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Histology of Rathke's Glands in the Razor-backed Musk Turtle, *Sternotherus carinatus* (Chelonia: Kinosternidae), with Comments on Lamellar Bodies

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Running Title: Rathke's Glands in the Razor-backed Musk Turtle, *Sternotherus carinatus* (Chelonia: Kinosternidae)

Abstract

I examined the histology and ultrastructure of Rathke's glands in two adult male razor-backed musk turtles (*Sternotherus carinatus*) collected in northeastern Arkansas. This species possesses two pairs of Rathke's glands that are embedded beneath marginal bones and are named according to their anatomical location (i.e., axillary and inguinal). These integumentary glands are similar anatomically to one another. Each gland is comprised of a single, highly vascularized secretory lobule, which is surrounded by a thin tunic of asymmetrically arranged, striated muscle. Two types of large secretory vacuoles characterize most of the holocrine cells produced by a relatively thin secretory epithelium. My results suggest that the chief secretory material of the smaller dark-staining secretory vacuole is a glycoprotein complex. The larger, mostly translucent secretory vacuole contains variously sized, multilaminar, osmophilic lamellar bodies, whose structural design is reminiscent of an epidermal lipid delivery system in vertebrates. The function of Rathke's glands in turtles remains unknown.

Introduction

Trauth and Plummer (2013) reviewed the literature on turtle Rathke's glands, which occur in members of 13 of the 14 living chelonian families (Waagen 1972; Ehrenfeld and Ehrenfeld 1973; Solomon 1984; Plummer and Trauth 2009; Trauth 2012). These exocrine integumentary glands, also known as musk or scent glands, number from one to five pairs (Waagen 1972) and release a musty, sometimes-malodorous secretion through external epidermal pores. The glands are named based upon the general location of their orifices (axillary and inguinal) and/or the proximity of the orifices to scutes (e.g., inframarginal). Most Rathke's glands consist of one or more lobules encased within a striated muscle tunic, and the secretory epithelium consists of ovoid-to-spherical holocrine cells (Ehrenfeld and Ehrenfeld 1973; Solomon 1984; Plummer and Trauth 2009; Trauth 2012; Trauth and Plummer 2013). Seifert et al. (1994) and Weldon et al. (2008) reported that the secretions released by these cells are primarily proteins and, to a lesser extent, lipids, as well as various acids. Lamellar bodies may also be present within the secretory vacuoles of these cells (Plummer and Trauth 2009; Trauth 2012; Trauth and Plummer 2013). The function of Rathke's gland secretions remains poorly understood. Few studies have focused on the histology and/or ultrastructure of Rathke's glands in chelonians (Stromsten 1917; Zangerl 1941; Ehrenfeld and Ehrenfeld 1973; Solomon 1984; Weldon and Tanner 1990; Weldon et al 1990; Rostal et al 1991; Plummer and Trauth 2009; Trauth 2012; Trauth and Plummer 2013).

Lamellar bodies are intracellular tubulo-vesicular organelles composed of concentric phospholipid bilayers. These osmophilic structures occur in epithelial cells (e.g., type II alveolar cells, corneocytes, and mesothelial cells) in humans (Schmitz and Müller 1991; Fartasch 2004; Kennish and Reidenberg 2005; Spener et al. 2006; Sato and Ghazizadeh 2009; Vanhecke et al. 2010) and in Rathke's glands of turtles (Ehrenfeld and Ehrenfeld 1973; Maltoltsy and Bednarz 1975, Alibardi and Toni 2006, Plummer and Trauth 2009; Trauth 2012; Trauth and Plummer 2013). Lipid storage and secretion are presumably the primary roles of lamellar bodies. Moreover, the functional characterization of lamellar bodies is mostly restricted to descriptive studies using electron microscopy (Spener et al. 2006). Plummer and Trauth (2009), Trauth (2012), and Trauth and Plummer (2013) illustrated the multilaminar structure of lamellar bodies in turtles using transmission electron microscopy. Other than a study by Mahmoud and Alkindi (2008), which showed the ultrastructure of a lipoidal body within the corpus luteum of the snapping turtle, no additional ultrastructural studies have depicted lamellar bodies in turtles.

