MORPHOLOGY AND FUNCTIONAL HISTOLOGY OF THE LOWER RESPIRATORY SYSTEM OF RETICULATED PYTHON (*Malayopython reticulatus*)

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Abstract. Respiratory tract disease (RTD) is one of the most common syndromes affecting pythons worldwide. Treatments may appear inefficient due to improper knowledge of the anatomy of the system, itself. Moreover, to date, there is no functional histology of reticulated python (RP) reported. Thus, our aim was to provide the histomorphology of the RP lower respiratory system (LRS) in relation to its function. Six (6) RP carcasses were obtained opportunistically for this experiment. The gross anatomy of the LRS organs was measured and recorded before samples were processed accordingly for routine histology. It was observed that the LRS comprised the trachea, lungs, and air sacs with the diaphragm abstinent. The lungs observed were elongated spongy cylindrical structures connected with the diaphanous and hollow air sacs, which interestingly appeared longer on the right side. The lumen was found to be lined by honeycomb appearances of trabeculae. Histologically, the lungs parenchyma comprised the ediculae and faveolae. The air sacs were lined by the pseudostratified ciliated columnar epithelium. The possible functional significance of these structures was also discussed. Thus, this study provides information about the histomorphology of the LRS of RP that could be useful for veterinarians and for future reference.

Keywords: Reticulated python (*Malayopython reticulatus),* respiratory system, faveolae, ediculae, functional histology

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Introduction

Jacobson [1] stated that respiratory disease is a major cause of mortality and morbidity in reptiles. Since the late 1990s, veterinarians have been aware of respiratory tract disease as a common syndrome affecting ball pythons [2]. Hence, knowledge of the physiological and anatomical differences between the reptilian and the mammalian respiratory systems should be recognized and studied. It is an important concept that impacts husbandry changes for prevention and therapeutics [3].

Reticulated Python (RP)

RP is scientifically known as *Malayopython reticulatus* [4,5]. It is a native species of South-East Asia. RP is famously known for its repetitive black X patterns trailing along its back, which creates an illusion of a diamond-like pattern. On the dorsal aspect of its body, it appears light yellowish to brownish, with black lines trailing along from the ventral part of its eyes down diagonally towards its snout [6]. The RP is also known for being the world's longest snake, reportedly having 10 m length individuals existing; although, the average length is about 4 m [7]. These pythons can be found making their homes in rainforests, grasslands, and woodlands [8]. Their diet consists of small mammals, birds, pigs, deer, and dogs [9].

Malayopython reticulatus is one of the seven species of the genus *Python* [10], family *Pythonidae*, which means that it is non-venomous; suborder of *Serpents*, which is under the order of *Squamata.* The special feature of this order is the ability to open the mouth wide to consume its prey. The python is in the class of *Reptilia,* since it is a cold-blooded animal and having its body covered with scales, under the phylum of *Chordata.* It is in the domain of *Eukarya* since at cellular level a nucleus is contained within a membrane-bound cell [4]. From the main species of *Malayopython reticulatus reticulatus,* there are two other subspecies currently recognised, namely *M. r. saputrai* and *M. r. jampeanus* [10]. They are native to Thailand but occur throughout mainland Southeast Asia. Outside these regions, they are also reportedly seen in parts of Assam (northeast India) and Bangladesh [4]. Like all other snakes, it is a cold-blooded animal that thrives most in areas where the temperature is between 26 °C to 33 °C [9].

The lower respiratory system of a snake is composed of incomplete cartilaginous rings of trachea, which bifurcated into short bronchi, elongated lungs, and air sacs [11]. Air first enters the oral cavity of the snake, then travels along the trachea until it reaches the bifurcation, located near the region of the heart. The bifurcation diverts the air into two openings, the right bronchus and the left bronchus. The right bronchus extends to the right lung, which is elongated. The cranial portion of the lungs is vascularized and functions in gas exchange, whereas the caudal half of the lung is made up of an avascular air sac, which leads into the tail section. The left bronchus extends to the left lung, which is said to be completely vestigial, meaning that the left lung is small, non-functioning, and has degenerated. The air sac in snakes acts as a pressure regulator inside the body cavity, performing hydrostatic functions in most snakes. Since snakes do not have diaphragms, air enters and leaves the lung due to the harmonious movement of the ribs and the body muscles [12].

Respiratory Physiology

The evolution of lizards into snakes has resulted in snakes possessing elongated lungs and air sacs. This might be a reason for the reduced degree of mixing between the lung gases and inhaled air [13]. Bartlet et al. [14] reported that breathing in snakes is not solely dependent on respiratory system mechanics or neuromechanical feedback, but it is mainly a centrally programmed process.

