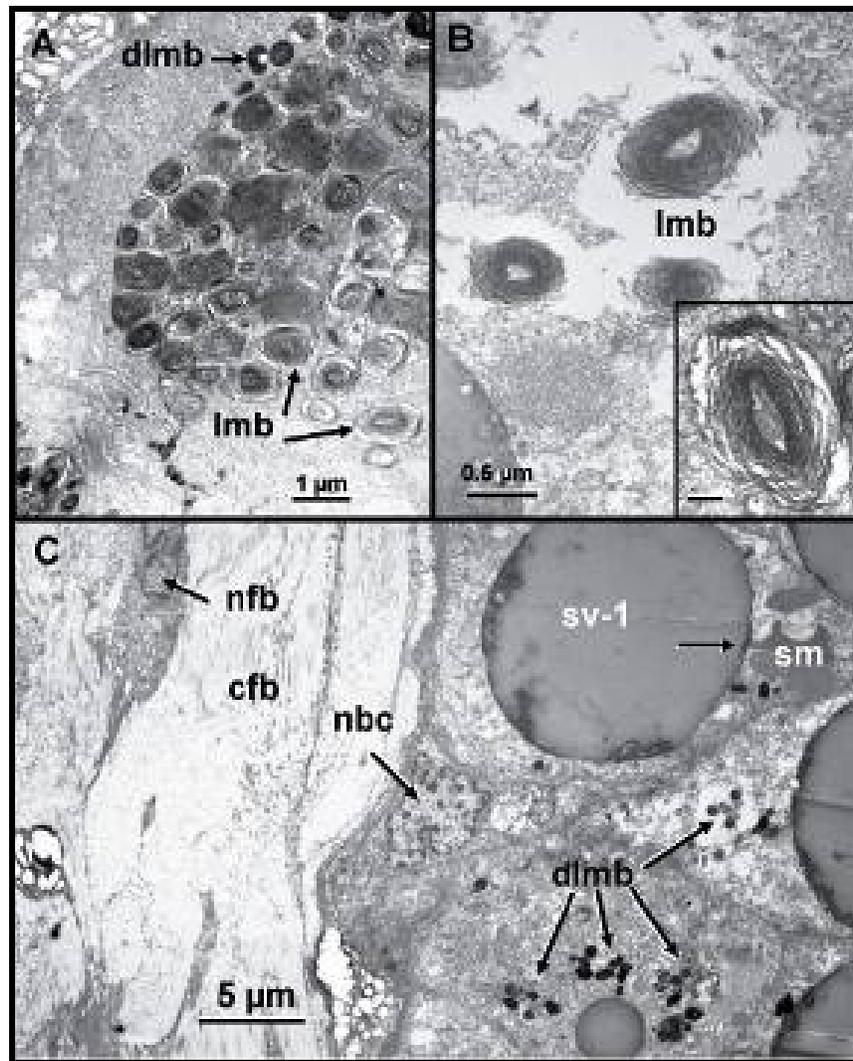


FIGURE 8. Transmission electron micrographs of the secretory epithelium of the right axillary Rathke's gland of *Apalone mutica* (ASUMZ 30748). (A) Large cluster of electron-dense lamellar bodies along with developing lamellar bodies (lmb). (B) Formation of lamellar bodies; note individual layering of lamellar body (inset box); line = 0.25 μm . (C) Interface between thick layer of connective tissue and the basal cell layer of the epithelium. Conspicuous aggregates of dense lamellar bodies are present in basal cell cytoplasm. Arrow within Type 1 secretory vacuole points to cytoplasmic portal for secretory material (sm); nfb = nucleus of fibroblast cell; nbc = nucleus of basal cell; cfb = collagen fibrils.

inset). The granules appear to be mostly-round, electron-dense lamellar bodies (dlmb), each ranging approximately $\sim 2 \mu\text{m}$ in diameter (Figs. 7B; 8). Many dlmb clusters aggregated within sv-2 vacuoles (Fig. 7B and F). Formation and growth of dlmb apparently occurs through the successive addition of concentric lamellar membranes with the eventual condensing of these membranes giving rise to compact granules (Fig. 8A–C). Early developmental stages of lamellar bodies are less conspicuous (less electron dense) when compared to the dense, highly osmophilic granules.

