Light microscopic and immunohistochemical study of the trachea of the broad-snouted caiman (*Caiman latirostris*)

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ABSTRACT: The purpose of this study was to examine the tracheal structure of the crocodile *Caiman latirostris* using light microscopy, histochemical and immunocytochemical techniques. The tracheal epithelium of *C. latirostris* consists of a ciliated pseudostratified columnar epithelium with goblet cells. The respiratory epithelium also includes endocrine cells immunoreactive to serotonin. The histochemical techniques demonstrated the presence of neutral and sulphated mucins secreted by goblet cells. The lamina propria consists of connective tissue with many reticular fibres. The elastic fibres are interspersed among collagen bundles, forming the border between the mucosa and the submucosa. The submucosal layer consists of connective tissue similar to that found in the lamina propria. Serous or mucous glands were not observed. The predominant characteristic in the adventitia is the presence of an incomplete hyaline cartilage ring, in the form of a circle. Dense connective tissue fills the space between the extremities of each cartilage ring. Serotonin-immunoreactive cells frequently had an apical cytoplasmic process directed towards the lumen, and were therefore classified as open type. The α-actin immunohistochemistry revealed smooth muscle cells only in blood vessel walls, confirming the absence of a tracheal muscle.

Keywords: reptiles; morphology; respiratory tract

The crocodilians are members of the Crocodylia order and 28 species and subspecies are divided into four subfamilies: the Alligatorinae, Crocodylinae, Tomistominae, and Gavialinae (Mader, 2006). Members of the Crocodylia order are found in most tropical areas throughout the world. They are the last survivors of the archosaurs and are the largest living reptiles in the world today. Broad-snouted caimans (*Caiman latirostris* Daudin 1802, Crocodylia: Alligatoridae) are widely distributed in South American aquatic ecosystems (Yanosky, 1990; Verdade, 1995). Caimans spend a large portion of their lives in the water. They are long-lived animals and are at the top of the food chain (Stoker et al., 2008).

The trachea in reptiles is long, bifurcated and in some species consists of incomplete cartilaginous rings. The structure of the tracheal epithelium of reptiles was described by Tesik (1981, 1982, 1986) and Pastor et al. (1987a). These studies demonstrate that the inner surface of the trachea in snakes, crocodiles and monitor lizards is lined with an epithelium consisting mainly of ciliated cells. Granular and goblet cells are in the minority, occurring either singly or in clusters of varying sizes (Tesik, 1992).

In the respiratory system of vertebrates, endocrine-like cells which secrete amines and regulatory peptides are a normal component of the epithelium (Beorlegui et al., 1994). The study of these endocrine cells and their distribution provides supporting data on the putative role of peptides in the control of normal bowel and respiratory function (Polak and Bloom, 1986). Despite its strategic evolutionary position, there is little information on the immunocytochemical characterization of the reptilian diffuse neuroendocrine system in extrapulmonary airways. However, endocrine cell immunoreactivity has been reported in vari-

ous reptilian respiratory systems: Serotonin in *Testudo graeca* and *Pseudemys scripta* (Pastor et al., 1987a,b) and *Mauremys caspica* and *Lacerda lepida* (Pastor et al., 1988); Calcitonin in lizards (Galan Galan et al., 1981); Calcitonin gene-related peptide (CGRP); and Enkephalins in the red-eared turtle (Adriaensen et al., 1991).

The aim of this study was to characterize the tracheal structure of *Caiman latirostris* using light microscopic, histochemical and immunocytochemical techniques to provide morphofunctional data that may contribute to clinical treatment of wild animals and to captive breeding of valuable commercial crocodiles.

