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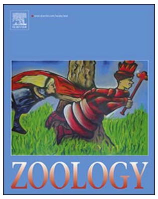

Deformation of avian middle ear structures under static pressure loads, and potential regulation mechanisms

Raf Claes[a,b,*], Pieter G.G. Muyshondt[c], Joris J.J. Dirckx[c], Peter Aerts[a,d]

[a] *University of Antwerp, Laboratory of Functional Morphology, Universiteitsplein 1, B-2610 Antwerp, Belgium*
[b] *Vrije Universiteit Brussel, Department of Mechanical Engineering, Pleinlaan 2, B-1050 Brussels, Belgium*
[c] *University of Antwerp, Laboratory of BioMedical Physics, Groenenborgerlaan 171, B-2020 Antwerp, Belgium*
[d] *University of Ghent, Department of Movement and Sport Science, Watersportlaan 2, B-9000 Ghent, Belgium*

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ABSTRACT

Static pressure changes can alter the configuration and mechanical behavior of the chain of ossicles, which may affect the acoustic transfer function. In mammals, the Eustachian tube plays an important role in restoring ambient middle ear pressure, hence restoring the acoustic transfer function and excluding barotrauma of the middle and inner ear. Ambient pressure fluctuations can be potentially extreme in birds and due to the simple structure of the avian middle ear (one ossicle, one muscle), regulation of the middle ear pressure via reflexive opening of the pharyngotympanic tube appears all the more important. In this study the deformations of the chicken (*Gallus gallus domesticus*) middle ear structures, as a result of middle ear pressure alterations, are quantified, using micro-CT scanning. It was experimentally tested whether reflexive opening of the pharyngotympanic tube to restore ambient middle ear pressure is present in chicken and mallard (*Anas platyrhynchos*) and whether this mechanism depends on sensing middle ear pressure indirectly via deformations of the middle ear components or sensing the middle ear pressure directly. A translation of the columella footplate was observed when middle ear pressure was kept at 1 kPa and −1 kPa relative to ambient pressure. Deformation of the tympanic membrane was larger than the columella footplate translation. Bending and deformation of the extracolumella was observed. Opening of the pharyngotympanic tube occurred at random pressure for both chicken and mallard when middle ear pressure was raised and lowered by 1.5 kPa relative to ambient pressure. We also did not find a difference in middle ear venting rate when middle ear pressure was held constant at 0.5, 1, 1.5, −0.5, −1 and −1.5 kPa for chickens and at 1, 2, 4, −1, −2 and −4 kPa for mallards. As a result, no statement can be made about pressure within the avian middle ear being measured directly or indirectly. Our experiments do not support the presence of a short-loop reflexive control of pressure equilibration via the pharyngotympanic tube. However, it is still possible that triggering this loop requires additional sensorial input (e.g. visual, vestibular) or that it occurs voluntarily (being controlled at a higher brain level).

1. Introduction

When tetrapods made the transition from water to land the development of the middle ear (ME) structures was essential to match the acoustic impedance between the outside air and the fluid-filled inner ear. Without the mechanical impedance match 99.9% of sound energy would be lost due to reflection (Møller, 1974). The ME ossicles are contained in an air-filled cavity with a mostly rigid wall and one opening is sealed off by the tympanic membrane. A connection between the ME cavity and the nasopharynx exists both in mammals (Eustachian tube) and in birds (pharyngotympanic tube). In mammals it has been shown that the Eustachian tube is closed most of the time. In normal conditions it occasionally injects small volumes of nasopharyngeal gas into the middle ear. When, mostly due to external circumstances, very large pressure differences develop between the ME cavity and the ambient pressure, it has the function of a protective valve (Dirckx et al., 2013) and can release over- or underpressure. The main pressure regulation mechanism in the mammal ear is a complex interplay between eardrum deformations, Eustachian tube gas injection and, most importantly, gas exchange over the mucosa covering the inner walls of the ME cavity and the air cells in the mastoid. In birds, a mucosa-lined mastoid is not present. However, the ME cavity and air cavities in the skull (connecting the two MEs) are covered with mucosa so gas exchange can be present. It could be that the pharyngotympanic tube

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[*] Corresponding author at: University of Antwerp, Laboratory of Functional Morphology, Universiteitsplein 1, B-2610 Antwerp, Belgium.
E-mail addresses: raf.claes@uantwerpen.be (R. Claes), pieter.muyshondt@uantwerpen.be (P.G.G. Muyshondt), joris.dirckx@uantwerpen.be (J.J.J. Dirckx), peter.aerts@uantwerpen.be (P. Aerts).

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might play a more prominent role in ME pressure regulation.

The mammalian ME contains three bony ossicles (malleus, incus and stapes), two muscles (tensor tympani muscle and stapedius muscle) and some ligaments (anterior and superior mallear ligament; posterior incudal ligament and the annular ligament) to transmit (and modulate) the sound waves from the tympanic membrane to the inner ear. The ME cavity is also enclosed within one single bony structure and the only connection to the pharynx is via the Eustachian tubes, one at each side of the head (Møller, 1974; Rosowski, 1996; Ades et al., 2012). The mammalian ME is subjected to slow and sudden pressure changes due to changes in ambient pressure (e.g. changing altitude, diving in water etc.). These pressure changes may cause a pressure differential over the tympanic membrane resulting in deformations of the tympanic membrane, thus altering the configuration and mechanical behavior of the chain of ossicles. This may affect the acoustic transfer function (Murakami et al., 1997; Teoh et al., 1997; Lee and Rosowski, 2001; Dirckx et al., 2006). When pressure changes are extreme (cfr. underwater diving: 10.1 kPa/m), barotrauma of the ME and inner ear can occur (e.g. rupture of the tympanic membrane, the inner ear membranes, oval and round window) (Melamed et al., 1992). The articulated multiple ossicles, muscles and ligaments of the ME enable to (partly) compensate for these altered mechanics, but cancelling the pressure differentials over the tympanic membrane is a more direct way to restore the normal transfer.