DISCUSSION

Despite minor differences in details, Rathke's gland morphology in *A. mutica* and *A. spinifera* closely resembles previous anatomical descriptions of Rathke's glands in turtles (Rathke 1848; Waagen 1972; Ehrenfeld and Ehrenfeld 1973; Solomon 1984). Rathke's glands in *A. mutica* and *A. spinifera* are large exocrine glands derived from epidermal epithelium and consist of one of more secretory lobules encased in a muscle and connective tissue capsule. Axillary and inguinal glands are structurally similar. We have not directly observed mitotic basal cells replacing secretory cells, processes necessary to confirm the holocrine nature of Rathke's glands in *Apalone*. However, our observation that secretory lobules were usually filled with secretory vacuole-apoptotic cell complexes, secretion droplets, and cellular debris strongly suggests a holocrine function as previously interpreted for other turtles (Ehrenfeld and Ehrenfeld 1973; Solomon 1984). No previous reports of lamellar bodies in Rathke's glands exist; however, because Rathke's glands are epidermal derivatives and lamellar bodies are a component of turtle epidermis (Matoltsy and Bednarz 1975), including that of *A. spinifera* (Alibardi and Toni 2006), we were not surprised to find them in softshell Rathke's glands. Lamellar bodies are thought to be involved in the transport of extracellular lipids (Alibardi and Toni 2006). Unlike all non-trionychoid turtles, each axillary gland of *A. mutica* and *A. spinifera* has a single lengthy secretory duct that empties through a pore on the leading edge of the carapace.

Duct structure of *A. mutica* and *A. spinifera* contrasts with that of *Chelonia mydas*, which exhibit several short secretory ducts that empty into a large passive excretory duct (Solomon 1984). Intraepithelial glands of *A. mutica* and *A. spinifera* are similar to previous descriptions (Solomon 1984). In softshell turtles, however, these glands are exclusively within the distal region of the axillary secretory duct, whereas intraepithelial glands are within the short secretory ducts in *C. mydas* (Solomon 1984). Solomon (1984) identified two types of intraepithelial cells (granule- and mitochondria-rich) and

speculated that granular cells discharged acidic mucosubstances. He considered mitochondria-rich cells to be structurally similar to salt secretory lacrimal glands of marine turtles (Abel and Ellis 1966). We did not examine the ultrastructure of intraepithelial glands or their cell types in *A. mutica* and *A. spinifera* nor did Ehrenfeld and Ehrenfeld (1973) in *Sternotherus odoratus* and *Lepidochelys olivacea*.

Rathke's glands may be specialized for the production and forceful extrusion of a secretion onto the body surface (Waagen 1972; Ehrenfeld and Ehrenfeld 1973). Because gland structure in *A. mutica* and *A. spinifera* is similar to that of other turtles, a similar function is plausible. Indeed, the muscular tunic of the gland spasmodically contracts if the nerve leading to it is physically stimulated. Furthermore, by squeezing between the thumb and forefinger the leading edge of the carapace at the axillary pore of *A. mutica*, one can induce the gland to extrude a semi-solid secretion of up to 1 cm in length. When captured and removed from water, *Carettochelys*, the closest relative of softshells, can forcefully extrude a substance of up to a meter (Sean Doody, pers. com.); however, we did not observe voluntary forceful extrusion in *A. mutica* or *A. spinifera*. None of the previous terms used to describe the nature of turtle Rathke's gland secretions such as yellow, bright yellow, light brown, white, turbid, milky, fluid, ooze, thin stream, oily, and powerfully malodorous (Dahlgren and Kepner 1908; Goode 1967; Waagen 1972; Ehrenfeld and Ehrenfeld 1973; Eisner et al. 1977; Solomon 1984; Radhakrishna et al. 1989) describe *Apalone* secretions. The rope-like semi-solid secretions of *A. mutica* are pale green in color and have no noticeable odor (MVP, pers. obs.).