MATERIAL AND METHODS

Animals and tissue preparations

Specimens from ten adults of *Caiman latirostris* from Bonsucesso Farm (Nossa Senhora do Amparo, Barra Mansa, Rio de Janeiro, Brazil) were taken at slaughter in the Acquanature commercial abattoir (Itaguaí, RJ, Brazil). The breeder is registered with the Brazilian environmental agency (IBAMA) and has authorization from the local government, Rio de Janeiro state environmental agency (FEEMA) and Regional Board of Veterinary Medicine. The animals were subjected to hypothermia and slaughtered after exsanguinations. One cm square tracheal samples were collected and fixed by immersion in Bouin's solution for six hours. The samples were then processed and embedded in paraffin. 5µm-thick sections were cut and stained with haematoxylin and eosin and Masson's Trichrome Periodic acid-Schiff (PAS) and alcian blue (AB) at pH 1.0 and pH 2.5 (Kiernan, 1990). The silver technique was used for the reticulin (Kiernan, 1990) and Weigert's Resorcin-Fuchsin staining after oxidation.

Immunohistochemistry

The standard avidin biotin conjugate (ABC) immunostaining procedures, with appropriate positive and negative controls, were used to detect serotonin endocrine cells and smooth muscle cells. Briefly, sections from Bouin's – fixed for six hours and paraffin-embedded samples were dewaxed, hydrated in a graded series of ethanol solutions of decreasing concentrations until the solution was all water and then washed in phosphate buffered saline (PBS, 0.01M, pH 7.4) for 5 min. The sections were treated for 30 min with a 3% hydrogen peroxide solution in methanol to block endogenous peroxidase activity. The sections were washed with three drops of PBS, incubated in a humidified chamber at 37 °C for 30 min with 1% goat serum, and then incubated at 4 °C in a humidified chamber with rabbit polyclonal anti-serotonin (S 5545 – Sigma-Aldrich, Inc., Saint Louis, USA) and diluted to 1 : 6000 and monoclonal anti-smooth muscle α-actin (cat. No.08-0106, Invitrogen, California, USA) diluted to 1 : 600 for 12–14 hours. Subsequently, the sections were incubated with biotinylated "Universal" secondary antibody diluted to 1 : 200 (PK 7200, Vector Laboratories, Inc., U.K.) for 30 min, then with ABC, diluted at 1 : 200, for 30 min (both from PK 6200, Vector Lab. Inc.). The sections were again washed in three drops of PBS and treated with 3, 3'-diaminobenzidine tetrahydrochloride (Dakocytomation 003222, California, USA) prepared according to the kit instructions, washed in distilled water, dehydrated in increasing concentrations of ethanol and mounted using Entellan (Merck). The negative controls were processed by replacing the anti-serotonin (5-HT) and anti-smooth muscle α actin antibody with PBS.

RESULTS

Light microscopy

Observation under a light microscope showed that the tracheal epithelium of *C. latirostris* consists of a ciliated pseudostratified columnar epithelium with goblet cells (Figure 1A). The histochemical techniques with PAS and Alcian Blue pH 1.0 and 2.5 demonstrated the presence of neutral and sulphated mucins, respectively, secreted by goblet cells (Figure 1B,C). The lamina propria consisted of loose connective tissue with many reticular fibres (Figure 1D). The lamina propria was vascularized and contained lymph follicles. Elastic fibres were interspersed among collagen bundles, forming the border between the mucosa and the submucosa (Figure 1E).

The submucosal layer consists of connective tissue similar to that found in the lamina propria. We did not observe any serous or mucous glands. The submucosa ended with the perichondrium of the tracheal cartilages.

