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Growth pattern of the middle ear in the gray short-tailed opossum, *Monodelphis domestica*

Festschrift in Honour of Professor Dr. Wolfgang Maier
Edited by Ingmar Werneburg & Irina Ruf

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Abstract

We studied the mass growth trajectories of middle ear ossicles and tympanic membrane and oval window area in 19 specimens of postnatal ages 30–180 days of the gray short-tailed opossum *Monodelphis domestica*. We weighed the skull mass and the mass of the three middle ear ossicles with appropriate balances. Using a binocular microscope provided with a grid, we measured the length of malleus and incus, as well as the longest axis and the one perpendicular to it on both the tympanic membrane and the stapes footplate. The size variation was studied with least squares regression analyses between various measurements. The incus and stapes change little in mass after 40 days of postnatal life, while the malleus does, reaching maximum mass at around 100 PND (postnatal days). This modularity in growth trajectory is in contrast with the shared evolutionary origin of malleus and incus from branchial arch 1. The maturation of the middle – and as indicated by previous work, that of the inner ear – is coupled with the improvement of hearing sensitivity at low and high frequencies after the initial onset of hearing at 29 PND.

Key Words

incus, malleus, Marsupialia, modularity, ontogeny, stapes

Introduction

The ontogeny of the definitive mammalian middle ear (DMME) has received much attention, as it serves to understand one of the classic morphological transformations of vertebrate evolution during synapsid evolution (McClain 1939; Maier 1990; Luo 2011; Luo et al. 2016). The change of the cranio-mandibular articulation, with a gradual reduction of postdentary elements, involved the acquisition of a new ossicular chain in the middle ear. How the structures that became the malleus (articular), incus (quadrate) and ectotympanic (angular) changed, and how they did so in relation to other middle ear and cranial structures related to mastication and hearing, is

a rich subject that has been approached from several perspectives, such as biomechanics, development, and phylogeny (Maier and Ruf 2016a). The disappearance of Meckel's cartilage may have occurred independently in numerous mammalian lineages from the Late Jurassic to the Late Cretaceous, whereas detachment of what became the middle ear bones from the postdentary trough may have occurred once each in the ancestors of therians and monotremes (Ramírez-Chaves et al. 2016). The study of the morphological transformation leading to the DMME in ontogeny and phylogeny can be approached from a conceptual perspective of modularity and evolvability (Navarro-Díaz et al. 2019; Le Maître et al. 2020).

Functional aspects considered, the mammalian middle ear functions as an impedance matching device between the surrounding medium and the inner ear cochlea, forming a chain between the tympanic membrane and the oval window. In placental mammals (Rosowski 1992; Hemilä et al. 1995; Nummela 1995), as well as marsupial mammals (Nummela and Sánchez-Villagra 2006), the adult middle ear is isometric within functionally important parameters. Based on this isometry, the high-frequency hearing limit of both these mammalian groups can be predicted from the middle ear parameters, mainly from the ossicular mass (Hemilä et al. 1995; Nummela and Sánchez-Villagra 2006). However, this model for predicting hearing limits is not applicable to our non-adult specimens.

The small South American didelphid *Monodelphis domestica* has been a prominent subject in evolutionary morphology studies of mammalian middle ear development and evolution since the pioneering works of Maier (1987). The gray short-tailed opossum, like other marsupials, experiences postnatally morphological transformations that take place intrauterine in placental mammals, and as such offers an advantageous study system (Clark and Smith 1993; Spiekman and Werneburg 2017). This marsupial offers then the chance to understand perinatal adaptations, so important in developmental evolution (Maier 1993). *Monodelphis* is established as a model species in studies of developmental evolution of diverse organ systems, including the limbs (Doroba and Sears 2010), the skull (Maier 1989; Sánchez-Villagra and Forasiepi 2017) and the middle ear (e.g., Macrini 2004; Urban et al. 2017; Anthwal et al. 2020). *Monodelphis* is the first marsupial whose genome has been sequenced and the most commonly used laboratory marsupial, one of small size, nonseasonal breeding (Keyte and Smith 2008).

