Middle ear bones of a mid-gestation ruminant foetus extracted from X-ray computed tomography

Loic Costeur[a], Bastien Mennecart[a], Bert Müller[b], and Georg Schulz[b]

[a]Department of Geosciences, Naturhistorisches Museum Basel, 4051 Basel, Switzerland;
[b]Biomaterials Science Center, Department of Biomedical Enginnering, University of Basel, 4031 Basel, Switzerland

ABSTRACT

The timing of ossification of middle ear ossicles has been extensively studied in humans. This is an exception since it is vastly unknown in the +5000 extant species of placentals. As a preliminary approach, a cow foetus (around 115 days of gestation) was visualized using X-ray microtomography (μCT) and the ossicles including stapes, incus, and malleus could be extracted from the data set. All three bones have already undergone substantial ossification, which allow comparison to adult middle ear bones. Their ossification at this stage parallels ossification in humans at a comparable stage of gestation. While full ossification is not yet achieved almost all the morphological characters of the ossicles are observed. Bone tissue is still very porous, the stapes does not have the characteristic plate-like footplate, the lenticular process of the incus is missing and the manubrium of the malleus is very thin and not yet complete. Despite all this, the ossicles are articulate with each other and perfectly with the bony labyrinth. The stapes footplate is positioned on the oval window but is smaller than the latter while it should perfectly fit to transmit sound vibrations to the cochlea. All ossicles, especially the stapes, have not yet reached adult size, while the bony labyrinth already has. This is the first detailed description of a set of middle ear bones in a placental at mid-gestation based on high-resolution μCT. Similarities in ossification timing with humans encourage more work to be done on foetuses to understand if a general rule for placental mammals exists.

Keywords: Microtomography, laboratory X-ray source, Ruminantia, development, middle ear, fetal stages, stapes, incus, malleus

1. INTRODUCTION

The middle ear bones (stapes, incus, and malleus) are the smallest bones of the mammalian skeleton. They play a crucial role in the hearing chain transmitting the sound waves from the external ear to the inner ear through a contact at its surface on the membrane of the oval window (fenestra vestibuli). While fossil ossicles have extensively been studied because they represent a classical example of evolution in early mammals (e.g., [1–3]), their extant counterparts have been the subject of fewer interest, especially in the 20th century (see reference [4] for a review). Only the middle ear bones of some extant taxa have been published and most of our knowledge relies on excellent but old publications [5,6]. More recent works (see references [4,7,8] for a review), have focussed on several extant placental mammals showing the diversity of the shape of the stapes or malleus in particular. Following this, the phylogenetic potential of the middle ear bones has been put forward and its use at different taxonomical levels has proven to yield sound results (see [7] for a review).

Ruminant middle ear bones make up a significant part of the previously investigated ossicles and about 15% of the ca. 210 extant species of ruminants have been either illustrated or studied so far. Most of the work has relied either on prepared bones sometimes only briefly illustrated and described [5, 6, 9–11], on thin sections or CT-scanned specimens [7]. The latter have very rarely been reconstructed in three dimensions, being mostly studied on slices, so that few is known about their general morphology.

A foetal set of the cows (*Bos taurus*) middle ear bones were illustrated although its age attribution seems problematic [6]. Despite the interest represented by developmental data for evolutionary or phylogenetic studies,

Further author information: (Send correspondence to L.C. and G.S.) E-mail: loic.costeur@bs.ch, georg.schulz@unibas.ch, Telephone: +41 61 265 9125, Fax: +41 61 265 9699; www.bmc.unibas.ch

Developments in X-Ray Tomography X, edited by Stuart R. Stock, Bert Müller, Ge Wang, Proc. of SPIE Vol. 9967, 99671Q · © 2016 SPIE · CCC code: 0277-786X/16/$18 · doi: 10.1117/12.2238119

the foetal morphology of the ear region has rarely been investigated except in humans (e.g., [12–16]). While the origin of the middle ear bones is now well known through extensive embryologic studies (see reference [4]), less is known, again except in humans (e.g., [12]), about the timing of their ossification, about their growth in the foetus and about potential prenatal morphological modifications through the gestation period.