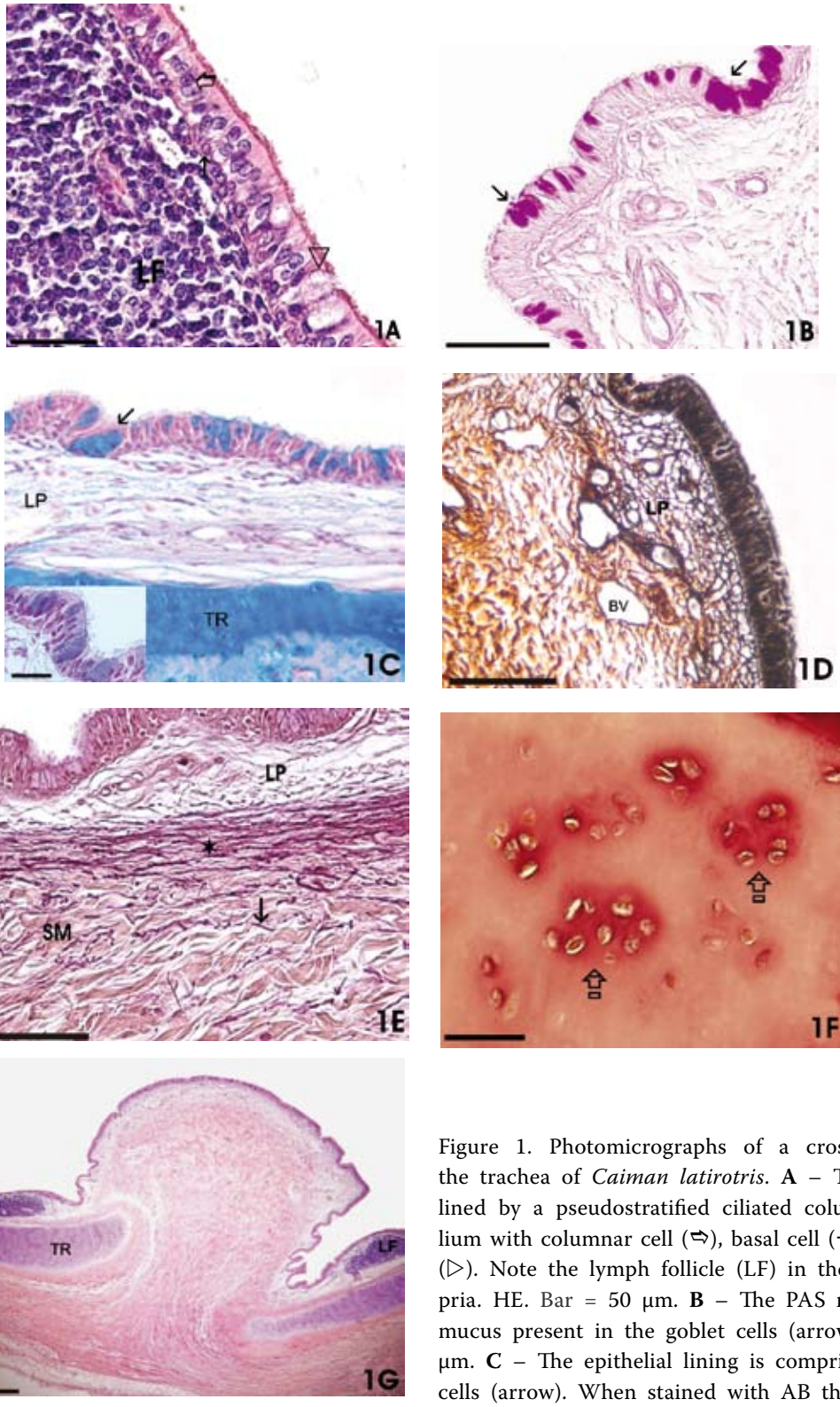

Figure 1. Photomicrographs of a cross section of the trachea of *Caiman latirotris*. **A** – The trachea is lined by a pseudostratified ciliated columnar epithelium with columnar cell (⇨), basal cell (→), goblet cell (▷). Note the lymph follicle (LF) in the lamina propria. HE. Bar = 50 μm. **B** – The PAS method stains mucus present in the goblet cells (arrows). Bar = 50 μm. **C** – The epithelial lining is comprised of goblet cells (arrow). When stained with AB they are clearly visible. Note the lamina propria (LP) and tracheal ring (TR) AB pH 1.0. Bar = 25 μm. **D** – In the lamina propria (LP) of the respiratory epithelium there is a concentration of reticular fibers. Note the blood vessel (BV). Silver technique. Bar = 50 μm. **E** – The elastic fibers (arrow) in submucosa (SM). Note the highest concentration of elastic fibres (*) between the lamina propria (LP) and SM. Weigert's Resorcin-Fuchsin. Bar = 50 μm. **F** – Hyalin cartilage with chondrocytes arranged in isogenous groups (arrows). Weigert's Resorcin-Fuchsin. Bar = 50 μm. **G** – The tracheal ring (TR) is fragmented. Note the evagination of the tracheal lumen and the lymph follicle (LF). Bar = 50 μm

The predominant characteristic in the adventitia is the presence of an incomplete ring of hyalin cartilage (Figure 1F), Alcian Blue pH 1.0 and 2.5 positive, in the form of a circle. In the region where the tracheal ring is incomplete dorsally, there is evagination of the mucosa and submucosal layer from the tracheal lumen (Figure 1G). Masson's trichrome stain revealed that dense connective tissue fills out the interval between the ends of each cartilage ring; therefore, smooth muscle was not present.

Immunohistochemistry

Serotonin-immunoreactive cells (5 HT-IR) were observed in the pseudostratified epithelium (Figure 2A). Their predominant shape was piriform (Figure 2B). The 5 HT-IR cells frequently had an apical cytoplasmic process directed towards the lumen, and we thus classified them as the open type (Figure 2B).