The effects of static pressure changes on the mammalian ME are well known and described (Hüttenbrink, 1988; Dirckx and Decreamer, 1991; Dirckx et al., 2006). During experiments conducted by Dirckx et al. (2006) on cadaveric rabbits' temporal bones, where ambient pressure was altered from −2.5 to 2.5 kPa, the umbo (of the tympanic membrane) was displaced by 0.165 mm while the stapes amplitude was only 0.034 mm. In humans the complex interplay between gas exchange processes, eardrum deformation and, to some extent, Eustachian tube action regulates the pressure differences between the ME cavity and ambient pressure (Dirckx et al., 2013). In the human middle ear, it has been shown that both over- and underpressure can develop over time and that normal Eustachian tube action does not reset the pressure to ambient conditions (Padurariu et al., 2016). Under normal conditions these Eustachian tubes are closed. During reflexive behaviors, including swallowing and yawning, muscles will enable gas transfer through the Eustachian tube between the ME and the pharynx (Siedentop et al., 1968; Rosowski and Merchant, 2000). Therefore, a baroreceptor function is premised at the level of the ME but the exact appearance and sites of these receptors are still under debate (Rockley and Hawke, 1992). For example, Nagai and Tono (1989) and Nagai et al. (1989) reported mechanoreceptors (Vater-Pacinian corpuscles) in the tympanic membrane and ME which are suggested to be sensitive to deformations of the tympanic membrane which may play a role in Eustachian tube action.

In birds, the ME contains one ossicle with a bony shaft (the columella) and a cartilaginous, trifurcated distal end (the extracolumella), some ligaments (ascendens ligament, drumtubal ligaments, Platner's ligament and annular ligament) and one muscle (stapedius muscle) (Smith, 1904; Starck, 1995; Saunders et al., 2000) (Fig. 1A). The MEs at both sides of the head are connected to each other by intracranial air-filled cavities and the interaural pathway which is part of the Y-shaped pharyngotympanic tube (Fig. 1B). Increasing the pressure outside one ear makes the tympanic membrane of the contralateral ear bulge out (Wada, 1923; Schwartzkopff, 1955; Counter and Borg, 1979) (Fig. 2). The avian MEs are also connected with the pharynx via the pharyngotympanic tube, which is also closed (Saunders et al., 2000) (Fig. 2). Under normal conditions ME pressure slowly decreases 20 Pa below ambient pressure. When this pressure is reached, venting of the ME (opening of the pharyngotympanic tube) occurs. As such, depending on the species, regular venting of the ME occurs at constant ambient pressures, every 20–180 s (Larsen et al., 1997, 2016).

However, much larger pressure differentials than a few tens of Pa/s are likely to occur as a result of birds' behavior. Many species frequently climb and descend several hundreds of meters in a relatively short time span. The bar-headed goose (*Anser indicus*), for instance, can bridge altitude differences of 6000 m starting at sea level in one flight (total pressure drop of ± 54 kPa (Hawkes et al., 2011), and stoop-dive flights of peregrine falcons, reaching speeds as high as 100 m/s (e.g. Ponitz et al., 2014), go along with pressure rates of change above 1 kPa/s (own estimates). In case of plunge-diving birds, such as gannets, these rates of change may even rise to several tens of kPa/s (based on, e.g., Brierley and Fernandez, 2001; Capuska et al., 2011). Due to the simple structure of the ME (one ossicle, one muscle), the capability to compensate for the inherently affected mechanical transfer by adjusting the properties of the chain connecting the tympanic membrane with the inner ear seems to be much less in birds, compared to the mammalian ME. Therefore, given the potentially large and fast ambient pressure fluctuations, the possibility to effectively regulate the ME pressure via reflexive opening of the pharyngotympanic tube appears all the more important.

If reflexive pressure equilibration via pharyngotympanic tube ventilation is present in birds, there is also a need for a system that can sense the pressure changes in the ME. Whatever the sensor is, from the mechanical point of view it could theoretically function in two different ways: either it senses the ME pressure directly, or it relies on the stress or strain changes that emerge when the mechanical chain of the ME

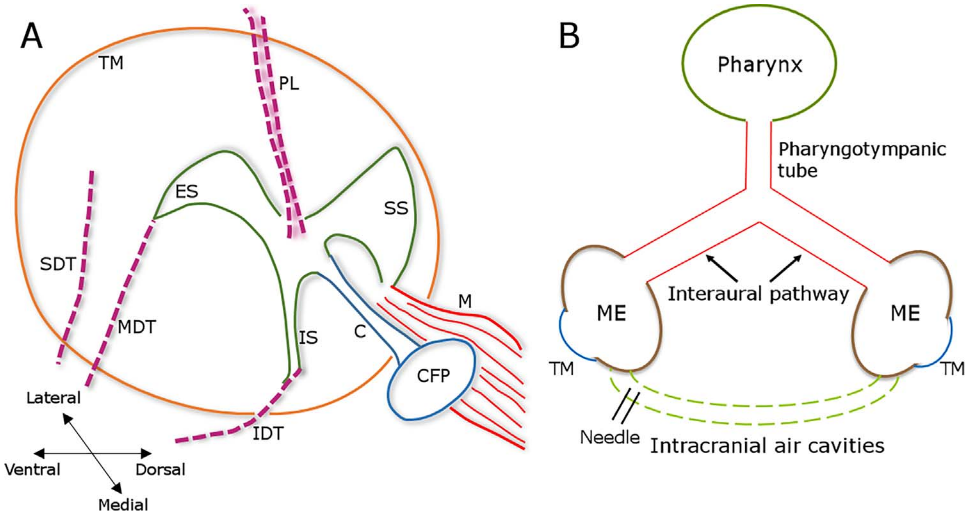

Fig. 1. (A) Medial view of the middle ear (ME) components: tympanic membrane (TM), columella (C), columella footplate (CFP), extrastapedius (ES), infrastapedius (IS), suprastapedius (SS), Platner's ligament (PL), superior drumtubal ligament (SDT), medial drumtubal ligament (MDT), inferior drumtubal ligament (IDT) and tympanic muscle (M). Deduced from Smith (1904). (B) Schematic overview of the connections between pharynx and middle ears (ME), with pharyngotympanic tube, interaural pathway and intracranial air cavities.