My objectives in the present study were to examine the histology and ultrastructure of Rathke's glands in the

adult male razor-backed musk turtle (*Sternotherus carinatus*) and report on the presence of lamellar bodies.

Materials and Methods

I prepared the Rathke's glands from two adult male razor-backed musk turtles collected from northeastern Arkansas (one on 2 March 2012 and the other on 4 April 2016) for light microscopy (LM-plastic) and transmission electron microscopy (TEM) in the lab at Arkansas State University. These two voucher specimens were deposited in the Arkansas State University herpetological collection (ASUMZ 31996 and 33475, respectively). Carapace (CL) and plastron (PL) lengths were measured prior to sacrificing with an intra-pleuroperitoneal injection of sodium pentobarbital following the university's established IACUC protocol for reptile euthanasia.

A Dremel Multi-Max™ oscillating tool was used to extract Rathke's glands from beneath the turtle carapace (Fig. 1). Glands were immediately placed into vials of 2% glutaraldehyde (GTA) solution buffered with 0.1 M sodium cacodylate at a pH of 7.2 and allowed to fix for 2 h. For postfixation, I used 1% w/v osmium tetroxide, buffered as above, for 2 h. I have previously described the methods used to prepare tissues for LM-plastic (Trauth 2012). In brief, I dehydrated glands in 20 min increments into increasing concentrations of ethanol (70-100%) and then placed the glands in a 50/50% acetone/plastic mixture for overnight infiltration via rotation. For thick sectioning (approximately 1 µm in thickness) and staining, I used glass knives on an LKB Ultrotome (Type 4801A) with Ladd® multiple stain (LMS), respectively. For photomicroscopy, I used a Nikon Eclipse 600 epi-fluorescent light microscope with a Nikon DXM 1200C digital camera (Nikon Instruments Inc, Melville, NY). A Canon T4i digital single lens reflex camera fitted with a macro lens was also used to photograph macroscopic images of the turtle carapace and internal glands.

Plastic-embedded samples prepared for light microscopy were also utilized for TEM. Trimmed tissue blocks were sectioned on a diamond knife. Sections were picked up with 150 - 200 mesh copper grids, stained with uranyl acetate (3% aqueous) and lead citrate for 30 min each. Grids were examined with a JEOL 100 CX-II transmission electron microscope (JEOL USA, Inc., St. Louis, MO) at 60 kV (55 µA). Positive digital images were generated by scanning developed TEM negatives using an Epson Perfection 4990 scanner (Epson America, Inc., Long Beach, CA).

I followed the descriptive terminology for Rathke's glands used by Ehrenfeld and Ehrenfeld (1973), Solomon (1984), Plummer and Trauth (2009), Trauth (2012), and Trauth and Plummer (2013). In addition, the descriptive ultrastructure for lamellar bodies followed previous investigations on snapping turtles and hatchling three-toed box turtles (Trauth 2012; Trauth and Plummer 2013).