Anti-smooth muscle α-actin

Immunohistochemistry revealed smooth muscle cells only in blood vessel walls. These blood capillaries appeared at high concentrations in the region below the epithelial layer (Figure 3A). There were no smooth muscle cells in the connective tissue in the area where the tracheal rings were incomplete, confirming the absence of a tracheal muscle (Figure 3B).

DISCUSSION

The ciliated pseudostratified columnar epithelium with goblet cells of *C. latirostris* has a structure typical of the respiratory epithelium. This same type of epithelium was also identified in the turtles *T. graeca* and *P. scripta* (Pastor et al., 1987a) and *Caretta caretta* (Fleetwood and Munnell, 1996). However, in the lizards *Lacerta vivipara* and *Lacerta agilis*, a stratified epithelium was described (Tesik, 1984). These lizards have a tracheal epithelium of differing thickness and stratification, depending on the area (over the cartilaginous or over the membranous zones).

The histochemical techniques with PAS, employed to detect neutral glycoconjugates (Martoja and Martoja-Pierson, 1970), and Alcian Blue pH 1.0 and 2.5 to demonstrate carboxylated and sulphated acidic glycoconjugates, gave positive reactions in the goblet cells of the epithelium of *C. latirostris*. The glycoconjugates secreted by goblet cells of the mammalian extrapulmonary airways have been widely studied using a variety of classical histochemical techniques (Castells et al., 1990). Castells et al. (1990) reported the existence of marked differences in the glycoconjugates of the extrapulmonary airways of tetrapode vertebrates, and only sialic acid residues appeared to be constant constituents of the glycoconjugates of the airways of all species studied. Pastor et al. (1987a), in a study of turtles, found differences in the composition of these glycoconjugates: goblet cells in the tracheal epithelium of *Testudo graeca* were posi-