Snakes have an active and passive component to their respiratory cycle [15]. Experiment by Clark et al. [16] proved that snakes have a biphasic ventilation (outflow-inflow-pause) pattern, rather than a triphasic ventilation (outflow-inflow-outflow-pause) pattern. Respiration in the poikilothermic reptile tends to be controlled by oxygen partial pressure, carbon dioxide, and temperature. As temperature increases, so does the body's oxygen demand. This demand is generally met by an increased tidal volume, rather than an increased respiratory rate. Likewise, hypercapnia, an increase in carbon dioxide, also causes an increase in tidal volume, whereas hypoxia causes an increase in respiratory rate. In reptiles, the low blood oxygen concentration is the stimulus to breathe. This mechanism, however, has limits. During significant pulmonary pathology, the ability of the snake to increase its functional tidal volume is disrupted by the loss of normal tissue elasticity, cellular infiltrates, and inflammatory debris. An interesting feature that reptiles exhibit is the ability to function with anaerobic metabolism. Reptiles are able to handle the accumulation of lactic acid and hydrogen ions due to their unique buffering system, which is present within their circulatory system. The accumulation of lactic acid and hydrogen ions occurs due to periods of anaerobiosis. However, this might conceal any severe respiratory disease that has advanced to a level beyond the compensatory abilities of the reptile [13].

Among articles which have been published on snake respiratory anatomy and physiology [17,18], none has explained and/or described the histological functions of the lung and air sac of RP, including those in Malaysia. Once the basic understanding of the functional histology of the respiratory system has been established, new ideas and techniques can be studied to treat future respiratory diseases more effectively. Thus, this study aimed to describe the gross morphology and histology, and to understand the functional histology of the lower respiratory system of the RP.

Materials and Methods

Animals

The carcasses of adult six (6) pythons, which consisted of four (4) males and two (2) females were obtained opportunistically after the permit was granted from the Department of Wildlife and National Parks of Peninsular Malaysia (PERHILITAN). In addition, the details on the source of the pythons and the premise where the samples were obtained had to be disclosed to PERHILITAN.

Post-mortem and Gross Examination

The entire internal organs were taken out and placed onto a clean background. The organs were carefully stretched out and exposed, in order to take a pictograph that would show the location and position of organs in relation to the respiratory system *in situ.* Then, the entire respiratory system was carefully removed from the thoracic cage using scalpel blades and a pair of scissors. Next, the weight and length of the trachea, the right and left lungs, and the right and left air sac were measured and recorded as mean ± SD.

Collection and Samples Processing

After all the documentation was done, small segments (around 1 cm^3) of each portion of the respiratory system were collected and fixed in 10 % neutral buffered formalin solution. Since the lungs were prone to float and not be fully submerged in the formalin solution, an absorbent cloth was used to cover the surface of the solution. Then, the samples were processed accordingly and stained with routine Haematoxylin and Eosin (H&E) staining and Toluidine blue stain for the lung. Histological structures of the trachea, lungs and air sacs were examined under a light microscope, from low to higher magnifications. All histological structure findings were studied and correlated with recent studies on lung function [14].

Results and Discussion

Gross Morphology

The gross morphology of the lower respiratory tract is seen to comprise of a trachea, a pair of lungs and the air sacs (Figure 1).

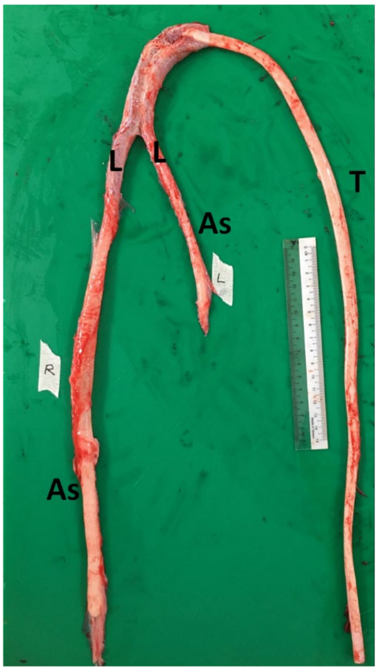

Figure 1: Pictograph of the *ex-situ* of the respiratory system of an adult male *Malayopython reticulatus*. Air Sac (As), Lung (L) and Trachea (T). Ruler used is 15 cm in length

The trachea is found to be a long white flimsy incomplete-ringed structure. The trachea begins at the glottis and runs caudally along the upper body cavity (Figure 2(A)) until it bifurcates into two bronchi. The bifurcation occurs just behind the apex of the heart (Figure 2(B)). The average trachea length for a male python is 62.53 cm ± 0.35, which is 20.2 % of the total snout-to-vent length; while for a female it is 62.9 cm ± 0.13, which is 0.2 % of the total snout-to-vent length. The average weight of the trachea of male pythons is 12.73 g ± 0.63, whereas, for females it is 13.19 g ± 0.72. The percentage of trachea weight to the total body weight for both male and female pythons is 0.1 %.