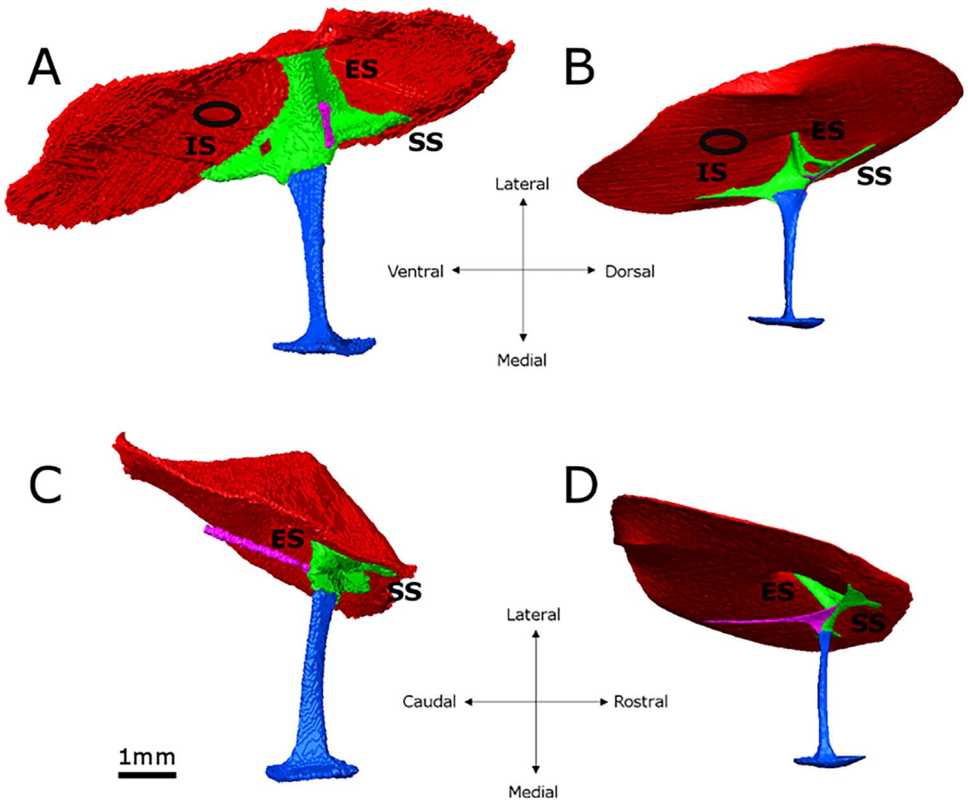

Fig. 2. Comparison of the ME mechanical chain of chicken (A and C) and mallard (B and D). (A and B) Frontal view; (C and D) lateral view. Tympanic membrane (red), extracolumella (green), columella (blue) and Platner's ligament (purple). Black oval: position and diameter of the tympanic membrane perforation. Abbreviations: IS, infrastapedius; ES, extrastapedius; SS, suprastapedius. Scale bar = 1 mm.

deforms as a result of pressure alterations. As for mammals, a 'candidate' organ can be premised. Birds (and also some other tetrapods such as juvenile alligators and one species of bat) have a small organ in the ME, the paratympanic organ (PTO), which is suggested to function as a baro- and altimeter (Von Bartheld, 1994; Von Bartheld and Giannessi, 2011), although the exact function of the PTO is still under debate.

In this study we first quantify the form changes of the sound transmission chain (i.e. the tympanic membrane, the extracolumella and the columella) as a result of ME pressure alterations (mimicking differentials with ambient pressure) using micro-CT (μCT) scanning. Next we test (i) whether reflexive opening of the pharyngotympanic tube to restore the neutral configuration of the ME-mechanical chain does occur in birds when exposed to pressure changes, and (ii) whether this relies on a mechanism of direct pressure sensing in the ME or on the deformation of the ME structures itself. The results are discussed in the context of the PTO function.

2. Materials and methods

2.1. Study species

Many experimental and morphological studies on the ME are carried out on domestic chickens (*Gallus gallus domesticus*) (Pohlman, 1921; Giannessi and Pera, 1985; Saunders, 1985; Von Bartheld, 1990; Von Bartheld and Giannessi, 1994; Giannessi et al., 1996; Mills and Zhang, 2006). Therefore, the present study focusses on chickens to quantify the deformation of the ME mechanical chain by means of μCT scanning. However, chickens are adapted to a terrestrial lifestyle and do not experience large ambient pressure fluctuations as compared to diving or flying species. For this reason, pressure experiments testing for the presence of reflexive opening of the pharyngotympanic tube and, eventually, for the nature of the triggering signal, are also performed on mallards (*Anas platyrhynchos*), a species which encounters higher static and dynamic pressure fluctuations due to its lifestyle (crossing altitudes of 1 km very fast).

2.2. Effects of pressure on middle ear components in chicken

μCT scans were made of the head of both chicken and mallard post mortem to compare the ME anatomy. To visualize the possible effects of ambient pressure fluctuations on the tympanic membrane, columella and extracolumella, one rooster head was used for μCT scanning. Before scanning a thin hollow needle (Ø = 0.8 mm) was inserted into the caudal part of the skull and was used to alter pressure within the intracranial air cavities, interaural pathway, and hence the MEs as they are connected with the intracranial air cavities and interaural pathway (Fig. 1). To ensure this system was airtight, glue was put around the entrance point of the needle into the skull. Three μCT scans were made with the Environmental Micro-CT (EMCT) of the Centre for X-ray Tomography at Ghent University (Dierick et al., 2014) to study the possible effects of pressure changes on the tympanic membrane, columella and extracolumella. During the first scan, the needle was opened, so ambient and ME pressure were identical. Secondly, a rubber tube connected to a custom-made pressure generator was placed on the needle and ME pressure was held at −1 kPa below ambient pressure, which corresponds to a descent in altitude for instance from 83 m to sea level. The custom-made pressure generator was used to change pressure in the intracranial air cavities, interaural pathway and MEs. After calibration, the system holds the pressure at the desired value with a precision better than 20 Pa. For the third scan the ME pressure was held 1 kPa above ambient pressure, which corresponds to a rise in atmospheric pressure from sea level to 83 m. All samples were scanned across an angle of 360° with the X-ray source set at 90 kV and 24.75 μA. The exposure time was set at 40 ms and the total scanning time per sample was 17 min. The reconstructed slice images had a voxel size of 30 μm.

A three-dimensional image processing software package (AMIRA 5.4.4; 64-bit version) was used to assign the voxels corresponding to the tympanic membrane, columella, extracolumella, Platner's ligament and the bony semi-circular canals of the inner ear. Segmentation was performed by automatic thresholding based on gray-scale values in combination with a manual correction in the three orthogonal views. The segmented outlines were smoothed and a surface model was created. The bony semi-circular canals of the inner ear were used to align the models so a comparison could be made between the three conditions.

The anatomy of the chicken ME, as deduced from the μCT-scans, was compared with that of the mallard. The mallard head was scanned with the High-Energy CT system Optimized for Research (HECTOR), also at Ghent University, with a resolution of 7.5 μm (Masschaele et al., 2013).