The human ossicles are observed from the second month of gestation but are still cartilaginous [12,13]. They do not ossify before the 16^{th} week of gestation [13, 15], so within the fourth month of gestation, slightly before mid-gestation. This timing parallels the timing of ossification of the inner ear [16]. A recent study investigated the prenatal ossification of the inner ear in a ruminant (the cow, *Bos Taurus*, [17]) and came to similar conclusions as in humans. This work focussed on a series of foetuses starting early in the fourth month of gestation at around 95 days of gestation up to about three weeks before birth. It shows that both the petrosal bone and thus bony labyrinth are not ossified before the onset of the fourth month of gestation and that ossification takes place rapidly within the fourth month to attain adult proportions especially in the bony labyrinth a few weeks before birth. The growth of the petrosal bone is constant throughout the gestation and starts in the fourth month with a very porous bone that is merely a capsule surrounding the bony labyrinth [17]. The question arose as to how ossified would be the middle ear ossicles at such a developmental stage or even if they would actually already be ossified at all. We here expand this work to investigate these questions. We were able to identify the three middle ear ossicles in a mid-gestation foetal stage of the cow (about 115 days old) for which we could reconstruct them from computed tomography-generated data. The non-invasive nature of this technology enables us to reconstruct the middle bones in anatomical position and in contact with the bony labyrinth. We here describe them in comparison with published data of the adult morphology and show their developmental state.

2. MATERIALS AND METHODS

2.1 Specimen preparation and methods

For the study a 115 days old cow foetus (specimen NMB 3038, species: *Bos taurus*) was chosen. The age estimation was performed according to skull length and based on [18]. The specimen is an historical dried skeletal specimen and thus few cartilaginous structures have been preserved. Nomenclature follows references [8] and [19]. The middle ear bones are the smallest bone of the skeleton, and are especially delicate, fragile and porous in the case of fetal specimens. Classical taxidermical preparation to isolate middle ear bones is possible but is an invasive method that takes all the skull bones apart. It does not allow articulated bones to be studied in their anatomical position. We have thus decided to use X-ray microtomography which is a non-destructive method, particularly appreciated in the case of historical and rare museum's specimens. It gives the possibility to illustrate the middle ear bones in articulation to one another and in particular in articulation with the petrosal bone and its embedded bony labyrinth. It then allows studying the relationships of the different structures with one another. The oval window of the bony labyrinth is the articulation surface on which the stapes contacts the inner ear. Investigating this relationship in anatomical position in an early foetal stage has the potential to bring insights into the developmental timing of both structures.

2.2 X-ray microtomography

The X-ray μCT scans were performed using a nanotom® m (phoenix|x-ray, GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany). The nanofocus X-ray source has a maximal acceleration voltage of 180 kV (15 W). For the scan the acceleration voltage of 120 kV and a beam current of 200 μA were chosen. The mean photon energy of the X-ray spectrum was increased by adding a 0.25 mm Cu filter between source and specimen. Over 360° 1440 equiangular radiographs were taken with a resulted pixel length of 25 μm. The whole scanning time of one specimen took about 2 hours.

2.3 Data treatment

The projections were reconstructed by the manufacturer's software phoenix datos|x 2.0.1 - RTM (GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany) using a cone beam filtered back-projection algorithm. Before further data treatment, the data sets were converted into DICOM format using VGStudio MAX 2.1 (Volume Graphics GmbH, Heidelberg, Germany). For the 3D representation of the middle ear ossicles (stapes, incus, and malleus) the structures were segmented using AVIZO 7.0.