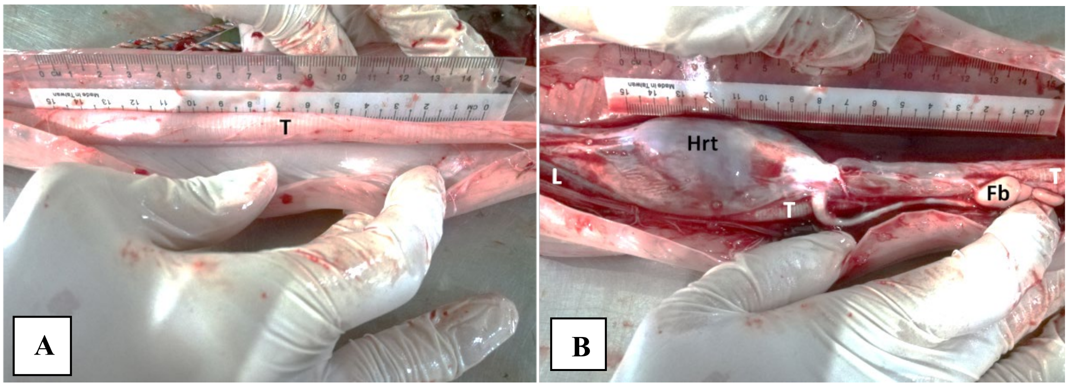

Figure 2: *In-situ* trachea of *Malayopython reticulatus*. (A) Trachea in the cervical region and (B) Junction between trachea and lungs. Fat body (Fb), Heart (Hrt), Lung (L) and Trachea (T)

The lungs consist of the right and left lungs, which are elongated spongy cylindrical structures with multichambered branching from the main bronchi. The liver is located in between the lungs and dorsally in the body (Figure 3). For the lungs a central lumen that is surrounded by many trabeculae is observed (Figure 3(C)). In male pythons, the average length of the right lung is 28.73 cm ± 0.45, which is 9.3 % of the total snout-to-vent length. The average weight of the right lung is 39.12 g ± 0.22, which is 0.4 % of the total body weight. For the left lung, the average length and weight are 19.68 cm ± 0.76 and 14 g ± 0.21, respectively. In percentage, if compared to the total snout-to-vent length and the total body weight, they are 6.3 % and 0.1 %, respectively. For female pythons, the average length of the right and the left lungs are 34.8 cm ± 0.45 and 28.1 cm ± 0.53, respectively. The percentage of the average length of the right and left lung to the total snout-to-vent length are 10.7 % and 8.6 %, respectively. Besides that, the average weight of the right and left lung for females are 31.55 g ± 0.87 and 13.13 g ± 0.92, respectively. The percentage of the average weight of the right and left lung to the total body weight are 0.3 % and 0.1 %, respectively.