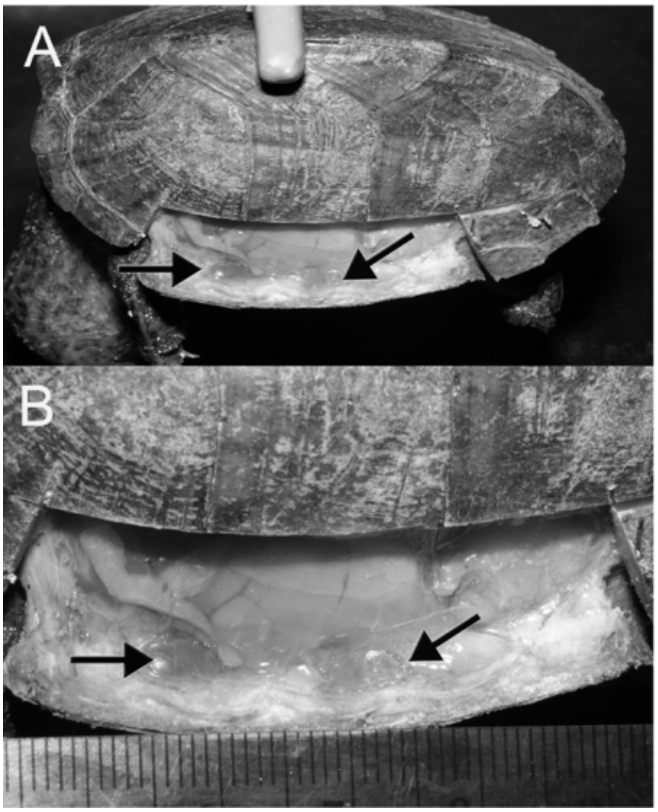

Figure 1. Rathke's glands in a small adult male *Sternotherus carinatus* (ASUMZ 31996; CL = 88 mm). A. Dissection of glands begins with the dorsal and lateral intrusion into carapace and marginal regions. B. Left arrow points to the left axillary gland and right arrow to the left inguinal gland (metric scale in mm).

Results

Gross Morphology

From a dorsal perspective, the axillary pair of Rathke's glands lie beneath the posterolateral edge of costal scute 1 and extend into the anterior portion of costal scute 2 (Fig. 1A and B). The inguinal glands are situated beneath the anteriolateral edge of costal scute 3 (Fig. 1.) Internally, the glands are positioned within slight depressions of the interior marginal bones. The glands' dimensions are variable according to turtle body size, but fall between 6 - 10 mm in length and 4 - 6 mm in width.

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Light Microscopy

Both the axillary and inguinal glands in *S. carinatus* are comprised of an elongated, circular lobule, whose lumen is filled with opaque secretory material (Fig. 2A and B) and/or secretory vacuoles. The secretory epithelium rests upon a thin basement membrane (Fig. 3B). A thin-to-moderately thick layer of dense connective tissue is contiguous with the basal lamina. In general, the secretory epithelium is comprised of a thin, basal, generative single cell layer of holocrine cells (Fig. 3). These epithelial cells proliferate outward into an expansive lumen (Fig. 2). The external wall of each gland is made of a uniformly thick muscular tunic (Fig. 3B). At some point following their release from the apical region of the secretory epithelial cell surface, secretory cells lose their structural integrity and degenerate, dumping their cellular contents into the glandular lumen. Eventually, a flocculent conglomerate (a more or less homogenous cellular fluid and debris mixture) becomes the material that is eventually passed into an excretory duct that leads to the exterior.

Two different types of secretory vacuoles (Type 1 and Type 2) were observed in the secretory epithelium in all Rathke's glands (Figs. 2 and 3). Type 1 secretory vacuoles are generally smaller than Type 2 and normally appear as single, dark-staining spherical or oval masses (Fig. 3). Their matrix is not removed during tissue preparation. In contrast, Type 2 secretory vacuoles are large circular-to-oblong organelles, when fully distended, and generally appear mostly devoid of material. These vacuoles are normally referred to as lipid droplets. Irregularly shaped osmophilic, lipoidal membrane-bound structures are clustered unevenly within Type 2 secretory vacuoles. Soluble lipids found in these lipoid droplets are removed from these vacuoles during histological preparation (Fig. 4).

Transmission Electron Microscopy

The ultrastructure of lamellar bodies of Rathke's glands is shown in Figure 4. Individual lamellar bodies may exhibit numerous bilayered membranes that may surround an electron-dense core region (Fig. 4C). Lamellar bodies are conspicuous dark entities observed in Type 2 secretory vacuoles when viewed with light microscopy (Fig. 3). The arrangement of the circular lamellar bodies varied, but they were observed scattered along the distal inner membrane surface of the Type 2 secretory vacuole (faintly apparent in vacuoles shown in Fig. 3A and C).