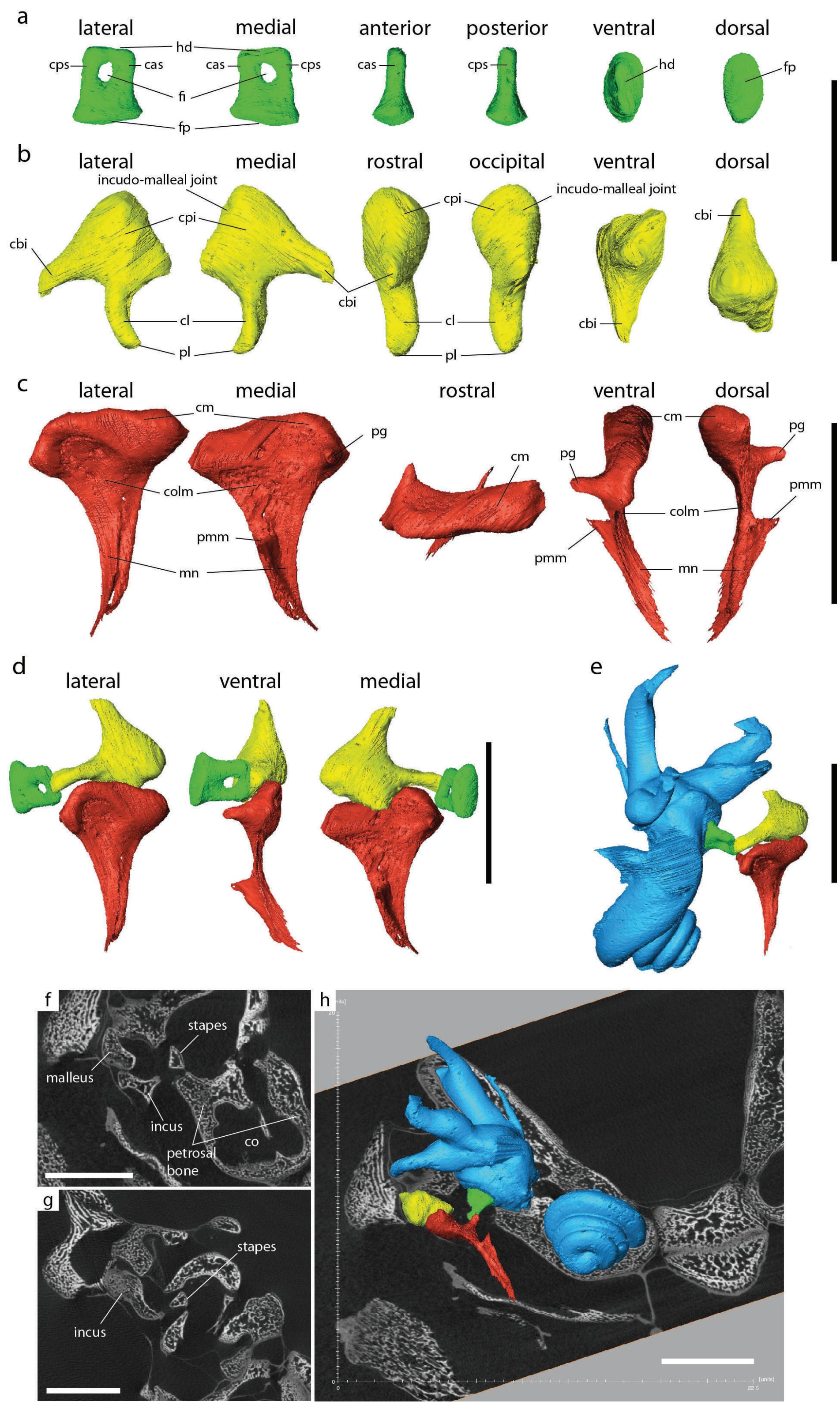

Figure 1. 3D models of the stapes (a, green), incus (b, yellow), and malleus (c, red) in different views. d, three views of the three middle ear bones in connection; e, the three reconstructed middle ear bones in connection with the reconstructed

bony labyrinth (note the absence of ossification of the semi-circular canals and the oval window larger than the stapesfootplate); f-g, two slices through the middle ear bones (note their very porous nature); h, a view of the reconstructed middle ear bones in connection with the bony labyrinth and embedded within a slice through the skull. Abbreviations: stapes: **hd**, head (or capitulum stapedis); **cas**, crus anterius stapedis; **cps**, crus posterius stapedis ; **fi**, foramen intercrurale; **fp**, footplate (or basis stapedis). Incus: **cbi**, crus breve incudes; **cl**, crus longum; **cpi**, corpus incudes; **pl**, processus lenticularis. Malleus: **cm**, caput mallei (or head); **colm**, collum mallei (or neck); **mn**, manubrium (or handle); **pmm**, processus muscularis mallei; **pg**, processus gracilis. All scale bars correspond to 5 mm.

3. RESULTS

The segmented middle ear bones are shown from different views in Fig. 1.

3.1 Stapes

The stapes is quadrangular in shape with an almost as broad head as the footplate (in lateral and medial views, Fig. 1a). It is slightly asymmetrical with a slightly longer crus posterius stapedis (cps) than the crus anterius stapedis (cas). It is 2.16 mm in height. The cas seems slightly thicker than the cps. Both crura do not seem to be internally excavated, or gully-shaped such as is the case in adult stapes. The head is flat and elongated antero-posteriorly. The foramen intercrurale is small, rather ovoid in shape and ventrally positioned. No processus muscularis stapedis for the stapedial muscle is visible below the head and on top of the cps. The footplate is not complete, it is bulged and rounded and do not end in the characteristic plate-like structure of the adult stapes (e.g., [6]). It measures 1.94 mm in length and 1.11 mm in width. A detailed inspection of Fig. 1e, f, and g shows that the stapes does not fill in the full space of the oval window where it comes in contact with the inner ear. A measure of the largest width of the oval window directly on the bony labyrinth yields 2.55 mm in length. Calculating the body mass of the animal from the length of the foetal stapes' footplate gives an estimate of about 67 kg, or 72 kg with the width of the footplate, using the equations of [8] based on adult stapes (Fig. 2). Estimating the body mass of the cow using the length of the oval window measured on the bony labyrinth yields a result of about 400 kg.