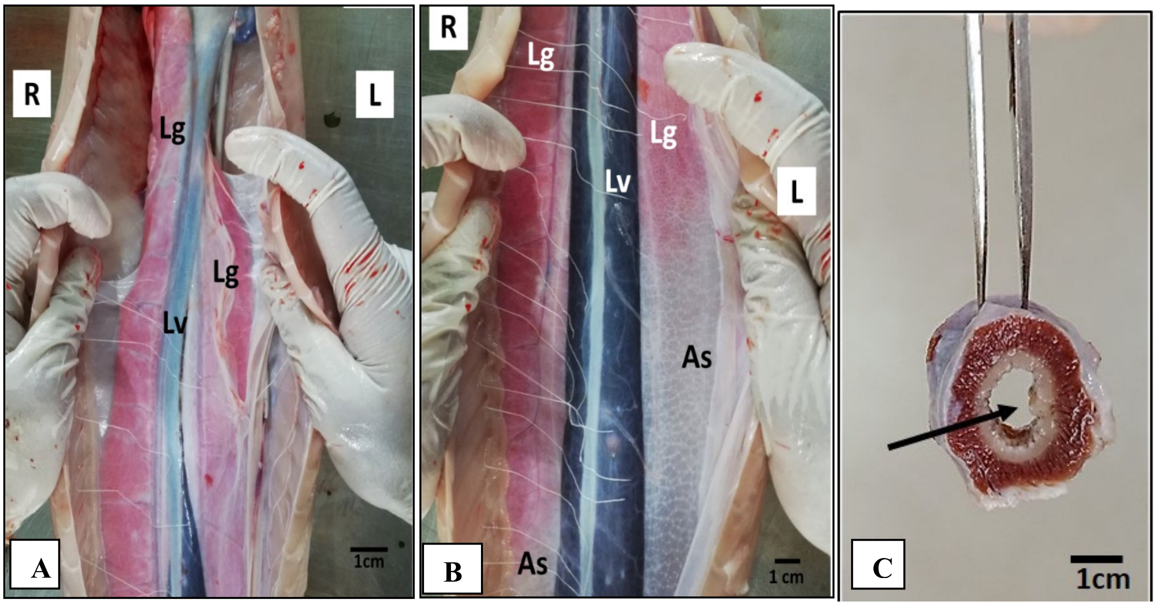

Figure 3: Gross view of the lungs of an adult male *Malayopython reticulatus*. (A) *In-situ* view of lungs in the thoracic region, (B) *In-situ* view of the junction between lungs and the air sacs and (C) Transverse cut surface of the lungs. Air sacs (As), Lungs (Lg) and Liver (Lv). Left (L) and Right (R). The arrow in (C) shows the lumen of lungs

The air sacs structure consists of the right and left air sacs. As the lungs progress caudally, each slowly transforms into the air sacs (Figure 3(B)). The air sacs are seen as translucent diaphanous balloons with a single layer of honeycomb inner surface (Figure 4(A) and (B)). The right air sacs structure starts halfway at the right liver lobe and ends cranial to the pancreas, gall bladder, and spleen. The left air sacs structure starts almost a quarter of the left liver lobe and ends a few centimetres away from the caudal end of the liver. In males, the average lengths of the right and left air sacs are 49.15 cm ± 0.41 and 11.73 cm ± 0.79, respectively. In relation to the total snout-to-vent length, the percentage of the right and left air sacs are 15.9 % and 3.8 %, respectively. For females, the average lengths of the right and left air sacs are 47.15 cm ± 0.63 and 19.2 cm ± 0.37, respectively. In relation to the total snout-to-vent length, the percentage of the right and left air sacs are 14.5 % and 5.9 %, respectively.

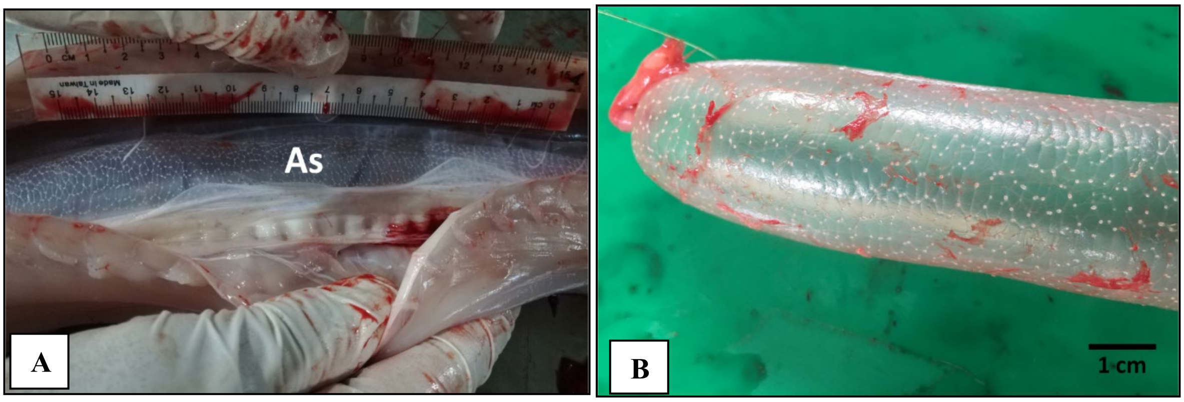

Figure 4: Gross view of air sac of an adult male *Malayopython reticulatus*. (A) *In-situ* view of air sacs and (B) *Ex-situ* view of the inflated pouch-end of air sacs. Air sacs (As)

Histology of Respiratory System of Reticulated Python

Histological findings of the various structures (i.e., trachea, lungs, and air sacs) making up the respiratory system could be observed under light microscopy. All these structures demonstrate four layers, with mucosa layer being the innermost and closest to the centrally located lumen and followed sequentially by sub-mucosa layer, cartilage or muscularis externa layer, and the tunica adventitia as the outer most layer.

The trachea is observed as being composed of incomplete cartilaginous rings consisting of the four layers mentioned (Figure 5). The trachea could be seen as having two distinct regions, which are the cartilaginous and the non-cartilaginous regions (Figure 6(A)). In both regions, the mucosa layer is comprised of ciliated pseudostratified columnar epithelium with the presence of goblet cells (Figure 5). The submucosa layer in both regions consists of loose connective tissues with the presence of blood vessels. The next layer in the cartilaginous region is the hyaline cartilage with large ovoid and polygonal chondrocytes, which could secrete the cartilage matrix. In comparison the layer after the submucosa of the non-cartilaginous region is the muscularis layer, specifically the longitudinal smooth muscle layer (Figure 6(B)). Observed as the final layer for both regions is the tunica adventitia, comprising the areolar connective tissues.