The Rathke's gland secretion among turtles may contain lipids, glycoproteins, enzymes, and various acids as seen in Stinkpots (Eisner et al. 1977), *Chelodina longicollis* (Eisner et al. 1978), Loggerhead Sea Turtles (Weldon and Tanner 1990), Kemp's Ridley's Sea Turtles (Weldon et al. 1990; Chin et al. 1996; Krishna et al. 1995), Common Mud Turtles (Seifert et al. 1994), and other species (Ehrenfeld and Radhakrishna et al. 1989). In addition, various bacteria may be associated with the secretions (Weldon et al. 1990). Lipids occur in Rathke's gland secretions of *Sternotherus odoratus* (Dahlgren and Kepner 1908; Ehrenfeld and Ehrenfeld 1973), *Kinosternon subrubrum* (Seifert et al. 1994), *Caretta caretta* (Weldon and Tanner 1990), *Lepidochelys kempfi* (Weldon et al. 1990), and *A. mutica* and *A. spinifera* (this study), but not in *Chelonia mydas* (Ehrenfeld and Ehrenfeld 1973) or *Chelodina longicollis* (Eisner et al. 1978). The apparent differences in the overall nature and consistency of *Apalone* secretions from that of other turtles beg further study. The overall similarity in the structure of Rathke's gland and the evolutionary conservation of secretion proteins among

turtles (Seifert et al. 1994; Chin et al. 1996) suggest that Rathke's glands are homologous among all turtle lineages (Ehrenfeld and Ehrenfeld 1973).

The function of Rathke's glands in turtles is unknown, but some suggest that the gland secretions contribute to shell maintenance (Legler in Kool 1981), have antimicrobial (Eisner et al. 1977) and pheromonal properties (Legler 1960; Lewis et al. 2007), warn or repel predators (Neill 1948; Eisner et al. 1977; Kool 1981; McCord et al. 2001), and function in excretion (Weldon and Tanner 1990; Rostal et al. 1991; Krishna et al. 1995). The small number of duct openings and absence of Rathke's glands in some terrestrial turtles argue against a shell maintenance function (Ehrenfeld and Ehrenfeld 1973; Krishna et al. 1995). The occurrence of functional glands in hatchlings and juveniles and the apparent lack of seasonal and sexual variation in gland size and activity oppose a reproductive function (Waagen 1972); however, the nuzzling and chasing behavior often seen during turtle courtship, including that of *Apalone mutica* (Plummer 1977), implies the involvement of chemical signals (Mason 1992). Some male turtles, including the malodorous Stinkpot (*Sternotherus odoratus*), recognize females by their odor, but we do not know if the specific odor recognized by the male is a Rathke's gland secretion (Lewis et al. 2007). Phenylacetic acid, identified in the Rathke's gland secretions of *S. odoratus* (Eisner et al. 1977), can function as a scent-marking pheromone in Mongolian Gerbils (*Meriones unguiculatus*; Thiessen et al. 1974). If chemical signaling via Rathke's gland secretions is involved in turtle courtship, the chemical diversity of the known glycoproteins in Rathke's gland secretions could provide a basis for species-specific recognition (Ehrenfeld and Ehrenfeld 1973).

Because Rathke's glands open directly to the environment, the most reasonable function is some form of chemical signaling and/or excretion (Krishna et al. 1995). Considering the widespread occurrence of Rathke's glands, their capability of rapid localized episodic secretion, and their presence in all age and sex classes, a function useful to all turtles having the glands is chemical defense against predators (Ehrenfeld and Ehrenfeld 1973), but there is no hard evidence supporting this function. The surprisingly forceful extrusion (>0.5 m) of malodorous Rathke's gland secretion in response to disturbance by the Australian "stinker" (*Chelodina longicollis*; Goode 1967; Eisner et al. 1978) is consistent with a defense function; however, in behavioral trials, investigators failed to show a strong avoidance by potential predators to *C. longicollis* Rathke's gland secretion (Kool 1981). Likewise, the highly malodorous Rathke's gland secretion produced in response to disturbance by the North American Stinkpot (*Sternotherus odoratus*; Eisner et al. 1977) suggests a defense function; however, the minute quantity secreted