3.2 Incus

The incus is a bulky bone with a short process (crus breve incudis, cbi on Fig. 1b) and a long process (crus longum, cl on Fig. 1b). The latter has a reduced or incomplete lenticular process (processus lenticularis, pl in Fig. 1b) in comparison to an adult stapes of the same species [6] where it should be more quadratic and prominent to articulate with the large head of the stapes. The body (corpus incudis, cpi in Fig. 1b) is triangular in shape and quite massive. The head of the incus, or articular surface for the malleus at the incudo malleal joint, is flat to slightly depressed to accommodate the head of the malleus (Fig. 1b).

3.3 Malleus

The malleus is the largest of all three middle ear bones. It is broad in lateral and medial views (Fig. 1c). Its head (caput mallei, cm on Fig. 1b) is rounded but not ball-shaped and slightly ventro-dorsally elongated. It represents a large and slightly wavy articular surface for the incus. The handle of the malleus (manubrium, mn in Fig. 1c) is long, very thin, and broad rostrally. The processus gracilis is small and faces medially. An incomplete processus muscularis mallei is present but is very thin (pmm in Fig. 1c). Fig. 1d shows how all three bones articulate, it is thus particularly evident that the lenticular process of the incus is not yet complete building an incomplete articulation with the stapes. The incudo-malleal joint seems more complete but both the incus and malleus are not as imbricated as in adult forms (see [5]). Fig. 1e shows how the middle ear articulates with the bony labyrinth and shows the incomplete size of the stapes leaving the oval window not fully obliterated by the footplate of the latter.

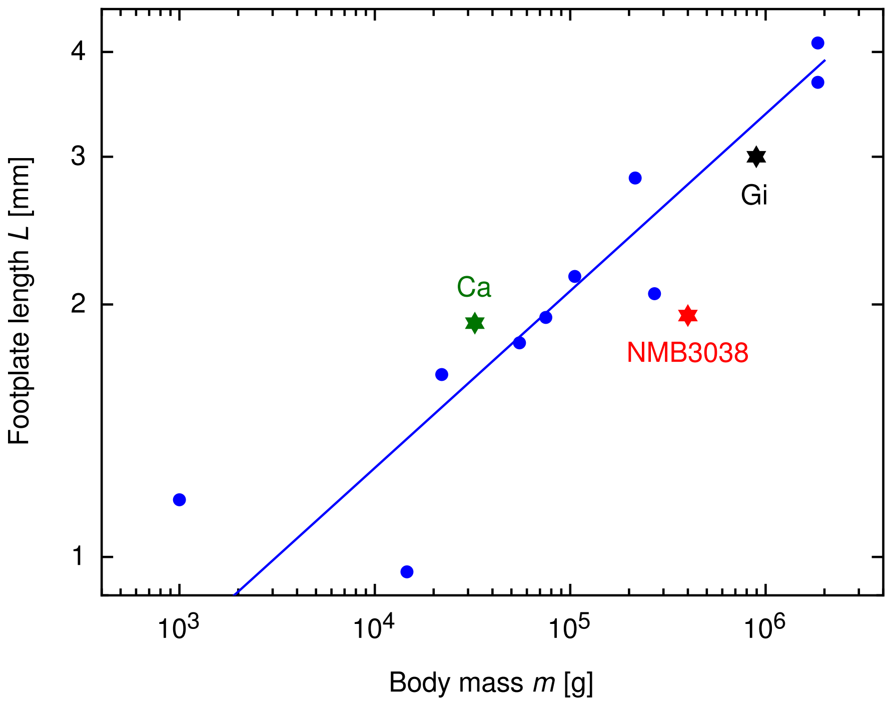

Figure 2. Log-Log bivariate plot of footplate length versus body mass in artiodactyls (modified from [8]). The red star indicates the studied cow foetus NMB3038. For comparisons with other ruminants: *Ca*: the roe deer Capreolus capreolus, *Gi*: the giraffe *Giraffa Camelopardalis*