Possible displacement of the tympanic membrane was measured at the apex where the extracolumella pushes the membrane outwards. The potential translation of the columella was assessed by allocating four anatomical points on the rim of the footplate. Of these coordinates the mean was calculated. The difference in position between the means of the three conditions was calculated. Potential angular displacement of the columella was calculated as the rotation of the tip of the bony shaft about the position where the bony shaft is connected to the columella footplate. Linear displacement of the tips of the three arms of the extracolumella was measured. After aligning the columellas of the three different conditions, exctracolumella deformation was quantified as rotation angles of the tips of the three arms of the extracolumella (infra-, supra- and extrastapedius) around the transition of the cartilaginous extracolumella and the bony columella (Figs. 1 and 2).

2.3. Pharyngotympanic tube response on fluctuating middle ear pressure

Three hens and three female mallards were used for these experiments. Three experiments were conducted on each individual. In the first experiment, the left tympanic membrane of the bird was perforated with a needle (Ø = 0.8 mm), so only a small puncture was present and the shape of the tympanic membrane was preserved (Fig. 2A, B). A plastic hollow plug was glued in the outer ear canal. This plug was connected via a rubber tube to our custom-made pressure generator (see above in Section 2.2). In this way pressure in the MEs, intracranial air cavities and interaural pathway could be altered above and below ambient pressure (see below). Secondly, the right tympanic membrane was also perforated with a needle in the same individual and the outer ear canal was sealed off. Again, ME pressure was altered. Thirdly, to confirm if free airflow was present within the MEs, the intracranial air cavities and the interaural pathway of the right ME were ventilated by removing the seal of the right outer ear. During these three experiments ME pressure was measured via a pressure transducer connected to the pressure generator tube. A sudden drop or increase in ME pressure indicated opening of the pharyngotympanic tube, hence ME ventilating (Figs. 4 and 5). Pressure in the ME was raised and lowered in chickens and mallards by 1.5 and 2 kPa, respectively, in steps of 50 Pa every 2 seconds. ME pressure was also held constant in chickens for 300 s at 0.5, 1 and 1.5 kPa, which corresponds to a sudden increase in altitude, for instance from sea level up to 40, 83 and 125 m, respectively. In mallards, ME pressure was held constant for 150 s at 1, 2 and 4 kPa, which corresponds to an increase in altitude from sea level to 83, 168 and 338 m, respectively. ME pressure was also decreased and held constant in chickens for 300 s at −0.5, −1 and −1.5 kPa, which simulates, for instance, a sudden decline from altitudes of 40, 83 and 125 m to sea level. In mallards, ME pressure was decreased and held constant at −1, −2 and −4 kPa for 150 s which corresponds to a decline from altitudes of 83, 168 and 338 m to sea-level or submerging the head 0.1 m, 0.2 m and 0.4 m during diving, respectively.

Leaving the right tympanic intact and alternatively perforating this membrane allowed us to test whether the pressure sensor acts as a direct pressure sensor or whether it senses the stress or strain changes that emerge when the mechanical chain of the ME deforms as a result of pressure alterations. No conclusion can be drawn from leaving the right tympanic membrane intact alone. However, when a reaction could be observed when the right tympanic membrane was intact and when the right tympanic membrane was perforated, it could be assumed that the sensor is a pressure sensor because no strain is present on both left and right eardrum. If a reaction could be observed when the right tympanic membrane was perforated, but not when the right tympanic membrane was intact, the sensor might be seen as a tension sensor because the tension of the tympanic membrane changes when the tympanic membrane is intact.

We measured the pressure at which the pharyngotympanic tube is opened for the stepwise in/decrease of pressure. For the constant pressure sequences, the frequency of pharyngotympanic tube opening was considered and compared with the 0.5 and −0.5 kPa treatment which was taken as a baseline.

After the experiments the animals were euthanized using carbon dioxide. The experiments were approved by the Ethische Commissie Dierproeven (ECD) (code: 2013-65).

2.4. Statistical analysis

All tests were carried out with R 2.15.1 (R Core Team, 2012). A Shapiro-Wilk normality test was used to test if the data were distributed normally ($W > 0.9$). When normality was confirmed, an F-test was used to compare variances ($p < 0.05$: variances not equal). Normality and equal variance were met for all data. Afterwards, a repeated measure ANOVA was conducted to test the null hypothesis (no differences between the conditions) with $p < 0.05$. No statistical analysis could be conducted on the data of the mallards due to the small sample size (see Section 3.3.2).

3. Results

3.1. Comparison of the ME of chicken and mallard

Fig. 2 shows great similarity between the ME of chickens and mallards; however, some differences can be observed. Both infra- and suprastapedius of the extracolumella are more elongated in mallards. The tympanic membrane of chickens is more conical than in mallards as the angle between the columella and the extrastapedius is smaller than in chickens. When comparing both columellas, it can be observed that the connection of the columella to the columella footplate and the bony shaft of the columella are wider in chickens. Overall, it can be stated that the ME of chickens is more robust than the ME of mallards.

3.2. Effects of pressure on middle ear components of chicken

When ME pressure was raised by 1 kPa, total lateral linear columella displacement was 0.197 ± 0.0 mm. Lateral linear displacements of the infrastapedius, suprastapedius and extrastapedius were 0.40 ± 0.01 mm, 0.42 ± 0.08 mm and 0.50 ± 0.07 mm, respectively. A lateral change in position of the conical tip of the tympanic membrane of 0.595 ± 0.061 mm was measured. A caudal columella rotation of 0.8 ± 0.8° was observed. The infrastapedius was deformed by 7.3 ± 2.9° and suprastapedius deformation was 7.0 ± 2.7°.

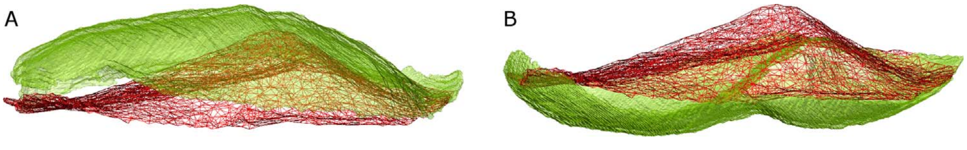

Fig. 3. Color code: green = increased and decreased ME pressure; red = ambient ME pressure. Edge view of the right tympanic membrane of a chicken. (A) Comparison of ambient ME pressure and increase of ME pressure by 1 kPa. (B) Comparison of ambient ME pressure and decrease of ME pressure by 1 kPa.