by *S. odoratus* may be insufficient to deter many predators (Eisner et al. 1977). Furthermore, small fish deter feeding in the presence of *S. odoratus* (Eisner et al. 1977). Eisner et al. (1977) suggested the Rathke's gland secretion of *S. odoratus* more likely may serve as a general aposematic signal that alerts a predator to other undesirable qualities of the turtle, such as distastefulness or pugnacity. For *Apalone mutica* and *A. spinifera*, Rathke's gland secretion is neither particularly malodorous (to humans) nor voluntarily forcefully extruded turtles during handling (MVP, pers. obs.); anecdotal observations suggest a possible link with courtship (Plummer 1977). The question of the function of Rathke's glands in softshells, and turtles in general, remains largely unknown and continues to offer a challenging opportunity for behavioral and chemical ecologists.

Acknowledgments.—Scientific Collecting Permits issued by the Arkansas Game and Fish Commission facilitated collection of turtles. Field collection and laboratory holding procedures followed standard guidelines (Anonymous 1987) and were approved by the Harding University Animal Care Committee. We thank Bill Duellman for permitting MVP to take tissue samples from *A. mutica* in the KUNHM Herpetological Collection. We thank Mac Hardy for lab work on the KU specimens in the early stages of the study, Bob Helsten for translating portions of the German literature, and David Sever, Jo Goy, and Joe Milanovich for comments on the manuscript. A Faculty Development grant from Harding University partly funded this study.

LITERATURE CITED

- Abel, J.H. Jr., and R.A. Ellis. 1966. Histochemical and electron microscopic observations on the salt secretory lacrymal glands of marine turtles. *American Journal of Anatomy* 118:337–358.
- Alibardi, L., and M. Toni. 2006. Skin structure and cornification proteins in the soft-shelled turtle *Trionyx spiniferus*. *Zoology (Jena)* 109:182–195.
- Anonymous. 1987. Guidelines for Use of Live Amphibians and Reptiles in Field Research. American Society of Ichthyologists and Herpetologists, The Herpetologists' League, and the Society for the Study of Amphibians and Reptiles. <http://www.asih.org/files/hacc-final.pdf> (last accessed 28 August 2009).
- Bozzola, J.J., and L.D. Russell. 1992. *Electron Microscopy: Principles and Techniques for Biologists*. Jones and Bartlett, Sudbury, Massachusetts, USA.
- Chin, C.C.Q., R.G. Krishna, P.J. Weldon, and F. Wold. 1996. Characterization of the disulfide bonds and the N-glycosylation sites in the glycoprotein from Rathke's gland secretions of Kemp's Ridley Sea Turtle