In *Monodelphis*, the onset of hearing occurs at 29 PND, and the maturation of hearing at around 40 PND. Between these two ages, the hearing threshold decreases, improving the sensitivity at the frequencies of best hearing, and the overall frequency range of hearing widens both towards lower frequencies and higher frequencies (Reimer 1996). We discuss the reasons responsible for these changes.

The postnatal development of mammals is part of the whole ontogeny of an individual, and one that is usually understudied. What transformations occur after birth until achieving adult anatomy? In this paper we examine this question in the middle ear ossicles of *Monodelphis*

domestica. We studied the mass growth trajectories of ear ossicles, and other middle ear parameters in the gray short-tailed opossum *Monodelphis domestica* in the postnatal life. We aim at understanding if there are changes in these parameters at times of changes in hearing function. Furthermore, we can also test if the tempo and mode of change in those parameters is uniform or if instead there are differences and modules of change that may reflect phylogenetic history or functional demands.

Materials

Skulls of 19 gray short-tailed opossums (*Monodelphis domestica*) with information about their age were collected from Kathleen Smith's Duke University *Monodelphis* facilities (Table 1). The skulls were prepared according to standard maceration methods. The age of the material ranges between 30 and 908 postnatal days (PND). The middle ear ossicles were removed from the middle ear cavity with great care under a light microscope. In the two youngest specimens, both of PND 30, the ossicles were still quite fragile, and in one specimen they could not be saved, and hence could not be used in our study. In the other specimen, the ossicles could be saved but the malleus broke so that its lever arm length could not be measured. Two of our specimens were of age PND 180 (specimens 97001 and 98011 in Table 1), and a third one was of age PND 908 (specimen 98005 in Table 1). Given that *M. domestica* reaches adult age by PND 180 (Keyte and Smith 2008), the age PND 180 was used for all these three adult specimens in the analyses and in Table 1.

For all specimens in which preservation made this possible, we measured the condylobasal length of the skull (CBL, in mm), the skull mass (in g), and the masses for the malleus, incus and stapes (when available; M, I, and S, respectively, in mg). All the measurements were taken three times, and a mean of them was then used. For the CBL we used a caliper, for weighing the skulls (ranging between 0.107 g and 1.578 g) we used appropriate balances, and for weighing the ossicles we used a micro balance Cahn T-28 (Cahn Instruments, Cerritos, CA) with a measuring range of 1 μg –1000 mg.

Further, for the tympanic membrane area, based on the tympanic ring, we measured two diameters of this area, the longest one (2a) and the one perpendicular to that (2b; see Table 1). The area (A_1, in mm^2), was calculated using the equation for the area of an ellipse: $A_1 = \pi ab$, where a and b are the two semi-axes of the ellipse. The oval window area was acquired directly by measuring the stapes footplate area (this is a common practice in middle ear research, see e.g., Henson 1961; Fleischer 1973). The longest axis (2c) of the footplate and the one perpendicular to it (2d) were measured, and the area was obtained as $A_2 = \pi cd$ (see Table 1).

We also measured the lever arm lengths for malleus and incus (L_1 and L_2, respectively, in mm) as the shortest distance between the pivot axis and the tip of the ossicle;

Table 1. Skull and middle ear data for the sampled 19 specimens of *Monodelphis domestica*.