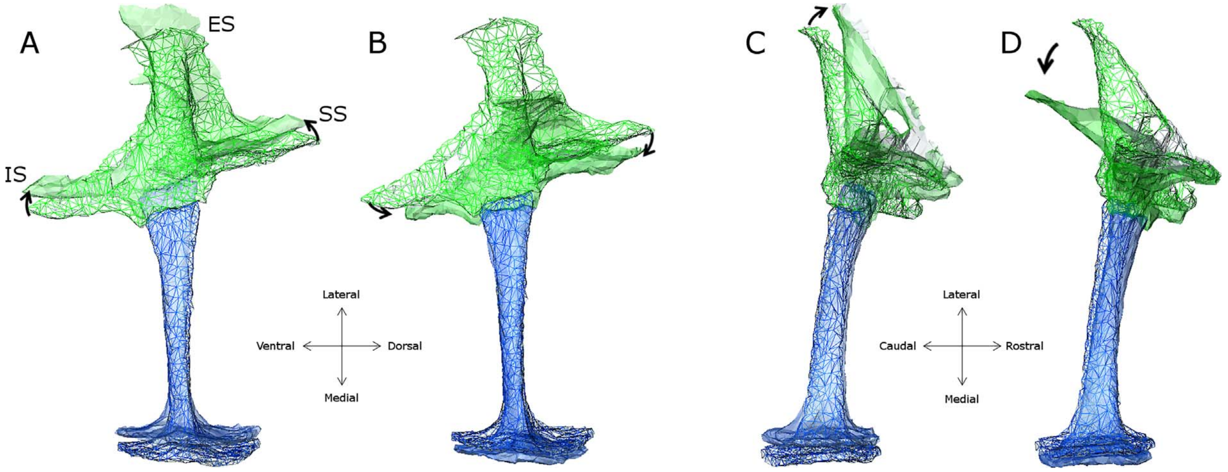

Fig. 4. View of the right columella–extracolumella complex (CEC) in a chicken ME. Color code: blue = columella; green = extracolumella. Mesh structures show CEC with a ventilated ME, solid structures visualize the deformations of the CEC when ME pressure is raised or lowered. (A) Frontal view of CEC, comparison ambient ME pressure and increase of 1 kPa of ME pressure. (B) Frontal view of CEC, comparison of ambient ME pressure and decrease of ME pressure by 1 kPa. (C) Side view of CEC along SS, comparison of ambient ME pressure and increase of ME pressure by 1 kPa. (D) Side view of CEC along SS, comparison of ambient ME pressure and decrease of ME pressure by 1 kPa. Abbreviations: IS, infrastapedius; ES, extrastapedius; SS, suprastapedius. Black arrows indicate the direction of rotation of extracolumella deformations.

Extrastapedius deformation was smaller, with a magnitude of 3.7 ± 0.3° (Figs. 3A and 4 A).

When ME pressure was lowered by 1 kPa, total linear columella medial displacement was 0.137 ± 0.014 mm. Medial linear displacements of the infra-, supra- and extrastrapedius were 0.52 ± 0.04 mm, 0.39 ± 0.10 mm and 1.05 ± 0.03 mm, respectively. A substantial medial displacement of the conical tip of the tympanic membrane of 1.055 ± 0.011 mm was observed. No angular displacement was measured for this condition. Extracolumella deformation was measured as an infrastapedius rotation of 5.8 ± 0.4°, a suprastapedius rotation with a magnitude of 8.6 ± 2.9° and a large extrastapedius deformation of 27.1 ± 1.2° (Figs. 3B and 4 B).

3.3. *Pharyngotympanic tube response to fluctuating middle ear pressure*

Fig. 5A shows the opening of the pharyngotympanic tube at random pressure intervals in chickens when ME pressure was slowly increased and decreased by 1.5 kPa with the right tympanic membrane intact. When the right tympanic membrane was perforated, ventilation of the ME was only observed for individuals C1 and C2, and only once and at random pressure. When ME pressure was decreased when the right tympanic membrane was perforated, no opening of the pharyngotympanic tube could be observed (Fig. 5B) (Table 1).

When ME pressure was increased by 2 kPa for mallards during both experiments (increased and decreased pressure), only one instance of ventilation of the ME could be observed. When ME pressure was lowered by 2 kPa, opening of the pharyngotympanic tube was observed at random pressure. Individual M1 did not ventilate its ME during the experiments (Table 1).

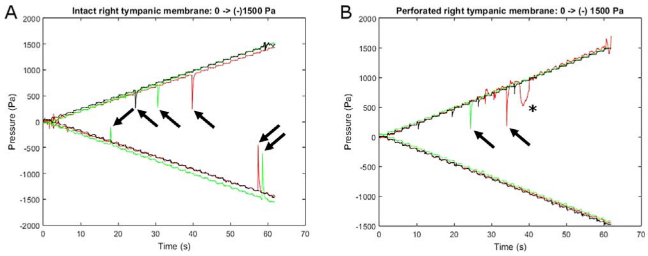

Fig. 5. ME pressure in chickens for stepwise increase/decrease with (A) intact right tympanic membrane and (B) perforated right tympanic membrane. Black arrow indicates opening of pharyngotympanic tube. * head movement artefact. Different colors indicate the different individuals.

Table 1

Middle ear (ME) pressure for both chicken (C) and mallard (M) at which the pharyngotympanic tube is opened for the stepwise increase/decrease of ME pressure for both intact and perforated tympanic membrane (TM). /, no opening of pharyngotympanic tube observed.

	Right TM intact		Right TM perforated	
	0 → 1.5 kPa	0 → –1.5 kPa	0 → 1.5 kPa	0 → –1.5 kPa
C1	610	–430; – 1497	820	/
C2	740	–1335	610	/
C3	895	/	/	/
	0 → 2 kPa	0 → –2 kPa	0 → 2 kPa	0 → –2 kPa
M1	/	/	/	/
M2	/	–1631	/	–895
M3	/	/	1397	–567

Fig. 6 shows an example of a readout of ME pressure from which the time intervals between ventilation of the ME could be extracted (note that the time intervals differ from those in Fig. 5). Results are summarized in Figs. 7 and 8 for chickens and in Figs. 9 and 10 for mallards.