- (*Lepidochelys kempfi*). *Anal. of Biochemistry* 233:181–187.
- Dahlgren, U., and W.A. Kepner. 1908. *A Text-book of the Principles of Animal Histology*. Macmillan, New York, New York, USA.
- Dawes, C.J. 1988. *Introduction to Biological Electron Microscopy: Theory and Techniques*. Ladd Research Industries, Inc., Burlington, Vermont, USA.
- Deraniyagala, P. 1939. *The Tetrapod Reptiles of Ceylon*. Dulau & Co. Ltd., London, England.
- Ehrenfeld, J.G., and D.W. Ehrenfeld. 1973. Externally secreting glands of freshwater and sea turtles. *Copeia* 1973:305–314.
- Eisner, T., W.E. Conner, K. Hicks, K.R. Dodge, H.I. Rosenberg, T.H. Jones, M. Cohen, and J. Meinwald. 1977. Stink of stinkpot turtle identified: □-phenylalkanoic acids. *Science* 196:1347–1349.
- Eisner, T., T.H. Jones, J. Meinwald, and J.M. Legler. 1978. Chemical composition of the odorous secretion of the Australian turtle, *Chelodina longicollis*. *Copeia* 1978:714–715.
- Gadow, H. 1923. Amphibia and reptiles. Pp. 312–411 *In* Cambridge Natural History. Harmer, S.F., and A.E. Shipley (Eds.). MacMillan, London, England.
- Goode, J. 1967. *Freshwater Tortoises of Australia and New Guinea*. Lansdowne Press, Melbourne, Australia.
- Hervet, S. 2006. The oldest European ptychogasterid turtle (Testudinoidea) from the lowermost Eocene amber locality of Le Quesnoy (France, Ypresian, MP7). *Journal of Vertebrate Paleontology* 26:839–848.
- Kool, K. 1981. Is the musk of the Long-necked Turtle, *Chelodina longicollis*, a deterrent to predators? *Australian Journal of Herpetology* 1:45–53.
- Krishna, R.G., C.C.Q. Chin, P.J. Weldon, and F. Wold. 1995. Characterization of γ -glutamyl transpeptidase from the Rathke's gland secretions of Kemp's Ridley Sea Turtles (*Lepidochelys kempfi*). *Comparative Biochemistry and Physiology* 2:257–264.
- Legler, J.M. 1960. *Natural history of the Ornate Box Turtle, Terrapene ornata Agassiz*. University of Kansas Publications, Museum of Natural History 11:327–669.
- Lewis, C.H., S.F. Molloy, R.M. Chambers, and J. Davenport. 2007. Response of Common Musk Turtles (*Sternotherus odoratus*) to intraspecific chemical cues. *Journal of Herpetology* 41:349–353.
- Loveridge, A., and E.E. Williams. 1957. Revision of the African tortoises and turtles of the suborder Cryptodira. *Bulletin of Museum of Comparative Zoology*. Harvard College 115:163–556.
- Mason, R.T. 1992. Reptilian pheromones. Pp. 114–228 *In* Hormones, Brain, and Behavior, Biology of the Reptilia, Vol. 18, Physiology E. Gans, C., and D. Crews (Eds.). University of Chicago Press. Chicago, Illinois, USA.
- Matoltsy, A.G., and J.A. Bednarz. 1975. Lamellar bodies of the turtle epidermis. *Journal of Ultrastructural Research* 53:28–132.
- McCord, W.P., M. Joseph-Ouni, and W.W. Lamar. 2001. Taxonomic reevaluation of *Phrynops* (Testudines: Chelidae) with the description of two new genera and a new species of *Batrachemys*. *Review Biologia Tropica* 49:715–764.
- Müller, G. 1961. Die moschusdrusen von *Clemmys caspica* Gmelin 1774. *Zoologischer Anzeiger* 167:149–158.
- Neill, W.T. 1948. Use of scent glands by prenatal *Sternotherus minor*. *Herpetologica* 4:148.
- Peters, W. 1848. Ueber eigenthümliche moschusdrüsen du schildkröten. *Müllers Archive für Anatomy* 1848:492–496.
- Plummer, M.V. 1977. Notes on the courtship and mating behavior of the softshell turtle, *Trionyx muticus* (Reptilia, Testudines, Trionychidae). *Journal of Herpetology* 11:90–92.
- Presnell, J.K., and M.P. Schreibman. 1997. *Humason's Animal Tissue Techniques*. 5th Edition. Johns Hopkins University Press, Baltimore, Maryland, USA.
- Rainey, W.E. 1981. *Guide to sea turtle visceral anatomy*. NOAA Technical Memorandum. NMFS-SEFC-81:1–82. Panama City, Florida, USA.
- Radhakrishna, G., C.C. Chin, F. Wold, and P.J. Weldon. 1989. Glycoproteins in Rathke's gland secretions of Loggerhead (*Caretta caretta*) and Kemp's Ridley (*Lepidochelys kempfi*) sea turtles. *Comparative Biochemistry and Physiology* 94B:375–378.
- Rathke, H. 1848. Ueber die Entwicklung du Schildkröten. F. Vieweg und Sohn, Braunschweig, Germany.
- Rostal, D.C., J.A. Williams, and P.J. Weldon. 1991. Rathke's gland secretion by Loggerhead (*Caretta caretta*) and Kemp's Ridley (*Lepidochelys kempfi*) sea turtles. *Copeia* 1991:1129–1132.
- Seifert, W.E., S.W. Gotte, T.L. Leto, and P.J. Weldon. 1994. Lipids and proteins in the Rathke's gland secretions of the North American mud turtle (*Kinosternon subrubrum*). *Comparative Biochemistry and Physiology* 109B:459–463.
- Smith, M.A. 1931. *The fauna of British India, including Ceylon and Burma*. Vol. 1. Loricata, Testudines. Taylor and Francis, London, England.
- Solomon, S.E. 1984. The characterization and distribution of cells lining the axillary gland of the adult Green Turtle (*Chelonia mydas* L.). *Journal of Anatomy* 138:267–279.
- Stromsten, F.A. 1917. The development of the musk glands in the Loggerhead Turtle. *Proceedings of the Iowa Academy of Science* 24:311–313.
- Thiessen, D.D., F.E. Regnier, M. Rice, M. Goodwin, N. Issacks, and N. Lawson. 1974. Identification of a ventral scent marking pheromone in the male

