The macroscopical and microscopical characters of the trachea in different avian species: A comparative study

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Abstract

This study aimed to explain and compare the anatomical, histological, histochemical and histomorphometrical analyses of the trachea in different species of birds. This study includes 21 healthy birds from geese (Anser anser domesticus), cattle egrets (Bubulcus ibis) and house sparrows (Passer domesticus), 7 seven from each species. Anatomically, the trachea of the goose (the proximal and distal parts) was the longest of all the studied species, yet it had fewer cartilaginous rings than those of the cattle egret. Also, the tracheal length, beside the tracheal muscle in geese plays an important role in the phonation process. Histologically, the trachea is composed of four distinct tunicae: mucosa, propria submucosa, fibrocartilaginous, and adventitia. The epithelium that lined the trachea was ciliated, pseudostratified columnar epithelium (respiratory epithelium) with simple mucous tubuloalveolar glands in either proximal or distal parts depending on the species. The proximal part is made up of overlapped hyaline cartilaginous rings that partially ossified in geese and cattle egrets but did not ossify in sparrows. In the distal part, the overlapping faded in three investigated species.

Introduction

There are very important roles of the respiratory system in birds as gaseous exchange, regulation of body temperature, sound production, and immunity of birds (1). The trachea is a tube that extends from the larynx to the syrinx, with the proximal part ventral to the esophagus and the distal part passing through the thoracic inlet (2), beyond which it splits into two main bronchi near the syrinx (caudal larynx) (3). The most significant part of the tracheal anatomy is the cartilaginous rings (2). The number of cartilaginous rings in the trachea varies with the length of the bird’s neck, usually ranging from 108 to 126 cartilages (4). Chickens have three pairs of tracheal muscles: sternotrachealis muscles, trachealonygeodorsalis muscles, and trachealonygeoventralis muscles (5). Histologically; the tracheal wall is comprised of respiratory epithelium, beneath epithelium; the propria-submucosa, and the tracheal wall is supported by complete rings of hyaline cartilage enclosed by the tunica adventitia and serosa (6). The tracheal epithelium in birds is pseudostratified, ciliated columnar epithelium with either goblet cells or intraepithelial mucus glands (respiratory epithelium) (7). The cilia waves transport secretions and tricked particulate matter (8). There are collagen fibers arranged in large bundles, blood vessels, and numerous lymphocytes, as well as serous and mucous tracheal glands in the propria-submucosa, which were also rich in elastic fibers (9). The cartilaginous layer was made up of complete hyaline rings, whose diameters, ossification and overlapping differed with the types and ages of the birds (2). The trachea has been studied morphologically, physiologically, and histologically in numerous species. However, the gross morphology, histology, and histochemistry of the trachea in geese and cattle egrets appears to be not adequate and there is no study revealing the structure of the trachea in the house sparrow, which is an...
example of a passerine bird. The aim of this study was to clarify the morphological and histological characters of the trachea in three different species (geese, cattle egrets, and sparrows), to 1- compare between them and other birds. 2- Show if these features have role in phonation or respiration processes or not.

Materials and methods

Species
In this study, 21 healthy birds (seven per species of geese (Anser anser domesticus)) with the weight ranged from 2500 to 3000 g were purchased from a commercial poultry in zagazig city, Sharkia province. The other species, cattle egrets (Bubulcus ibis) and house sparrows (Passer domesticus), were caught from the local surroundings. Immediately after bringing the birds, they were trapped in well-aerated cages and then moved to the Department of Histology and Cytology, faculty of Veterinary Medicine, Zagazig University. Then, under observation for 2 days, the birds were kept before the beginning of tissues harvesting to enable them to adapt to their environment.

Animal preparation (ethical consideration)
All the birds were euthanatized, after which they were retained on their backbone, an opening made from the posterior part (vent) to the superior one (shoulder joint) laterally, then the related bones and muscles imitated, giving access to the viscera such that the whole respiratory system could be pictured, and its relationship with adjacent organs observed after retracting the trachea and getting rid of any tissues as adipose tissue to record structural measuring. This study protocol was evaluated and accepted by the Institutional Animal Care and Use Committee (IACUC) of Zagazig University, Egypt. (ZU-IACUC/ 2/F/67/2021).