Specimen #	Side	Age (PND)	CBL (mm)	Skull (g)	M (mg)	I (mg)	S (mg)	M+I (mg)	A$_1$ (mm^2)	A$_2$ (mm^2)	L$_1$ (mm)	L$_2$ (mm)
97141	left	30	15.57	0.096	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
97142	left	30	16.22	0.119	0.142	0.056	0.005	0.198	3.6191	0.0924	N/A	0.72
97143	right	35	20.15	0.107	0.239	0.086	0.006	0.325	4.5038	N/A	N/A	0.8
97140	right	40	21.61	0.205	0.497	0.097	0.018	0.594	4.7853	0.1307	2.16	0.8
98003	right	60	26.17	0.191	N/A	0.098	0.014	N/A	4.6445	0.1178	N/A	0.76
98001	right	63	27.16	0.272	0.474	0.098	0.014	0.572	4.9763	0.1178	N/A	0.8
98012	right	72	29.37	0.366	0.584	0.107	0.014	0.691	4.5038	0.1335	2.08	0.8
98006	right	80	31.23	0.452	0.561	0.119	0.013	0.68	5.2779	0.1272	N/A	0.76
98013	left	90	33.12	0.516	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
98009	left	100	32.65	0.546	0.342	0.111	0.011	0.453	4.9763	0.1178	N/A	0.8
98010	right	104	34.69	0.605	0.469	0.114	0.019	0.583	5.1271	0.1442	2.24	0.8
98007	right	104	35.45	0.65	0.625	0.114	0.023	0.739	4.9562	0.1307	2.24	0.8
98004	right	110	35.77	0.703	0.454	0.124	0.02	0.578	5.5795	0.1389	2.24	0.84
98002	right	120	33.67	0.628	0.385	0.115	0.019	0.5	4.6445	0.11	2.32	0.8
98008	right	120	34.84	0.619	0.314	0.1	0.016	0.414	5.2779	0.1527	2.24	0.8
98014	right	132	35.09	0.688	0.603	0.1	0.013	0.703	4.8104	0.1389	2.16	0.8
98005	right	180	37.36	1.011	0.436	0.109	0.014	0.545	4.9763	0.1178	2.24	0.8
97001	right	180	39.62	1.018	0.352	0.108	0.014	0.46	5.2779	0.1012	2.08	0.8
98011	right	180	43.4	1.578	0.501	0.114	0.013	0.615	5.4538	0.1084	2.24	0.8

PND, postnatal days; CBL, condylobasal length of the skull; Skull, skull mass; M, malleus mass; I, incus mass; S, stapes mass; M+I, combined mass of malleus and incus; A$_1$, tympanic membrane area; A$_2$, oval window area; L$_1$, malleus lever arm length; L$_2$, incus lever arm length

in malleus the tip of manubrium and in incus the tip of its long process, i.e., a point very close to the incudo-sta-pedial joint. A binocular dissection microscope provided with measuring grids was used for measuring the diameters of the tympanic membrane and the stapes footplate, as well as the lever arm lengths of the ossicles.

The data for the masses, areas and lengths measured on the skulls and the middle ear ossicles are presented in Table 1. Least-squares (LS) regression analyses were applied to the data using the function *lm* in R 4.1.2. (R Core Team 2021) and plotted using the package *ggplot2* (Wickham 2016). The plots in Fig. 2A–F are shown with the LS slope, in accordance with similar data on these same parameters from adult marsupial mammals in Nummela and Sánchez-Villagra (2006).

Results

The variation of ossicular mass related to the age is shown in Fig. 1 for malleus, incus and stapes. The malleus mass varies clearly, whereas the incus and the stapes masses show much less variation (see changes at ages PND 30 and 35).

The variation of the condylobasal length related to skull mass shows normal variation found among mammals. For our material, the least squares (LS) regression analysis gives $y=40.087x^{0.351}$, $R^2=0.93$. This suggests that the large variation seen in middle ear ossicle mass and parameters during the postnatal time is not caused by any deviation in the skull growth as such.

There is a rapid growth between the two specimens of ages PND 30 and 35 (specimens # 97142 and 97143, respectively), and the specimen # 97140 of age PND 40. By PND 40 the ossicles seem to have reached their adult size (mass), and it seems that the mass even drops down by the time they are adults.