3.3.1. *Chicken*

Fig. 7A shows there was no significant difference in ME venting

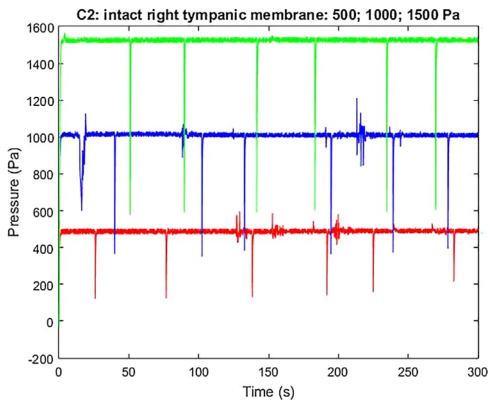

Fig. 6. Example readout of ME pressure in C2 with an intact right tympanic membrane at a constant applied pressure of 0.5, 1 and 1.5 kPa.

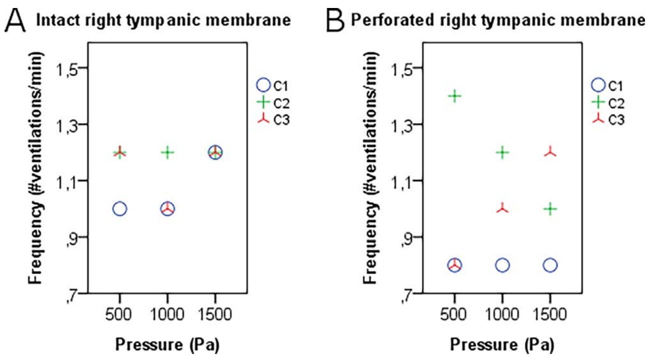

Fig. 7. Plot of ME ventilation frequency (ventilations/minute) in chickens C1–3 at a constant ME pressure of 500, 1000 and 1500 Pa for (A) intact right tympanic membrane and (B) perforated right tympanic membrane.

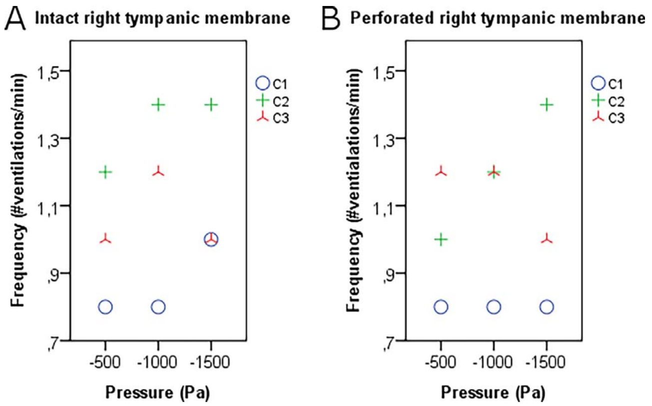

Fig. 8. Plot of ME ventilation frequency (ventilations/minute) in chickens C1–3 at a constant ME pressure of −500, −1000 and −1500 Pa for (A) intact right tympanic membrane and (B) perforated right tympanic membrane.

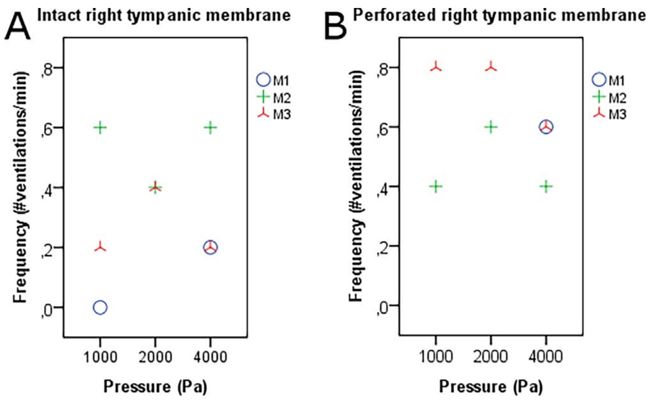

Fig. 9. Plot of ME ventilation frequency (ventilations/minute) in mallards M1–3 at an increase in ME pressure of 1000, 2000 and 4000 Pa for (A) intact right tympanic membrane and (B) perforated right tympanic membrane.

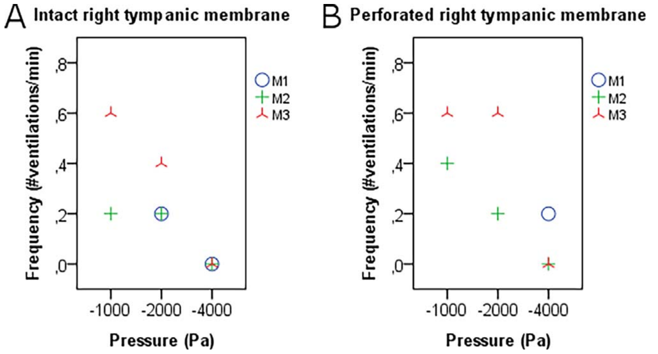

Fig. 10. Plot of ME ventilation frequency (ventilations/minute) in mallards M1–3 at an increase in ME pressure of −1000 and −2000 Pa for (A) intact right tympanic membrane and (B) perforated right tympanic membrane. (No ventilation of the ME was observed when ME pressure was lowered 4000 Pa below ambient pressure.).

frequency when ME pressure was raised to 1000 (1.1 ± 0.1; $p = 0.679$) and 1500 Pa (1.2 ± 0.0; $p = 0.679$) compared with the baseline measurement at 500 Pa (1.1 ± 0.1) when the right tympanic membrane was kept intact. No significant difference was observed in ventilation frequency between 1000 Pa and 1500 Pa ($p = 0.269$). Fig. 7B shows similar results when the right tympanic membrane was perforated, with ventilation frequencies for 500, 1000 and 1500 Pa being 1.0 ± 0.3, 1.0 ± 0.2 ($p = 1$) and 1.0 ± 0.2 ($p = 1$), respectively. No significant difference was observed in ventilation frequency between 1000 Pa and 1500 Pa ($p = 1$). No differences could be found in the ventilation of the MEs between an intact and perforated right tympanic membrane ($p = 0.171$).

When ME pressure was lowered by 500, 1000 and 1500 Pa, no differences could be observed in ventilation frequency when the right tympanic membrane was kept intact (1.0 ± 0.2, 1.1 ± 0.3 ($p = 0.797$) and 1.1 ± 0.2 ($p = 0.797$), respectively). When comparing −1000 and −1500 Pa, no significant difference was found ($p = 1$) (Fig. 8A). When t ne was perforated, again no differences could be found between the three conditions. Results were 1.0 ± 0.2 (–500 Pa,; 1.1 ± 0.2 (–1000 Pa; $p = 0.943$), and 1.1 ± 0.3 (–1500 Pa; $p = 0.943$). No significant difference was observed in ventilation frequency between −1000 and −1500 Pa ($p = 1$) (Fig. 8B). No significant differences could be found in ventilation frequency between an intact and a perforated right tympanic membrane ($p = 0.712$) (Fig. 8A and B).