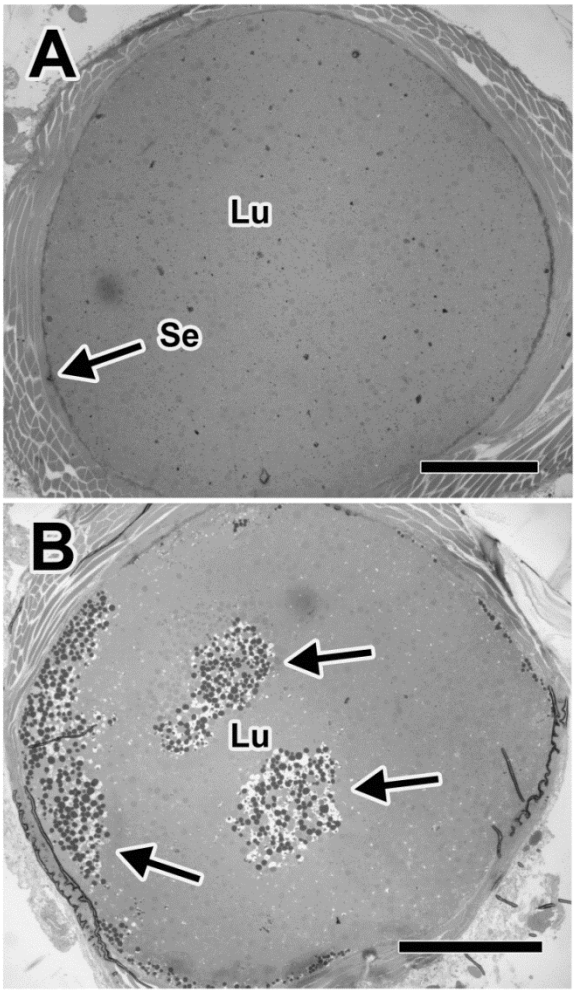

Figure 2. Light micrograph of left axillary (A) and right inguinal (B) Rathke's gland in *Sternotherus carinatus* (ASUMZ 31996). A. Transverse section through gland lumen (Lu) filled with opaque secretory material. Secretory epithelium (Se) exhibits few secretory vacuoles. B. Transverse section through gland lumen (Lu) exhibiting clusters of secretory vacuoles (ends of arrows). Scale bar = 50 μm for A and B.

Discussion

Rathke's glands of relatively few non-marine turtles have been studied anatomically or histologically in any detail; however, a number of common morphological and histological features occur among those species. For example, the glands of *Sternotherus odoratus* (Ehrenfeld and Ehrenfeld 1973), *Apalone mutica* and *A. spinifera* (Plummer and Trauth 2009), *Kinosternon subrubrum* (Webb 2010), and *Terrapene carolina* and *T. ornata* (Trauth and Plummer 2013) are comprised of either a single lobule or, in other cases, multiple lobules, which exhibit a thin to relatively thick layer of loose connective tissue immediately surrounding the secretory