Morphometrical analysis
Pieces of equipment were utilized in the morphometric investigation, such as Caliper, ruler, and a digital camera, to measure the following: (1) The respiratory system length (from the cranial termination of the larynx to the caudal margin of the lung) and the trachea length (from the first tracheal cartilage ring into the last one which connects with syrinx). (2) After combining formalin and 70% alcohol, the diameter of the trachea was measured. The diameter of tracheal cartilage was measured in two regions: (a) a proximal part (b) a distal pat. (3) Calculating the number of tracheal rings.

Histological study
The trachea was fixed in Bouin solution for 6-10 hours to protect the mucous secretory cells from damage (10), after which the samples were collected immediately and placed in 10% neutral buffer formalin overnight. The samples were decalcified by decalcifying agent (EDTA), dehydrated in a series of ascending grade of ethanol, cleared with xylene (three changes), infiltrated in soft melted paraffin in a hot air oven, and embedded in hard paraffin wax to form paraffin blocks. The specimens were processed to obtain a desirable paraffin section of 4-5 µm thickness by using a microtome. Some achieved sections were stained with usual staining: As a general stain the (H&E) Harris’s hematoxylin and eosin. Crossmon's trichrome stain for detection collagen fibers (11). (AB) Alcian Blue stain to detect acidic mucopolysaccharides (12). The Periodic Acid Schiff (PAS) technique is used to detect neutral mucopolysaccharides and some acidic ones (13). Orcein stain for elastic fibers (14). The handling with tissue and staining methods were conducted consistent with (15). All the stained sections were examined with a standard light microscope (Objective × 4, 10, 40 and 100) and photo’d with a digital cyber shot camera (Sony-Japan). And this carried out in Histology and Cytology Department, Zagazig University).

Histomorphometric analysis
Six birds per each species were used for the morphometry; the Image-J software was used for the quantitative measurements of the epithelial height using H&E-stained sections from both proximal and distal parts, and the cartilaginous layer thickness of both proximal and distal parts, also the AB and PAS optical densities in the secretory glands in AB- and PAS-stained sections at ×40 magnification, in addition to, the collagen fibers area % in sections stained with Crossman's trichrome stain.

Statistical analyses
The Anderson-Darling test was used to confirm that all numerical data was evaluated for normality. The SPSS software (v.16) was done for statistical assessment. The information was stated as mean±standard error (SEM). The Mann-Whitney U test was used to determine the significance of the disparities between in AB and PAS optical densities of among the three investigated species. For exposing significances between the three species in the epithelial height, the area percentage of the collagen fibers and the thickness of tunica fibrocartilage of both proximal and distal regions, the Kruskal- Wallis H test was done.

Results

Macroscopical examination of the trachea
In geese, cattle egrets, and house sparrows, the trachea appeared as an extended pliable tube. It generally appeared ventral to the esophagus along the right side of the neck and then returned to the mid-ventral position as it approached the thoracic inlet (Figure 1a, c, e). The mean length of tracheas of geese was 38.3±8 cm; in cattle egrets, was 21.2±5 cm; and in the house sparrow was 4.5±0.1 which accounts for
almost 79% in the first, 73.1% in the second and 60% in the third of the overall length of the respiratory system, whereas the length of the larynx, syrinx, primary bronchi and lungs (all another organs) form approximately 21% in the first, 26.9% in the second and 40% in the third (Table 1).

The tracheas of three studied species formed of proximal and distal parts (Figure 1a, c, e) with the proximal part starting at the cricoid cartilage of the larynx to the xiphoid process of the sternum. While the distal part runs from the xiphoid process to the tympanum (tracheosyringeal cartilages) of the syrinx. The mean length of the proximal part of geese, cattle egrets, and house sparrows was 31.8±1.013, 16.33±0.557, and 3.4±0.143 respectively. While the mean length of distal part in geese was 6.5±0.428, in cattle egret was 4.83±0.167 and finally in house sparrow was 1.167±0.115. Consequently, the trachea of geese was the longest either the proximal or the distal parts (Figure 1g, h).