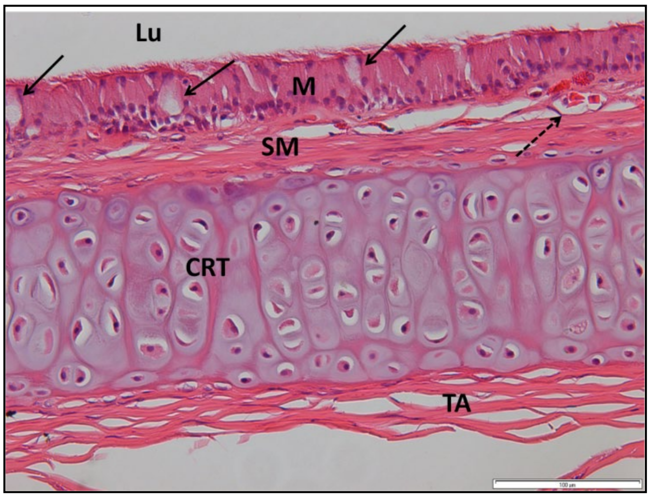

Figure 5: Photomicrograph of cross section of the cartilaginous region of trachea of *Malayopython reticulatus*. Hyaline cartilage layer (CRT), Lumen (Lu), Mucosa layer (M), Submucosa layer (SM) and Tunica adventitia layer (TA). Goblet cell (solid-line arrow) and Capillary (dotted-line arrow). (H&E, Bar = 100 µm)

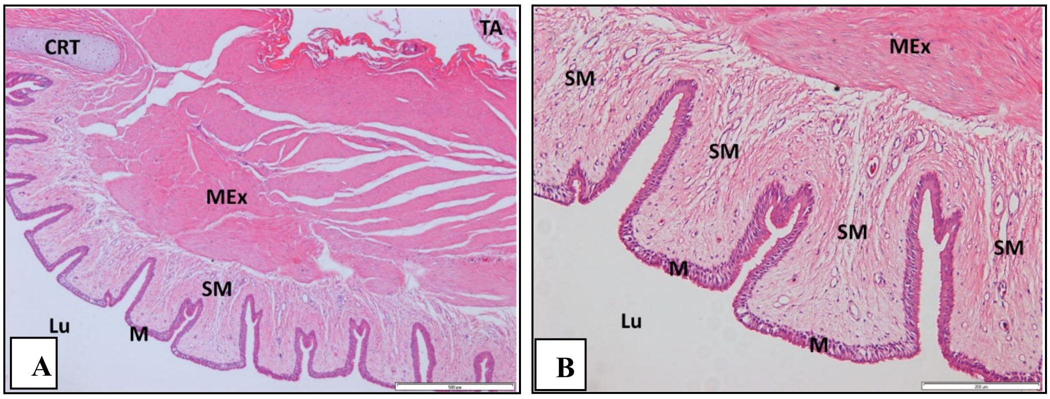

Figure 6: Photomicrographs of cross-section of the non-cartilaginous region of the trachea of *Malayopython reticulatus*. Hyaline cartilage layer (CRT), Lumen (Lu), Mucosa layer (M), Muscularis externa layer (MEx), Submucosa layer (SM) and Tunica adventitia layer (TA). (H&E, Bar (A) = 500 µm and Bar (B) = 200 µm)

From the findings of the study a lot of similarities could be found between the trachea of the reticulated python and the trachea of the mammalian and reptilian groups, with only minor differences. The incomplete C-shaped ring with a muscular layer joining the cartilage end-rings of the trachea has also been reported in the Viperine snakes and the broad-snouted caiman [19]. However, in the alligator, the trachea is reported to be made up of complete rings [19]. The trachea then bifurcates into two bronchi, with each entering a separate lung [20]. This is seen in most reptiles, such as the Eastern diamondback rattlesnake, Black mamba and Garden lizard [1].

The histologically four layered trachea is comparable to the trachea of most mammals and reptiles. The mucosal layer is lined by ciliated pseudostratified columnar epithelium with the distributed presence of goblet cells. In Eurasian lizards and Sand lizards, a non-ciliated stratified epithelial layer has been described [19]. Goblet cell secretion at the mucosa layer trapped small particles in the inspired air in a thin layer of surface mucous, which is propelled towards the glottis by the coordinated movement of the cilia [21]. As for the submucosa layer, it is made up of close connective tissues with an absence of sero-mucous glands, which is comparable to what has been reported for the broad-snouted caimans and tortoises [19]. The submucosa layer also contains blood vessels. The C-shaped rings of the hyaline cartilage are a layer just below the submucosa layer, which supports the trachea mucosa and prevents its collapse during inspiration [21]. The most external layer is the tunica adventitia. At the region where there is no cartilage layer, it is replaced with a muscular layer. This muscular layer is made up of bands of smooth muscles called trachealis muscle, which joins the free ends of the hyaline cartilage rings posteriorly [22]. Even though Viperine snake and broad-snouted caiman have incomplete tracheas, they do not have tracheal muscles. Instead, the muscular region is replaced by a thick layer of submucosa [19].