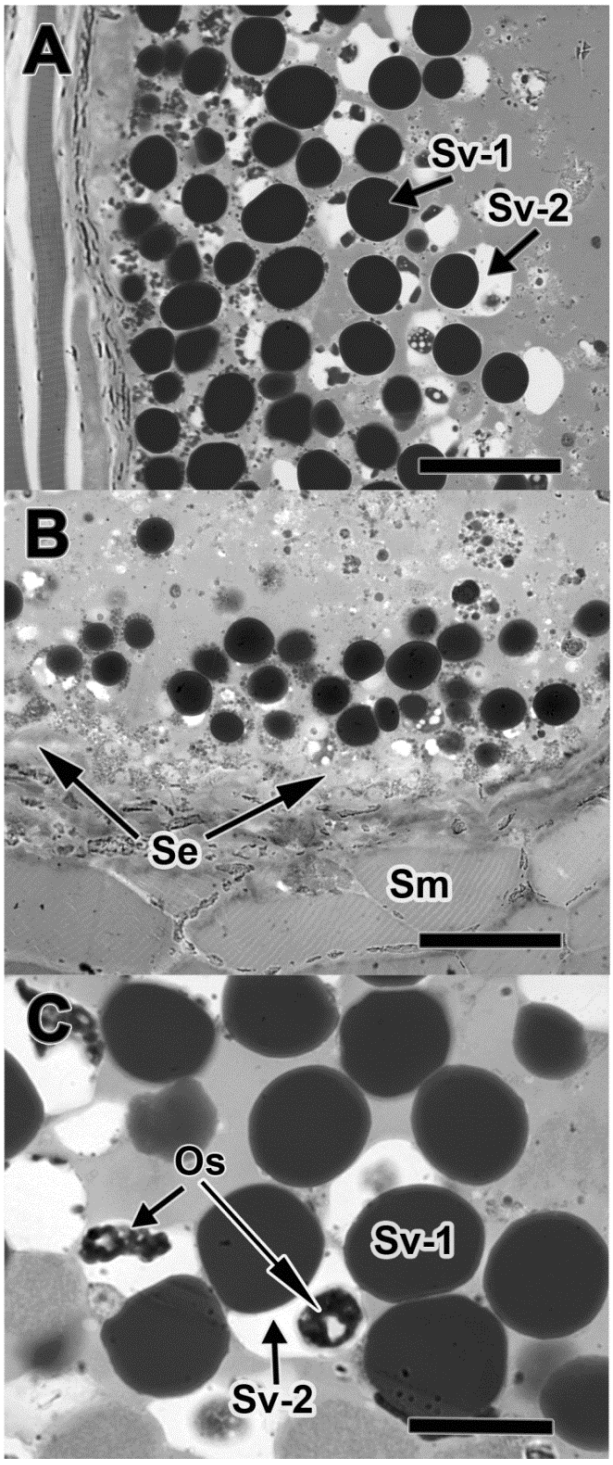

Figure 3. Light micrographs of axillary glands in *Sternotherus carinatus* (A and C, ASUMZ 31996; B, ASUMZ 33475).
A. Section showing secretory epithelium with numerous holocrine cells containing smaller, dark-staining, Type 1 secretory vacuoles (Sv-1) and larger, lipid droplets (clear spheres) characteristic of Type 2 secretory vacuoles (Sv-2). B. Section similar to A. C. Magnification of Type 1 and 2 secretory vacuoles; some Type 2 vacuoles contain osmophilic lamellar bodies (Os). Se = secretory epithelium; Sm = striated muscle. Scale bars in A and B = 50 µm; in C = 20 µm.

epithelium. All are also wrapped in a tunic of striated muscle, and all receive a rich supply of blood from capillaries that lie in close proximity to the basal lamina of the secretory epithelium. Despite these structural similarities, hatchling box turtles, for example, possess glands with holocrine cells that more closely resemble those of *Apalone* and *Kinosternon* than to those of *Sternotherus carinatus*. Although all these species studied thus far possess at least two types of epithelial cells (basal and secretory), *Sternotherus odoratus* differs from the others by possessing a third cell, best described as a holocrine cell containing a number of small lipoid droplets (Ehrenheld and Ehrenheld 1973). These lipoid cells are concentrated within the center of the glandular lumen. Trauth and Plummer (2013) identified solitary large Type 1 secretory vacuoles in box turtles, and these secretory vacuoles were also present in softshell turtles (Plummer and Trauth 2009) and in the mud turtle (Webb 2010). The secretory material of Type 1 secretory vacuoles was putatively identified as a glycoprotein complex in *Sternotherus odoratus* as shown by Ehrenheld and Ehrenheld (1973) based upon PAS+ staining results. The carbohydrate component of the glycoprotein comprised less than 4% of the total molecule in *Sternotherus*. We found similar staining results in the holocrine cells of box turtles and razor-backed musk turtles as did Webb (2010) for *Kinosternon subrubrum*.

Type 2 secretory vacuoles of the razor-backed musk turtle were generally large open spheres, which contained lamellar bodies various sizes and shapes. This type of microstructure was also apparent in *Sternotherus odoratus* (Trauth 2012). In general, lamellar bodies are similar to one another in all turtles studied thus far, although the lamellar membranes, for the most part, were more densely compacted and more numerous in both species of *Apalone* (Plummer and Trauth 2009) . Lamellar bodies may play a role in lipid transfer (Ehrenheld and Ehrenheld 1973), but their function remains unknown in Rathke's glands.