The tracheal cartilages (the basic structural unit of the trachea) were complete rings (O) in shape. The tracheal cartilages number in geese is approximately 130-140, in cattle egret 155-160 and in house sparrow 45-60. The diameter of the tracheal cartilage rings in geese is approximately unequal, starting with a steady decrease from the larynx to the syrinx, since the mean diameter of the trachea connection with larynx was 4.566±0.138 cm, but the connection with syrinx was 2.8±0.208 cm. While cattle egret measured 1.466±0.083 cm and 1.3±0.036 cm, house sparrow measured 0.4±0.036 cm and 0.45±0.042 cm. So, in cattle egret and house sparrow the diameter of the tracheal cartilage rings in two parts is roughly identical from larynx to syrinx and there is no noticeable variation between them. In three of the investigated species; there were two skeletal muscles laterally on the trachea, namely, 1. The Trachiolateralis muscle: - a pair of cylindrical thick skeletal muscles that extend from the caudalateral aspect of the body of the cricoid cartilage of larynx near the linkage between the trachea and the syrinx (bronchiosyringeal cartilages) (Figure 1a, c, e). 2-

The Sternotrachialis muscle: - a paired cylindrical thick and broad skeletal muscle that extend from the costal process of the sternum (craniolateral process) to the caudal rings of trachea to help ascending the caudal part of the trachea (Figure 1b, d, f).

Table 1: The Length of the whole respiratory system and its parts in three studied birds

<table>
<thead>
<tr>
<th></th>
<th>Respiratory system</th>
<th>Trachea</th>
<th>Other organs</th>
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<tr>
<td></td>
<td>Length (cm)</td>
<td>Percentage</td>
<td>Length (cm)</td>
</tr>
<tr>
<td>Geese</td>
<td>48.5</td>
<td>100%</td>
<td>38.3</td>
</tr>
<tr>
<td>Cattle egret</td>
<td>29</td>
<td>100%</td>
<td>21.2</td>
</tr>
<tr>
<td>House sparrow</td>
<td>7.5</td>
<td>100%</td>
<td>4.5</td>
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</tbody>
</table>

**Microscopical examination of trachea**

The H&E-stained sections of the trachea from proximal part in geese, cattle egrets, and house sparrows explained that the tracheal wall comprised of four distinct tunicae; mucosa, submucosa, fibrocartilage, and adventitia (Figure 2a, b, c). In all studied birds, the lamina epithelialis of the tunica mucosa was ciliated, pseudostratified columnar epithelium with great numbers and several sizes of acini of the simple tubuloalveolar mucous glands that pierced the epithelium to the tracheal cavity in geese (Figure 2a) and simple alveolar mucous glands in cattle egrets (Figure 2b). In house sparrows, the epithelium of proximal part contained mostly goblet cell (Figure 2c) with the mucous gland only appearing on rare occasions in the intraepithelial gland of geese and cattle egrets, the secretory epithelial cells were pyramidal cells with pale vacuolated acidophilic cytoplasm, mucous...
droplets, and basal flattened nuclei with hardly visible cell boundaries (Figure 3a, b). What took up most of the thickness of the epithelium compressing on the epithelial cells and converting them to a short cell layer. In addition, in house sparrows the goblet cell appears as secretory cell with wide apical part containing mucous droplets with basal flattened nuclei (Figure 3c). Statistically, the proximal tracheal epithelium of the geese was the highest (Figure 3d), (Table 2).