Microscopically, the honeycomb appearance of the trabeculae in the lungs is referred to as ediculae. The functional unit of the lungs are many smaller branching trabeculae found deeper than the ediculae and known as faveolae (shown in Figure 7(A) and (B)). The layers seen in each of these regions are the mucosa, submucosa, and muscularis mucosa. At the ediculae, which is the beginning of the luminal end of the trabeculae, the mucosa layer is made up of pseudostratified ciliated cuboidal and columnar cells, without the presence of non-ciliated secretory epithelial cells (Figure 7(C) and (D)). Next is the submucosa layer, which comprises of loose connective tissues, with an abundance of blood vessels. The next layer in the lungs is the muscularis mucosa, which is made up of longitudinal smooth muscles. This muscularis mucosa is also connected to the muscle layer of the faveolae (Figure 7(A) and (B)). At the faveolae region, the mucosa layer is made up of mostly Type I pneumocytes with a few Type II pneumocytes, as shown in Figure 8. Its submucosa layer is made up of a thin layer of connective tissues, with an abundance of capillaries. The next layer is the muscularis mucosa, which branches and connects from the muscularis mucosa of the ediculae. This layer is also made up of longitudinal smooth muscles. The last layer, which is the tunica adventitia, below the faveolae region is made up of areolar connective tissue.

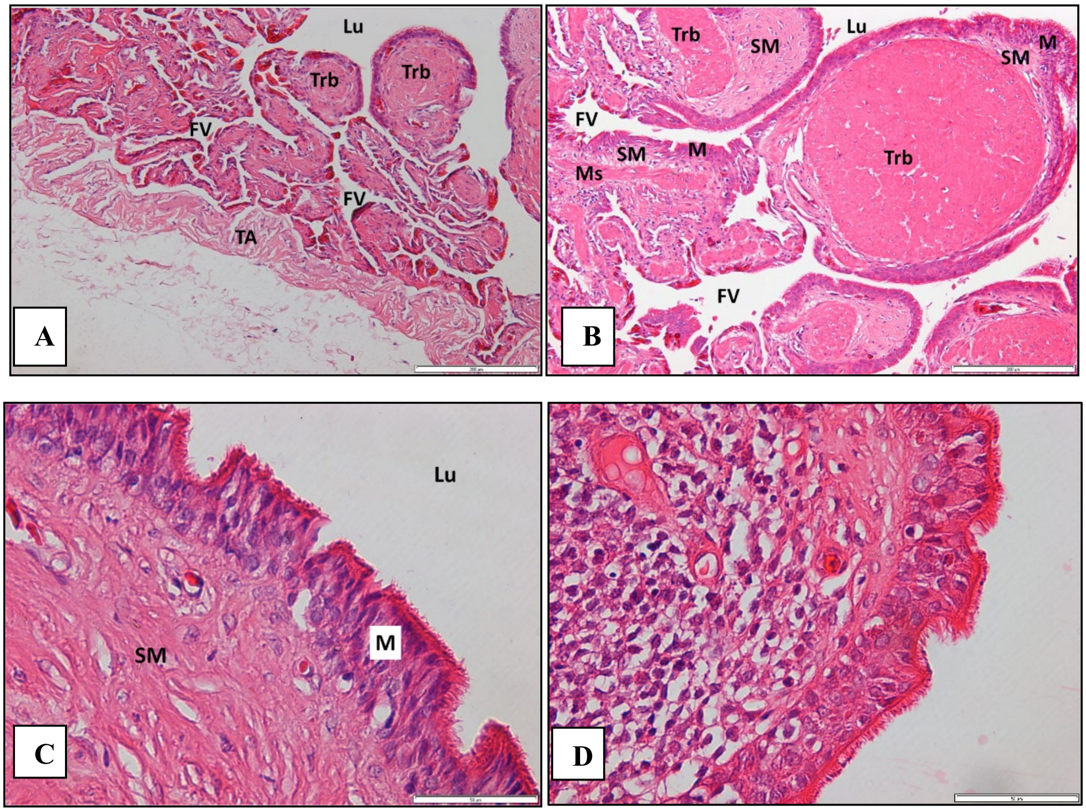

Figure 7: Photomicrographs of cross-section of the lungs of *Malayopython reticulatus* stained with H&E. Faveolae (FV), Lumen (Lu), Mucosa layer (M), Muscularis mucosa layer (Ms), Submucosa layer (SM) and Trabeculae (Trb). Bar (A) = 200 µm, Bar (B) = 200 µm and Bar (C and D) = 50 µm