3.3.2. *Mallard*

Fig. 9A shows an increase in mean ME venting frequency when comparing a ME pressure increase of 2000 Pa (0.4 ± 0.0) with an increase of 1000 Pa (0.3 ± 0.3) with an intact right tympanic membrane. ME ventilation frequencies did not change when ME pressure was increased to 4000 Pa (0.3 ± 0.2). When the right tympanic membrane was perforated, the mean of the ventilation intervals was similar for the three pressure conditions (Fig. 9B).

When ME pressure was lowered and held constant at −1000 (0.6 ± 0.3), −2000 Pa (0.3 ± 0.1) and −4000 Pa (0.0 ± 0.0), a decrease in ventilation frequencies of the MEs with an intact right tympanic membrane could be observed (Fig. 10A). With a perforated right tympanic membrane, a decrease in ME ventilation frequency could be observed when comparing decreases of ME pressure by −1000 Pa (0.5 ± 0.1) and −2000 Pa (0.4 ± 0.3) (Fig. 10B). At an ME pressure of −4000 Pa only one of the individuals ventilated the MEs in both experiments.

4. Discussion

In the present study we quantified the deformation of the ME mechanical chain as a result of pressure changes that are inherent to a bird's life style. Secondly, it was inquired whether the neutral configuration of the mechanical chain is reflexively restored by ME ventilation to ensure proper sound transmission and/or avoid damage.

Changes in ambient pressure resulting from an animal's daily activities may cause a pressure differential across the tympanic membrane which results in a deformation of the tympanic membrane. These deformations may cause altering in the configuration and mechanical behavior of the different ME components which may affect the acoustic transfer function (Murakami et al., 1997; Teoh et al., 1997; Lee and Rosowski, 2001; Dirckx et al., 2006). Extreme pressure changes can cause barotrauma in the ME and inner ear which can result in a permanent hearing loss (Melamed et al., 1992). For this reason, restoring ME pressure to ambient levels seems essential.

In the human middle ear, it has been shown that both over- and underpressure can develop over time and that normal Eustachian tube action does not reset the pressure to ambient (Padurariu et al., 2016). It is known that the mammalian ME is subjected to slow and sudden ambient pressure changes (e.g. when changing altitude, diving in water, etc.). An increase in ME pressure will cause an outward movement of both umbo and stapes while an inward movement is observed when ME pressure is decreased. In rabbits a stapedial footplate peak-to-peak amplitude of 34 μm was found when ME pressure was raised and lowered by 2.5 kPa above and below ambient pressure (Dirckx et al., 2006). Stapedial footplate displacement is 15 to 25 times smaller than umbo (tympanic membrane) movement when ME is lowered and raised by 2 kPa (Murakami, 1997; Hüttenbrink, 1998). Rahm et al. (1956) showed an 18 dB hearing drop at lower tones in cats with an increase and decrease of 1 kPa ME pressure and a 2–7 dB drop at higher tones. In humans an increase or decrease of 1 kPa reduced hearing at lower tones up to 1.8 kHz (Loch, 1942). In humans the Eustachian tube, together with the mastoid, is capable of regulating ME pressure (Gaihede et al., 2010, 2013) and hence plays an important role in restoring these temporary hearing losses. Gas exchange, tympanic membrane movement and occasional injection of small amounts of nasopharyngeal gas through the Eustachian tube continuously regulate pressure fluctuations. When large pressure differences develop, they are usually caused by sudden changes in ambient pressure. The Eustachian tube acts as a safety valve but under normal conditions these tubes are closed. During reflexive behavior muscles will open the Eustachian tube and enable for a small passive gas transfer between the ME and the pharynx (Siedentop et al., 1968; Rosowski and Merchant, 1999). Therefore a baroreceptor function is premised at the level of the ME.

In birds, ambient pressure changes can be even more extreme than in mammals and the mastoid is not present in birds (Rowe, 1988), so the pharyngotympanic tube in birds may have a more important role in ME pressure regulation than in mammals. Many species frequently climb and descend several hundreds of meters in a relatively short time span. Pressure changes non-linearly with height and depends on the meteorological conditions, but typically such differences in altitude represent pressure fluctuations of a few kPa. The pressure differential of 1 kPa used in the morphometric part of this study is equivalent to an altitude change of about 83 m above sea level. For most birds, this is still a modest altitude, yet the observed deformations of the ME mechanical chain are already considerable. As this height difference can easily be covered in matters of seconds (especially during descent), reflexive ventilation of the ME to restore a zero pressure differential across the TM seems necessary. Peregrine falcons can reach speeds as high as 100 m/s during stoop-diving (Ponitz et al., 2014) and may encounter changes of pressure rates above 1 kPa/s (own estimate). For plunge-diving birds (e.g. gannets) the rates of change may even amount to tens of kPa/s. It may be debated whether the ability of hearing is crucial during stoop- and plunge-diving, but other species of birds do rely on their hearing when they are climbing or descending. Some species of larks (e.g., the Eurasian skylark (*Alauda arvensis*) and the Oriental skylark (*Alauda gulgula*)) are climbing up to 100 m whilst singing (Geoffrey, 1985; Dave, 2005). Moreover, restoring ME pressure to ambient levels may still be important for species who do not depend on their hearing during stoop- and plunge-diving as barotrauma to the ME and inner ear may occur (e.g. rupturing of the tympanic membrane, oval and round window) (Melamed et al., 1992).

Despite the morphological differences between the mammalian and avian middle ear (shape of the tympanic membrane, articulated chain of three ossicles versus single columella, etc.), deformations of the tympanic membrane occur in both species when middle ear pressure is lowered and raised (Pohlman, 1921; Dirckx and Decreamer, 1991). In mammals, pressure alterations will cause deformations of the ossicle chain (Dirckx and Decreamer, 1991) and hence change the material mechanical stress in the connection to the inner ear, influencing sound transmission. In birds, the deformations caused by pressure alterations will occur at the three arms of the extracolumella (Pohlman, 1921), also changing the mechanical stress of the middle ear components, hence influencing sound transmission.