Table 2: Histomorphometry of the trachea

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Geese</th>
<th>Cattle egrets</th>
<th>Sparrows</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal part</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial height</td>
<td>225.06±1.50</td>
<td>185.20±1.10</td>
<td>216.60±1.30</td>
<td>0.001*</td>
</tr>
<tr>
<td>Optical densities of AB</td>
<td>38.80±0.70</td>
<td>10.70±0.50</td>
<td>10.90±0.50</td>
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<tr>
<td>Optical densities of PAS</td>
<td>63.40±0.40</td>
<td>12.80±0.40</td>
<td>22.90±0.70</td>
<td>0.007*</td>
</tr>
<tr>
<td>Area % of collagen fibers</td>
<td>47.70±0.50</td>
<td>18.07±0.50</td>
<td>25.60±0.60</td>
<td>0.002*</td>
</tr>
<tr>
<td>Epithelial height</td>
<td>115.83±1.10</td>
<td>139.22±1.80</td>
<td>124.2±1.40</td>
<td>0.005*</td>
</tr>
<tr>
<td>Optical densities of AB</td>
<td>12.83±0.20</td>
<td>12.97±0.40</td>
<td>11.37±0.20</td>
<td>0.002*</td>
</tr>
<tr>
<td>Optical densities of PAS</td>
<td>24.81±0.20</td>
<td>13.83±0.30</td>
<td>25.87±0.40</td>
<td>0.002*</td>
</tr>
<tr>
<td>Area % of collagen fibers</td>
<td>20.30±0.20</td>
<td>11.68±0.30</td>
<td>5.79±0.20</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Abbreviations: PAS= periodic Acid Schiff and AB= Alcian Blue. *Means a major variation in the directly above-cited factors, the p-value indicates whether or not there is a significant difference between the three distinct species in the same raw at P≤0.05.
Figure 4: AB & PAS-stained sections from tracheal proximal part. Photomicrograph of the intraepithelial glands or goblet cells from (a,b) geese, (c,d) cattle egrets and (e,f) sparrows. The completely stained mucous acini by AB (arrows) and PAS (dashed arrows). AB (black arrowhead) and PAS (yellow arrowhead) positively stained goblet cells. Scale bars all = 50µm. Bar charts representing the of AB (g) and PAS (h) optical densities. Records are stated as means±SEM from 6 bird per each species, (a-c) refer to statistically considerable differences (P≤0.05).

The propria submucosa, which is a loose connective tissue with no serous or mucous glands, was highly vascularized. Numerous aggregations of lymphocytes, which were also observed in all the studied species (Figure 2a, b, c), contained large bundles of collagen fibers that gave positive reaction to Crossman stain (Figure 5a, b, c). Statistically, the percentage of collagen fibers in geese was the highest (Figure 5d and Table 2). Also, sparrows had a higher percentage of elastic fibers than the other two groups that positively reacted to orcein stain (Figure 6a, b, c). The submucosa is closed by the tracheal cartilage’s perichondrium. In the proximal part of the tracheal wall, there were overlapped hyaline cartilaginous rings (Figure 2a, b, c). Statistically, the thickness of proximal part (overlapped part) of the geese was the highest (Figure 2d and Table 2). In geese the cartilage consists of plates (Figure 2a). The matrix (ground substance) of hyaline cartilaginous rings in proximal part contained the scattered lacunae which limited chondrocytes; these chondrocytes fill all lacunae and their sizes in geese and cattle egrets are less than those in sparrows (Figure 7a, c, d). The matrix in proximal part was partially ossified in geese (Figure 7b) and cattle egrets (Figure 7c) but there was no ossification in sparrows (Figure 7d). The Trachiolateralis muscles (skeletal muscles) tightly connected to the adventitia laterally on the left and right sides of the proximal part and about half of the distal part of the trachea (Figure 2 and 7). The tracheal wall of examined species had four distinct tunicae; mucosa, submucosa, fibrocartilage, and adventitia according to H&E-stained cross-sections of trachea from distal part (Figure 8a, b, c).

Figure 5: Collagen fibers in proximal part. Crossman’s trichrome stained sections of the tracheal proximal part from geese (a) cattle egrets (b) and sparrow (c) from. The collagen fibers are widely appeared in propria submucosa (arrows), between cartilage (double arrow) and between the muscle fibers (dashed arrows). Scale bars a = 100 µm; b, c =50 µm. (d): Bar chart from all studied species demonstrating the collagen fiber area %. Records are stated as means±SEM from six bird of each species, (a-c) refer to statistically considerable differences (P≤0.05).

Figure 6: Elastic fibers in proximal part. Orcein-stained sections of the tracheal proximal parts from (a) geese, (b) cattle egrets and (c) sparrows. The elastic fibers are more visible in propria submucosa (arrows), between cartilage (dashed arrows). Scale bars a = 100 µm; b, c =50 µm.
Figure 7: Ossification of proximal part. Photomicrographs of the tunica fibrocartilage of the proximal part from geese (a,b) cattle egrets (c) and sparrow (d) from H&E-stained sections. Showing chondrocyte (arrow), perichondrium (arrowhead), osteocyte (dashed arrow), spongy bone with bone marrow (BM), periosteum (yellow arrowhead) and the tunica muscosa (M). Scale bars all=50 µm.