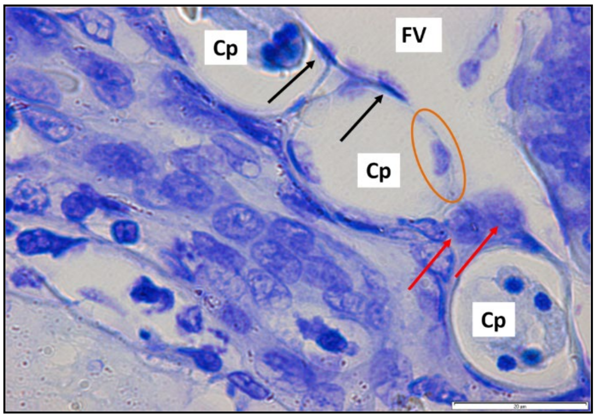

Figure 8: Photomicrograph of cross-section of the lungs of *Malayopython reticulatus* stained with Toluidine Blue. Capillary (Cp), Endothelial cell (orange circle) and Faveolae (FV). Type I Pneumocytes (black arrow) and Type II Pneumocytes (red arrow). Bar = 20 µm

The findings of the study concur with that by Maina [23], which reported that Boidae and Pythonidae families have two functional lungs. However, in the family Colubridae, the left lung is drastically reduced in size, whereas in Viperidae, the left lung is largely absent [23]. For reticulated pythons, the right lung appears longer than the left lung. Snakes have very elongated cylindrical lungs, and this situation differs greatly from the normal mammalian, avian, and amphibian lungs. It could be seen that each lung does not further divide into lobes, and this is commonly termed unicameral. Unicameral lungs can also be found in modern amphibians and *Sphenodon.*

Histologically, a distinct difference in the parenchyma of the lung is observed. The first parenchyma, which is the closest to the lumen of the lung, is the trabeculae. A single unit of trabeculae is made up of an ediculae, which is the second parenchyma. The third parenchyma is the faveolae, which is right below the ediculae. The faveolae is the functional unit of the respiratory system of snakes. The three parenchymal layers are also seen in other snakes, such as the Eastern diamondback rattlesnake, king snake, and boa constrictor [1,24]. The outer and final layer is the tunica adventitia. At the trabeculae, the mucosa layer is made up of pseudostratified ciliated cuboidal and columnar epithelium, without the presence of non-ciliated secretory epithelial cells. This differs from the findings of Jacobson [1], which showed that there are presence of non-ciliated secretory epithelial cells at the mucosal layer of the trabeculae of vipers and rattlesnakes. Just below the mucosa layer is the submucosa layer, which is made up of loose connective tissue with the presence of blood vessels. Next is the muscularis externa layer, which is made up of longitudinal smooth muscle bundles. These muscle bundles run along and connect to the muscularis externa layer of the faveolae. At the faveolae region, the layer above the muscularis externa layer is the submucosa, typically made up of loose connective tissue with numerous presence of blood capillaries.

The mucosal layer of the faveolae is made up of a majority of Type I pneumocytes, with fewer Type II pneumocytes, which is in agreement with Jacobson [1] findings. Since the reptiles (e.g., pythons) have nucleated red blood cells, it has been difficult to identify and differentiate the Type I pneumocytes and Type II pneumocytes from the red blood cells, when stained with H&E. Hence, Toluidine Blue was used to help differentiate these cells, since nucleus picks up the stain of Toluidine Blue more effectively. Type I pneumocytes appear to have small, dense, and flattened nuclei. In comparison, the Type II pneumocytes have large and plump nuclei, with the presence of fine unstained vacuoles in their plentiful cytoplasm. Since the Type I pneumocytes are found near the capillaries, it can be assumed that it follows the function of normal mammalian cells as a blood-gas exchange barrier. Type II pneumocytes secrete a surface-active material called surfactant, which reduces surface tension within the faveolae, preventing faveolar collapse during expiration. They can also differentiate into Type I pneumocytes in response to damage to the faveolar lining [21].

As the others, the air sacs structure is also made up of the four layers; namely, mucosa layer, submucosa layer, muscularis externa layer, and tunica adventitia layer (Figure 9(A)). The mucosa layer is seen to comprise of squamous and pseudostratified ciliated cuboidal epithelial cells (Figure 9(B)). Although the submucosa layer comprises of loose connective tissues, but there is only minimal presence of blood vessels. As for the muscularis externa layer, it is made up of longitudinal smooth muscles. The last layer is the tunica adventitia, which comprises of areolar connective tissue.