The size of the middle ear in *M. domestica* was studied with a bivariate plot of the ossicular mass (in mg) against the skull mass (in g; Fig. 2A). For the ossicular mass we used the combined mass of malleus and incus, as the relation of the stapes mass to the malleus plus incus mass is isometric (see below). The LS regression analysis gives $y=0.622x^{0.253}$, $R^2=0.32$. The relation of the ossicular mass in relation to the skull mass is negatively allometric, deviating from isometry. The low slope value (0.253) indicates that there is hardly any change in the ossicular mass, only the two smallest individuals differ from the rest of the group by clearly smaller ossicles. The low R^2 value (0.32) indicates that the variation in the ossicular mass cannot be explained by the skull mass; the ossicles do not seem to grow along with the skull**.**

The mass relation between malleus and incus was studied with a bivariate plot of the incus mass against the malleus mass (Fig. 2B). The LS regression analysis gives $y=0.145x^{0.382}$, $R^2=0.62$. This relation is also negatively allometric, the incus hardly grows at all, indicating that the increase seen in the combined mass of malleus and incus (Fig. 2A) is mainly brought about by the malleus.

For a bivariate plot of the stapes mass against the combined mass of malleus and incus (Fig. 2C), the LS regression analysis gives $y=0.025x^{0.951}$, $R^2=0.62$. A bivariate plot was done for the relation between the mass of the malleus plus incus, a three-dimensional value, versus
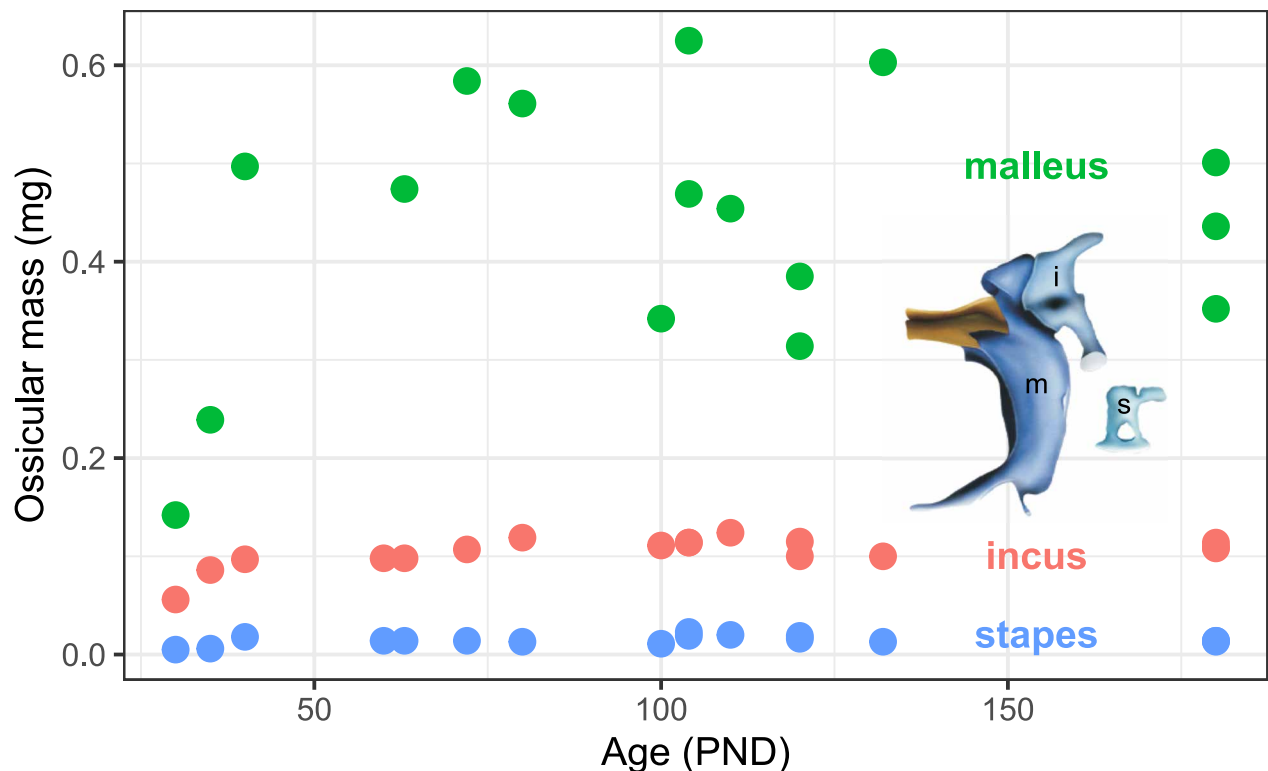
Figure 1. Variation of the ossicular mass along the postnatal age (postnatal days, PND) shown on linear axes. The inset shows a medial view of a three-dimensional model of the right middle ear ossicles of *Monodelphis domestica* at 30 PND. The stapes is illustrated disarticulated from the incus (adapted from Sánchez-Villagra et al. 2002: Fig. 2c). Abbreviations: i, incus; m, malleus; s, stapes.