4. DISCUSSION AND CONCLUSIONS

4.1 Comparison to adult morphology

The only two publications illustrating the middle ear bones of the cow are more than a hundred years old [5, 6]. They show the very characteristic quadrangular shape of the stapes, which is already present in the studied foetus NMB3038. A foetal set of middle ear bones illustrated a more triangular stapes [6]. The author indicates that the specimens are from a foetus in its "6^{th} week". Since embryological studies in humans have shown that the middle ear bones only start to form between the 6^{th} and 8^{th} weeks and start to ossify around week 16 [13], it is highly unlikely that Dorans specimen be that young. In addition, a recent study shows that ossification has not yet occurred in the ear region of the cow by the beginning of the 4^{th} month of gestation [17]. The published "foetal" incus is very similar to the one we describe here [6], with the exception of the lenticular process which does not seem to be that differentiated in our foetus NMB3038. The short process of the incus NMB3038 (cbi in Fig. 1b) faces ventrally while it is rostrally curved in most mammals where it is known and in particular in an adult cow [5, 6]. Its full length and final orientation are thus probably not yet attained. The malleus NMB3038 is again simpler built as in the adult cow [6] and the manubrium or processus muscularis mallei are both very fine and most probably not yet completely ossified. The malleus head (caput mallei, cm in Fig. 1c) seems less rostrally expanded than in an adult cow [6].

The described middle ear bones are not as fully formed as in adults but are identifiable at the species level, sharing many characteristics of the adult morphology.

4.2 Timing of ossification

The prenatal development of the middle ear bones in the cow is not known. Many studies have investigated the timing of formation and ossification of the three ossicles in humans [12, 13, 15] and observed that ossification first occurs in the incus around the 16^{th} week of gestation and is well underway in all three ossicles the 20^{th}

week [14]. The gestation period of the cow is about as long as in humans with 280 days in average. Our foetus NMB 3038 was estimated to be 115 days old so about 16.5 weeks old or a little more than four months. The three middle ear bones are already ossified but do not present the exact same morphology than those of an adult cow [6]. They are a little simpler with specific characters such as the lenticular process of the incus or the plate of the stapes footplate still lacking. Their internal structure is very porous too (Fig. 1f, g) such as that of the forming petrosal bone (Fig. 1f). The bony labyrinth in contact with the stapes is not yet completely ossified, the semi-circular canals being still mostly membranous (Fig. 1e). Full ossification of the labyrinth has recently been shown to occur around mi-gestation time probably within the $5th$ month of gestation in the cow ([17]. This study seems to indicate that the same is true for the middle ear bones mimicking the timing observed in humans. Very little is known as to the timing of ossification of the ossicles and bony labyrinth in placental mammals. This is too premature to hypothesize that full ossification of the ear bones and organs occurs as a general rule around mid-gestation.

4.3 Size of the stapes

Our measurements of the stapes footplate were used to estimate the body mass of the cow using the equations of reference [8]. The largely underestimated weight (67 kg to 72 kg) for an adult cow that would have a stapes of this size and the fact that the stapes does not cover the entire oval window measured on the bony labyrinth indicate that the foetal stapes has not yet reached an adult size. Using the length of the oval window itself yields an animal of about 400 kg which is more in accordance with an adult cow, for which body mass range is 450 kg to 1000 kg [20]. The absence of the typical plate-like footplate such as depicted earlier [6] for an adult cow confirms the smaller size of the foetal stapes in comparison to the adult size. The stapes will thus most probably continue to grow throughout the gestation period to fill in the space of the oval window. The same observation was made on humans where a 40 mm long human foetus had a stapes in which the footplate was smaller than the oval window of the inner ear, only occupying a portion of the oval window membrane [14]. It also indicates that hearing may not yet be fully effective in a 4 months old foetus, despite the adult size of the bony labyrinth itself [17]. The footplate is probably too small to effectively transmit sound vibrations to the membrane of the oval window.

While the membranous labyrinth of the cow seems to ossify only once full adult size has been reached [17], the middle ear ossicles seem to continue their growth throughout the gestation period. Remodelling of the middle ear bones in humans, especially the incus, has been observed in infancy but also throughout the life of an individual [13] showing constant modifications. These early studies [12, 13] report that the stapes final shape is acquired by the end of the gestation period, which makes sense since the oval window shape and size are already achieved largely before birth and do not change afterwards.