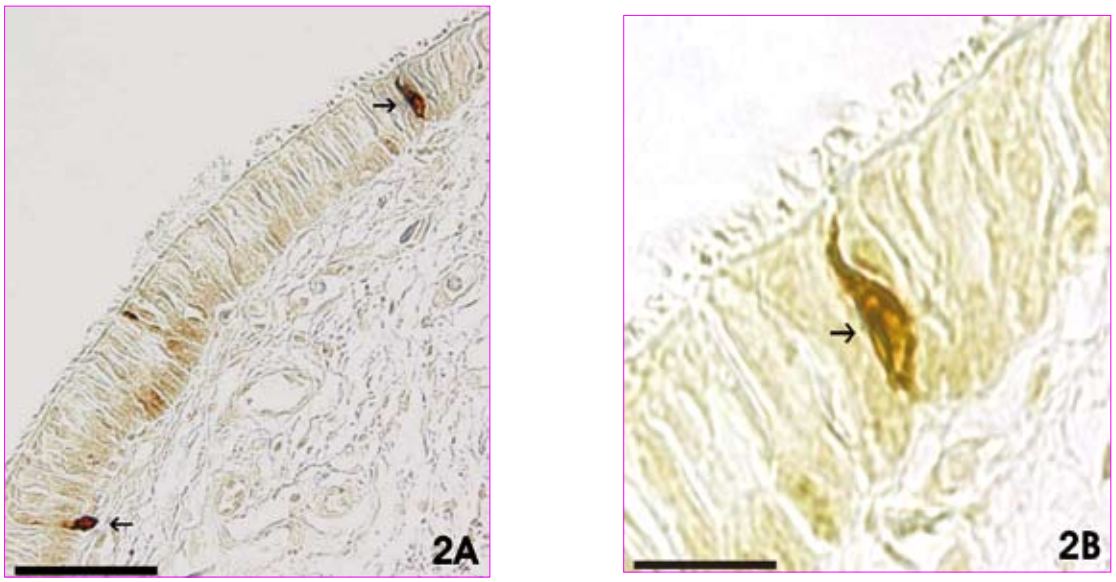

Figure 2. **A** – Serotonin-immunoreactive cells in the pseudostratified epithelium (arrows). Bar = 50 μm. **B** – Note the presence of an apical cytoplasmic process directed toward the lumen (arrow). Bar = 25 μm

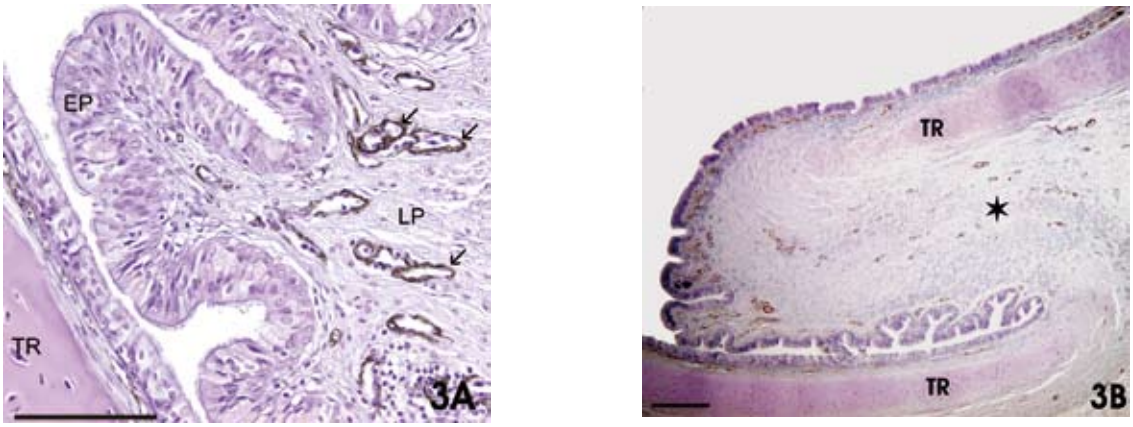

Figure 3. **A** – Smooth muscle cells in blood vessel walls (arrows). Capillaries in close contact with the epithelial layer in lamina propria (LP). Bar = 50 μm. **B** – Tracheal ring (TR). Note the absence of a tracheal muscle (*). Anti-smooth muscle α-actin. Bar = 50 μm

tive to PAS and AB pH 1.0 and 2.5, as in our study, where the goblet cells were positive for both histochemical techniques. However, the tracheal cells of *Pseudemys scripta elegans* were positive only to PAS and AB pH 2.5 (Pastor et al., 1987a). According to Naruse et al. (2005), the cilia and mucous secreted by goblet cells in the epithelium represent the first line of airway defence after exposure to infection, pollution, toxins or inflammation.