Figure 8: H&E-stained sections in tracheal distal parts. Cross section of the tracheal distal part from geese (a), cattle egrets (b) and sparrows (c) showing all layers of trachea; mucosa (lamina epithelialis (E) pseudostratified ciliated columnar epithelium with intraepithelial glands (arrow) or goblet cells (arrowhead), propria submucosa (PS), non-overlapped ossified cartilaginous rings (Bone (B)). Partially Ossified (PO) cartilaginous rings and tunica muscosa (M). Scale bars; a = 100 µm; b, c =50 µm. (d): Bar chart for three investigated species illustrating the epithelial height of the distal parts. Records are stated as means±SEM from 6 bird per each species, (a-c) refer to statistically considerable differences (P≤0.05).

Figure 9: The distal part lining epithelium. Photomicrographs showing the pseudostratified ciliated columnar epithelium with intraepithelial glands (dashed arrow) in cattle egrets (b) or with goblet cells (arrow) in geese (a) and house sparrows (c). Scale bars; a=50 µm while (b,c) =30 µm. (d): Bar chart for three investigated species illustrating the epithelial height of the distal parts. Records are stated as means±SEM from 6 bird per each species, (a-c) refer to statistically considerable differences (P≤0.05).

The thickness of the distal part of the geese was statistically the highest (Figure 8d and Table 2). The lamina epithelialis of the tunica mucosa was ciliated, pseudostratified columnar epithelium with goblet cells in geese (Figure 9a) and house sparrows (Figure 9c), but with intraepithelial glands in cattle egrets (Figure 9b) as the proximal part indicating a high number of mucous secretions secreted by trachea. The tracheal epithelium of the cattle egrets had the highest statistical value (Figure 9d and Table 2).

The intraepithelial gland and goblet cells in the lining secretory cells of the three investigated species secret neutral and sulfated mucins that were positively reacted to AB (Figure 10a, c, e) and PAS (Figure 10b, d, f). Conversely, the AB optical densities in three investigated species were relatively equal (Figure 10g and Table 2), while PAS optical densities in house sparrows were statistically higher than those in cattle egrets and geese (Figure 10h and Table 2). In geese, the propria submucosa was dense connective tissue (Figure 8a) but it was loose connective in cattle egrets (Figure 8b) and house sparrows (Figure 8c) with bundles of collagen fibers that gave positive reaction to Crossman stain.
(Figure 11a, b, c). Statistically, the collagen fiber area % of the geese was higher than cattle egret and house sparrow (Figure 11d and Table 2). Also, there were large amounts of elastic fibers that positively reacted to orcein stain (Figure 12a, b, c) highly detected in geese and sparrows than cattle egrets. The tracheal wall consisted of non-overlapped ossified cartilaginous rings in distal part (Figure 8). Statistically, the thickness of distal part (non-overlapped part) of the geese was the thickest (Figure 8d and Table 2). The ring matrix in the distal part ossified entirely in geese (Figure 8a) and house sparrows (Figure 8c) but in cattle egrets started in stage of ossification since matrix more acidophilic and osteocyte inside its lacuna (partially ossified) (Figure 8b).

Figure 10: AB&PAS-stained sections from tracheal distal part. Photomicrograph of the intraepithelial glands or goblet cells from geese (a,b), cattle egrets (c,d) and sparrows (e,f). The AB (black arrows) and PAS (red arrows) certainly stained goblet cells. AB (black arrowhead) and PAS (red arrowhead) positively stained mucous acini. Scale bars all = 50 µm. Bar charts showing the AB (g) and PAS (h) optical densities. Records are stated as means±SEM from 6 bird per each species, (a-c) refer to statistically considerable differences (P≤0.05).

Figure 11: Collagen fibers in distal part of trachea. Crossman’s trichrome stained sections of the tracheal distal parts from (a) geese, (b) cattle egrets and (c) sparrows. The collagen fibers are more visible in propria submucosa (arrows) as well as between the muscle fibers (dashed arrows), Scale bars a = 100 µm; b, c = 50 µm. (d): Bar chart for all studied species viewing the collagen fibers area %. Records are stated as means±SEM from 6 bird per each species, (a-c) refer to statistically considerable differences (P≤0.05).