X-Ray imaging in phase contrast mode allowing the 3D imaging of soft and hard tissues together with classical CT-scanners is rapidly developing [21]. It will allow investigating soft structures in early stages of middle ear bones formation or the membranous labyrinth without necessitating a Synchrotron scanning facility. This possibility will make more developmental data at disposal to gain new insights into the evolution of these structures and related taxa. This is likely to increase our understanding of the development of the ear complex in a large range of taxa, beyond humans, for which virtually nothing is known so far.

ACKNOWLEDGMENTS

Financial supports of the Swiss National Science Foundation in the frame of the R'equip initiative (316030_133802) and the SNF project 200021_159854/1 are especially acknowledged. The Stiftung zur Förderung des NMB and the Kugler-Werdenberg Stiftung are thanked for financial support.

REFERENCES

[1] Rich, T. H., Hopson, J. A., Musser, A. M., Flannery, T. F., and Vickers-Rich, P., "Independent origins of middle ear bones in monotremes and therians," *Science* **307**, 910–914 (2005).

[2] Luo, Z. X., "Transformation and diversification in early mammal evolution," *Nature* **450**, 1011–1019 (2007).

[3] Meng, J., Yuanqing, W., and Chuankui, L., "Transitional mammalian middle ear from a new Cretaceous Jehol eutriconodontan," *Nature* **472**, 181–185 (2011).

[4] Maier, W. and Ruf, I., "Evolution of the mammalian middle ear: a historical review," *J. Anat.* **228**, 270–283 (2016).

[5] Hyrtl, J., [*Verleichende-anatomische Untersuchungen über das innere Gehörorgan des Menschen und der Säugethiere*], Friedrich Ehrlich, Prague (1845).

[6] Doran, A. H. G., "Morphology of the Mammalian Ossicula auditus," *J. Linn. Soc.* **1**, 371–497 (1878).

[7] Maier, W. and Ruf, I., "The anterior process of the malleus in Cetartiodactyla," *J. Anat.* **228**, 313–323 (2016).

[8] Orliac, M. J. and Billet, G., "Fallen in a dead ear: intralabyrinthine preservation of stapes in fossil artiodactyls," *Palaeovertebrata* **40**, –e3 (2016).

[9] Cuvier, G., [*Leçons d'anatomie comparée*], Baudouin (1800).

[10] Fleischer, G., "Studien am Skelett des Gehörorgans der Säugetiere, einschließlich des Menschen," *Säugetierkd. Mitt.* **21**, 131–239 (1973).

[11] Nummela, S., "Scaling of the mammalian middle ear," *Hear. Res.* **85**, 18–30 (1995).

[12] Anson, B. J. and Cauldwell, E. W., "The developmental anatomy of the human stapes," *Ann. Otol. Rhinol. Laringol.* **51**, 891–904 (1942).

[13] Anson, B. J. and Bast, T. H., "Development of the incus of the human ear," *Q. Bull. Northwest. Univ. Med. Sch.* **33**, 110–119 (1959).

[14] Richany, S. F., Anson, B. J., and Bast, T. H., "The development and adult structure of the malleus, incus, and stapes," *Ann. Otol. Rhinol. Laryngol.* **63**, 394–434 (1954).

[15] Declau, F., Moeneclae, Y. L., and Marquet, J., "Normal growth pattern of the middle ear cleft in the human fetus," *J. Laryngol. Otol.* **103**, 461–465 (1989).

[16] Jeffery, N. and Spoor, F., "Prenatal growth and development of the modern human labyrinth," *J. Anat.* **204**, 71–92 (2004).

[17] Costeur, L., Mennecart, B., Schulz, G., and Müller, B., "Prenatal growth stages show the development of the ruminant bony labyrinth and petrosal bone," *J. Anat.* , in press (2016).

[18] Sweet, W. W., Matthews, C. A., and Fohrman, M. H., "Development of the fetus in the dairy cow," *US. Dep. Agric. Tech. Bull.* **964**, 1–34 (1948).

[19] Schwarz, C., "Phylogenetische und funktionsmorphologische Untersuchungen der Ohrregion bei Sciuromorpha (Rodentia, Mammalia)," *J. R. Soc. Interface* **7**, 1665–1676 (2010).

[20] Nowak, R. M., [*Walker's Mammals of the World*], The Johns Hopkins University Press (1999).

[21] Khimchenko, A., Schulz, G., Deyhle, H., Hieber, S. E., Hasan, S., Bikis, C., Schulz, J., Costeur, L., and Müller, B., "Non-destructive phase contrast hard X-ray imaging to reveal the three-dimensional microstructure of soft and hard tissues," *Proc. SPIE* **9797**, 97970B (2016).