In *C. latirostris*, we observed in lamina propria lymph follicles, but this was not observed in turtles by Pastor et al. (1987a). This region showed a rich vascularization in *C. latirostris* such as observed in the amphibian *Siphonops annulatus*. The rich vascularization of this organ in *S. annulatus* suggests its participation in blood-air gas exchange (Kuehne and Junqueira, 2000). We did not observe any serous or mucous glands in the lamina propria and submucosa. The presence of these glands is common in mammals; however, Pastor et al. (1987a) did not observe them in the turtles *T. graeca* and *P. scripta*.

According to Reese (1915), most of the tracheal rings are closed, as occurs in alligators, which have complete tracheal rings. At the cranial third of the trachea, the cartilaginous ring is horizontally flattened in the dorsal direction in all alligators, whereas at the middle and distal parts of the trachea they are circular (Lafortune et al., 2005; Cevik-Demirkan et al., 2007). However, in *C. latirostris* the predominant characteristic in the adventitia is the presence of an incomplete tracheal ring of hyalin cartilage in the form of a circle, as occurs in the snake *Natrix maura* (Pastor, 1990). The presence of a well-defined cartilaginous structure in this organ may demonstrate its importance in keeping the passageway open for air flow (Kuehne and Junqueira, 2000).

We did not detect a tracheal muscle in *C. latirostris*, as is usually present in mammals, connecting the open portions of the cartilaginous rings. In this region, there was connective tissue, as is the case in the amphibian *S. annulatus* (Kuehne and Junqueira, 2000) and the snake *N. maura* (Pastor, 1990). In American alligators the transverse muscle fibres that are found in the most anterior and largest of these breaks in the tracheal rings were found, in embryos, after the middle period of incubation (Clarke, 2005).

The endocrine cells in the tracheal epithelium have been the subject of particular attention from researchers, since in order to establish the evolutionary and functional features of the respiratory system in this class of vertebrates, knowledge of the distribution of regulatory peptides in a large number of species is required (Beorlegui et al., 1994). The serotonin-positive endocrine cells in *C. latirotris* are of the open type, which in other animals like the hamster (Sorokin, 1968) and amphibians (Goniakowska-Witalinska, 1997) may have some sensory function. This cell was found in the tracheal epithelium and interticial cells located along large blood vessels in the connective tissue of the Japanese salamander *Cynops pyrrhogaster* (Adrieaensen et al., 1994a). In the trachea of the reptile *Podarcis hispanica*, serotonin-positive endocrine cells were not found (Beorlegui et al., 1994). However, in the Tokyo salamander, *Hynobius nebulosus tokyoensis*, these cells were reported to be present (Gomi et al., 1994).

The presence of serotonin-secreting cells in the respiratory epithelium is related to the induction of

mucus secretion in the digestive system of mammals (Menguy, 1967; Plaisancie et al., 1998) and has been suggested to increase mucus secretion and ciliary beat frequency, as well as to be a potent vasoconstrictor in the respiratory system (Dey et al., 1981; Adriaensen et al., 1994a,b; Scheuermann, 1997a,b).

Serotonin immunoreactive cells in the respiratory system may possibly play an important role in the defense and repair of the ciliomucous epithelium, such as increasing mucus secretion, ciliary beat frequency and proliferation of epithelial cells (Naruse et al., 2005).

The tracheal morphology of *C. latirotris* showed specific variations when compared to other crocodilians, such as the absence of a tracheal muscle and incomplete tracheal rings. These differences might help us understand the evolution of the respiratory system in reptiles.

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