Figure 12: Elastic fibers in tracheal distal part. Orcein stained sections of the tracheal distal parts from (a) geese, (b) cattle egrets and (c) sparrows. The elastic fibers are prominent in propria submucosa (arrows). Scale bars (50 µm).

Discussion

The tracheas of geese, cattle egrets and house sparrows appear as empty, long, cylindrical flexible and cartilaginous tubes in the present study, which are consistent with the finding in birds in general (1). The average tracheal length was found to be longer in geese than cattle egrets and house sparrows in the current study. This variation in the tracheal length could be attributable to species difference in bird body size (16). The changes in the tracheal length might be successful in sound production (17). The mean length in
house sparrows was 4.5±0.1 which was noted in Bee-eater 5.087±0.21 cm and the length of trachea in budgerigars was approximately 5 cm (18), which could be due to these birds having similar body volumes (19).

Although geese have longer tracheae than cattle egrets, the latter have more cartilaginous rings than geese. This has been explained by the fact that geese have thicker cartilaginous rings than cattle egret. The mean total number of Cartilaginous rings forming trachea in geese 130-140 this result was partially in accord with (20) who found that There were 137-140 tracheal cartilage in trachea of geese. The total number of tracheal cartilaginous rings in cattle egrets was 155-160 this is in line with the finding of many studies (21,22). In long-legged buzzard, this number was 89-96; the mean number of cartilaginous rings was 64.2±1.2 in Kingfisher Birds and 151±12 in turkey. We found that the house sparrow had 45-60 cartilaginous rings in their trachea, that agrees with (23) who found 53-61 in budgerigars, 46-47 in canaries and other Passeriformes birds. This also came from species differences and variations in body volume and this result accept with (1) who said that the number of the tracheal rings ranged from about 30 in small passeriform birds to about 350 in long neck flamingos.

In three studied species, there were two tracheal muscles (sternotrachialis and Trachiolateralis muscles) which harmonized with (21,22,24,18) and inconsistence (20) who noted there were another tracheal muscle called (Ciliated muscle), as well as (25) who showed in the brown thrashers, the tracheal muscles were composed of Trachiolateralis dorsalis, Trachiolateralis ventralis, tracheobronchialis, besides the sternotrachialis. These muscles help in the process of inspiration and expiration especially the sternotrachialis muscles that are thought to have a role in the phonation process (17). These characteristics of the trachea, such as the presence of complete rings, length and strong muscles can be attributed to its role in adaptation of sounds and mucus secretion movement (26,27).

The tracheas in geese, cattle egrets and sparrows were lined by ciliated, pseudostratified columnar epithelium, typical respiratory epithelium, as well as large and numerous mucous glands in the proximal part and goblet cell groups in the tracheal distal part in geese. These results were well-matched with the finding of certain previous studies (6,9). In house sparrows, goblet cells were present in the epithelium and intraepithelial glands were observed which agreed with (7,20) in their goose study and (28) in guinea fowl. There were intraepithelial glands in both the proximal and distal parts of cattle egret, and this agree with (28) in coot birds who reported that the anterior part of the birds had the most and the posterior part had the least. This may be due to the environment that surrounds the birds adapted them to release huge amount of mucous. These features of tracheal epithelium as ciliated, mucus glands compensated by numerous of goblet cells and epithelial thickness, act as defensive elements of bird health, performing cleaning function by removing foreign bodies from the respiratory tract and helping in sound release (29).

The studied bird’s propria submucosa was loose connective tissue disagree with (30) who claimed that the Japanese quail trachea’s propria and submucosa was a dense connective tissue. Elastic fibers are abundant especially in sparrow which may help in sound production and singing. Blood vessels, and numerous aggregations of lymphocytes were also observed this harmonized with (30) in quails, (28) in coot birds and guinea fowl and (9) in turkeys.

Conclusions

This study concluded that the environment of bird plays an important role in structure of its respiratory system as in cattle egrets the lining epithelium is respiratory epithelium but with intraepithelial mucous gland in two parts of trachea and this refers to the high amount of mucous production. Also, the tracheal length is a species-specific structural variable that plays an important role in sound production as in geese have the longest proximal and distal parts and this explains the very loud sound of geese.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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