Plummer and Trauth.—Rathke's Glands in *Apalone*

- Mongolian Gerbil (*Meriones unguiculatus*). Science 184:83.
- Von Eggeling, H. 1931. Hautdrüsen, Pp. 633–692 In Handbuch der Vergleichenden Anatomie der Wirbeltiere. Bolk, I.L., E. Göppert, E. Kallius, and W. Lubosch (Eds.). Urban und Schwarzenberg, Berlin, Germany.
- Waagen, G.N. 1972. Musk glands in recent turtles. M.S. Thesis, University of Utah, Salt Lake City, Utah, USA. 64 p.
- Weldon, P.J., and M.S. Cannon. 1992. Histology and histochemistry of the Rathke's glands of Kemp's ridley Sea Turtles (*Lepidochelys kempfi*). Zoological Jahrbucher 122:67–78.
- Weldon, P.J., and E.S. Gaffney E.S. 1998. An ancient integumentary gland in turtles. Naturwissenschaften 85:556–557.
- Weldon, P.J., and M. J. Tanner. 1990. Lipids in the Rathke's gland secretions of hatchling Loggerhead Sea Turtles (*Caretta caretta*). Copeia 1990:575–578.
- Weldon, P.J., R.T. Mason, M.J. Tanner, and T. Eisner. 1990. Lipids in the Rathke's gland secretions of hatchling Kemp's Ridley Sea Turtles (*Lepidochelys kempfi*) Comparative Biochemistry and Physiology 96B:705–708.
- Zangerl, R. 1941. A series of lateral organs found in embryos of the Snapping Turtle (*Chelydra serpentina*). Papers of the Michigan Academy of Science Letters 26:339–341.



MIKE PLUMMER is Professor of Biology at Harding University where he teaches biostatistics, herpetology, and several team-taught courses. He holds a Ph.D. from the University of Kansas and a M.S. from Utah State University. His research focuses on the ecology and physiology of reptiles, especially snakes and turtles; his publications span more than three decades. (Photographed by Sarah Goy)



STAN TRAUTH is Professor of Biology at Arkansas State University in Jonesboro. He holds a Ph.D. from Auburn University and an M.S. degree from the University of Arkansas-Fayetteville. His research involves the reproductive anatomy and life cycles of amphibians and reptiles. He continues to investigate the reproductive ecology of plethodontid salamanders primarily within the genera *Plethodon* and *Desmognathus*. In reptiles, he has a wide variety of interests including lizard nesting ecology, snake reproductive anatomy, and turtle sperm morphometrics. (Photographed by Champ Williams)