Rathke's glands in razor-backed musk turtles normally exude a malodorous substance, as is the case in most turtles. For instance, the foul-smelling secretion may be present in both adult and hatchling *Terrapene* spp. (Neill 1948; Norris and Zweifel 1950; Legler 1960; Patton et al. 2004; Gangloff and Nash 2010). Gangloff and Nash (2010) detected a musk odor in 12 of 34 hatchling *T. ornata* and 2 of 48 adult *T. ornata*. Patton et al. (2004) detected musk odor in 315 of 1407 (22.4%) hatchling *T. carolina*, but did not detect odor in any individuals more than a few days old. Based on the human detection of a musky odor, Patton et al. (2004)

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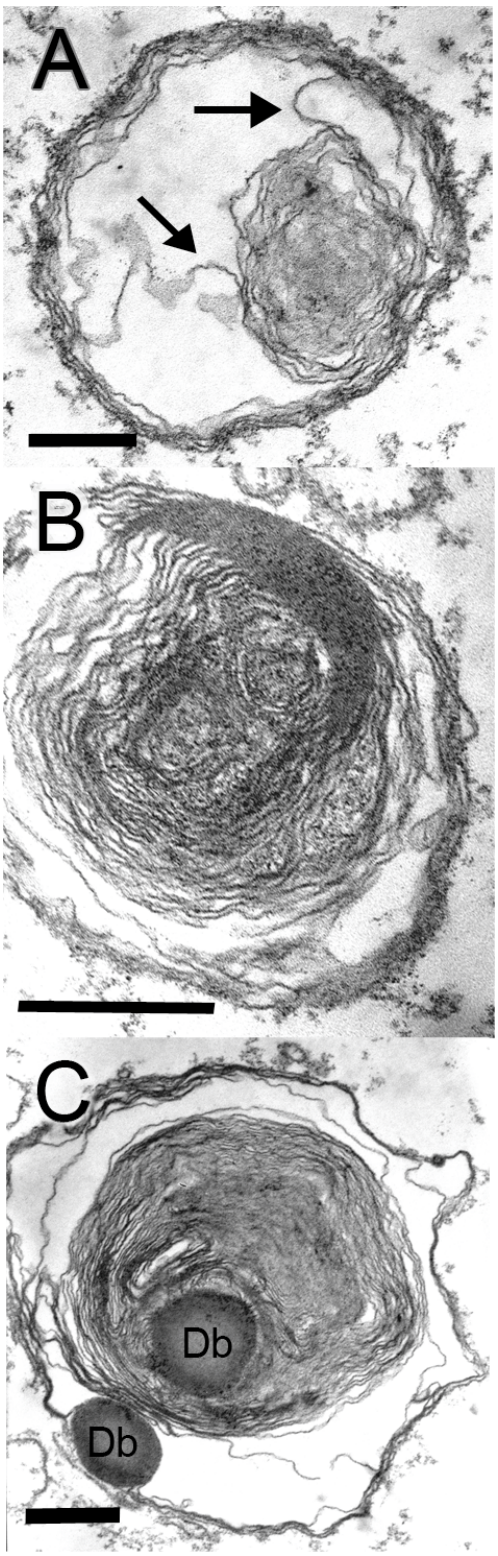

Figure 4. Transmission electron micrographs of lamellar bodies of Rathke's glands in *Sternotherus carinatus* (ASUMZ 31996).
A. Lamellar body showing formation of bilayers. (arrows) around an eccentric core. B. Lamellar body showing multilaminar bilayers C. Lamellar body with associated electron dense granules (Db). Scale bars = 0.5 µm for A-C.

concluded that relatively few *T. carolina* possess Rathke's glands at birth and in those that did possess the glands, function decreased with age. The incidence of siblings producing a musk odor within 503 different clutches varied from 4 to 54% (Patton et al. 2004). Corroborating the conclusion, based on behavior, that relatively few individuals possess Rathke's glands at birth, Waagen (1972) found the physical presence of Rathke's glands in only three of 16 (19%) dissected *Terrapene* individuals.

The presence of Rathke's glands is thought to be the basal condition for all turtles (Waagen 1972; Weldon and Gaffney 1998). Their absence is presumably an apomorphic condition. Terrestrial turtles (testudinoids and a few emydids--Ehrenfeld and Ehrenfeld 1973; Waagen 1972) generally lack the glands. Rathke's glands may be of less biological importance in the terrestrial environment.

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