A pressure differential of 1 kPa across the tympanic membrane is equivalent to an altitude change of about 83 m above sea-level. This rather modest change in altitude (for birds) already causes considerable deformations of the ME mechanical chain, hence changing the acoustic transfer function (see above in Section 1). We observed in chickens that, when ME pressure is raised, the surface of the tympanic membrane bulges outwards and the conical tip of the membrane also displaces outwards. The columella footplate shows a piston-like displacement away from the inner ear as only a linear translation and a very small rotation of the columella could be observed. This piston-like displacement is limited in magnitude by both the annular ligament which connects the columella footplate with the oval window, and also by Platner's ligament which becomes tense. Extrastapedius deformations are three times larger than columella footplate displacement, as was also described by Pohlman (1921). The extracolumella consists of hyaline cartilage so a bending movement is not only possible between the extracolumella and columella but also at the processi of the extracolumella (Saunders, 1985; Mills, 1994; Starck, 1995; Arechvo et al., 2011). Unlike Pohlman (1921), we did find an outward displacement of the infrastapedius when ME pressure was increased. When ME pressure is lowered, the tympanic membrane displaces inwards. In this condition, the columella footplate also undergoes a piston-like movement, as only a linear translation and no rotation was observed. Likely, the annular ligament provides a protective mechanism by limiting the displacement of the footplates so that no damage is caused by the columella penetrating too deep into the inner ear. Extrastapedius deformation was 8 times larger than columella footplate displacement. The smallest extracolumella deformation was found at the suprastapedius as displacement is limited by the close relation of this arm to the tympanic cavity wall. The largest deformation was found at the extrastapedius, which rotates inwards. These deformations and translations confirm the findings by Pohlman (1921). The deformations of the extracolumella and the difference in magnitude between the deformation of the tympanic membrane and the linear translation of the columella footplate indicate that the extracolumella is an effective buffer for static pressure changes. However, we argue that ME pressure differentials in nature may well exceed the applied experimental pressure differences and the possibility remains that the annular ligament and/or tympanic membrane may rupture. Similar deformations, as described for the chicken, can be expected in the mallard ME when subjected to the same altering ME pressures, as dimensions of the ME mechanical chain are similar in both chicken and mallard.

We showed that the displacement of the avian columella footplate as a result of pressure changes is larger than in mammals, which may be indicative for an even larger reduction of hearing than what is reported for mammals. Therefore, ME pressure regulation via the pharyngotympanic tube seems all the more important in birds. In birds it has been shown that under normal conditions of closed pharyngotympanic tube and constant ambient pressure, ME pressure slowly decreases to 20 Pa below ambient pressure (Larsen et al., 2016). When this pressure is reached, opening of the pharyngotympanic tube occurs every 20–180 s, depending on the species (Larsen et al., 1997, 2016). However, much larger pressure differentials than a few tens of Pa/s are likely to occur and the pressure differential over the tympanic membrane can change in a very short time span (see above in Section 1). We expected to observe a reflexive opening of the pharyngotympanic tube to restore ME pressure to ambient pressure levels, thus restoring optimal sound transmission and protecting the ME and inner ear from barotrauma, when a certain pressure gradient across the tympanic membrane was reached. However, during the experiments, when ME pressure was slowly lowered and raised above and below ambient pressure, no reflexive opening of the pharyngotympanic tube could be observed for both chicken and mallard. It could be expected that ME ventilation frequency would increase when ME pressure was increased or decreased even more. The ME ventilation rate in chickens is 1.1 times per minute with an intact right tympanic membrane and 1 time per minute with a perforated right tympanic membrane. In mallards, ventilation frequency is 0.3 times per minute with an intact right tympanic membrane and 0.4 times per minute with a perforated right tympanic membrane. These frequencies are within the range of 0.3 and 3 times per minute reported in other species (Larsen et al., 2016). When ME pressure was increased or decreased further, no change in ME ventilation frequencies could be observed. Also, no differences were observed between the two experiments. As a result, no statement can be made regarding the question whether the pressure sense organ (if present) in the avian ME acts as a tension or pressure sensor. Our pressure experiments do not support the presence of a short-loop reflexive control of pressure equilibration via the pharyngotympanic tube. It is, however, still possible that triggering reflexive ME venting requires higher middle ear pressure than used during our experiments. On the other hand, the pain threshold in humans lies around 2.8 kPa (Blackstock and Gettes, 1986) so it can be expected that the pressures used in this study (2 kPa) are sufficient to evoke pharyngotympanic tube response. It is also possible that ME venting requires additional sensorial input (e.g. visual, vestibular) or occurs voluntarily, i.e. being controlled at higher brain levels. This, however, could not be derived from our experiments. The ecology of the two species (chicken and mallard) used during the pressure experiments may explain the lack in venting of the middle ear. Other species which are subjected to more extreme ambient pressure fluctuations (e.g. gannets) may show venting behavior when subjected to middle ear pressure changes.

In Section 1 we put forward the paratympanic organ (PTO) as candidate for a sensor that triggers reflexive opening of the pharyngotympanic tube. The PTO is a mechanoreceptive sense organ situated in the medial wall of the tympanic cavity, close to the opening of the pharyngotympanic tube and dorsolateral to the columella in the avian ME (Von Bartheld, 1994). The PTO lumen is filled with a mucous fluid and its medial side contains a sensory epithelium covered with type II hair cells (Jørgensen, 1984; Giannessi and Pera, 1986; Giannessi et al., 1996; Von Bartheld and Giannessi, 2011). The PTO is connected to both the columella and the tympanic membrane, via Platner's ligament and the superior drum tubal ligament, respectively (Von Bartheld, 1994). It is assumed that ambient pressure fluctuations will significantly deform the tympanic membrane, resulting in movement of the fluid within the PTO either due to the direct ligamentous connections or the displacement/deformation of the columella. This fluid motion is probably the stimulus of the type II hair cells that may allow birds to sense tympanic membrane position or tension and hence register absolute or relative differences in air pressure (Jørgensen, 1984; Von Bartheld, 1994). Kreithen and Keeton (1974) have shown that pigeons are sensitive to small changes in atmospheric pressure of 100–200 Pa, equivalent to an altitude difference of about 10–20 m. As mentioned above, the results of the pressure experiments in our study suggest that the PTO is not involved in reflexively opening the pharyngotympanic tube, hence venting the ME, unless integration with information of other sensors is required. Our results, however, do not exclude that the PTO can function as a baro- or altimeter.

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