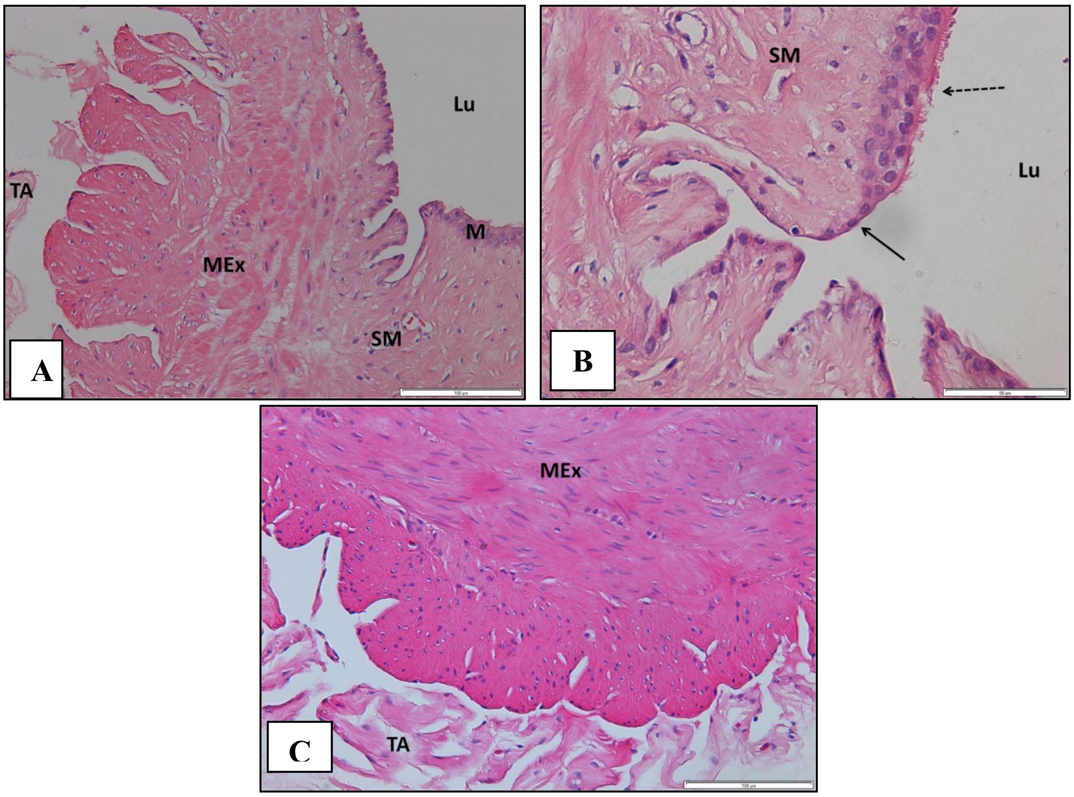

Figure 9: Photomicrographs of cross-section of the air sacs of *Malayopython reticulatus.* Lumen (Lu), Mucosa layer (M), Muscularis externa layer (MEx), Submucosa layer (SM) and Tunica adventitia layer (TA). Pseudostratified ciliated cuboidal epithelium (dotted-line arrow) and Simple squamous epithelium (solid-lined arrow). (H&E, Bar (A & C) = 100 µm and Bar (B) = 50 µm)

Continuing caudally, the right and left lungs, slowly transform into the air sacs. The right air sacs structure is much longer than the left air sacs structure, which is in agreement with findings by Jacobson [1]. The air sacs structure appears diaphanous, hollow, and balloon-like. Histologically, it is found that the air sacs also have the typical four layers as the other structures. The mucosal layer is found to be made up of squamous and pseudostratified ciliated cuboidal epithelial cells. This is different from what Jacobson [1] found in Neotropical rattlesnake, which has ciliated columnar epithelium. From the findings of the study, it could be seen that the submucosa layer is made up of loose connective tissues with blood vessels being rare. This proves that gas exchange does not occur in the air sacs [24]. The layer below the submucosa is the muscularis externa layer, which comprises of two types of muscle, i.e., the inner circular and outer longitudinal smooth muscles. The final layer is the tunica adventitia.

Conclusions

In this study, the gross morphology and histology of the lower part of the respiratory system of reticulated python were observed and described. Subsequently, the findings were correlated to the functional histology of the respiratory system of the reticulated python. We would like to recommend future studies using immunohistochemistry and electron microscopy so that certain immune cells can be highlighted, and detailed ultrastructure of the cells can be obtained.

Author contributions

All authors contributed toward sample collection, data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosure to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards

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