the tympanic membrane area A_1, a two-dimensional value (Fig. 2D). The LS regression analysis gives $y=0.017x^{2.135}$, $R^2=0.43$, indicating slightly positive allometry. Here, the stapes could be excluded, because its mass scales isometrically with the combined mass of malleus and incus, as shown above (Fig. 2C). As pointed out by Nummela (1995) and Nummela and Sánchez-Villagra (2006), for isometric bones with constant density a given cross-sectional area is related to the mass of the bone as $mass^{2/3}$, and in isometric middle ears this cross-sectional area ought to be proportional to the tympanic membrane area. Here, the density of these different-aged middle ear bones is unknown, given that their ossification process is not yet finished**.** A slope value of 1.5 would indicate full isometry here, and the slope 2.135 indicates a clearly positive allometry, although the R^2 value is low.

To investigate other basic structures responsible for the sound transmission in the middle ear, we used the tympanic membrane and the oval window areas to study the area ratio A_1/A_2, and the malleus and incus lever arm lengths to study the lever ratio L_1/L_2. A bivariate plot relating the oval window area A_2 to the tympanic membrane area A_1 shows negative allometry (Fig. 2E). The LS regression gives $y=0.045x^{0.631}$, $R^2=0.22$. The allometric relation is weak, as the R^2 value is low. The youngest specimen of PND 30 has a clearly smaller tympanic membrane area than the other specimens, and has the smallest oval window area, too. There is no isometric relation here found during this growth series.

A bivariate plot for the incus lever arm length L_2, in relation to the malleus lever arm length L_1, is shown in Fig. 2F. The LS regression gives $y=0.760x^{0.070}$, $R^2=0.03$. There is no allometry found here; the malleus lever arm length varies to some degree, but the incus lever arm length is quite equal among different-aged specimens. The relations of lever arm length/ossicular mass of both malleus and incus were not studied here, due to the lack of variation in L_1 and L_2.

Discussion

The incus and stapes change little in mass between 40 and 180 days of postnatal life, while the malleus does, reaching maximum mass at around 100 PND. Of note is also the strong variation of the malleus in this regard. This pattern of coupling in growth between incus and stapes (somewhat independent of the malleus) is unexpected if one considers that malleus and incus are linked functionally (Fleischer 1978) and in their developmental origin from pharyngeal arch 1, whereas the stapes originates from the pharyngeal arch 2 (see e.g., Liem et al. 2001). Our finding of the growth patterns of middle ear ossicles and other middle ear parameters suggests a modularity that contrasts with that of pharyngeal origins of these ossicles, showing a growth trajectory independent of the evolutionary history of the structures in question. Fleischer (1978) grouped mammalian middle ears in different types, mainly the ancestral, the transitional, and the freely-mobile type, based on the relative proportions of
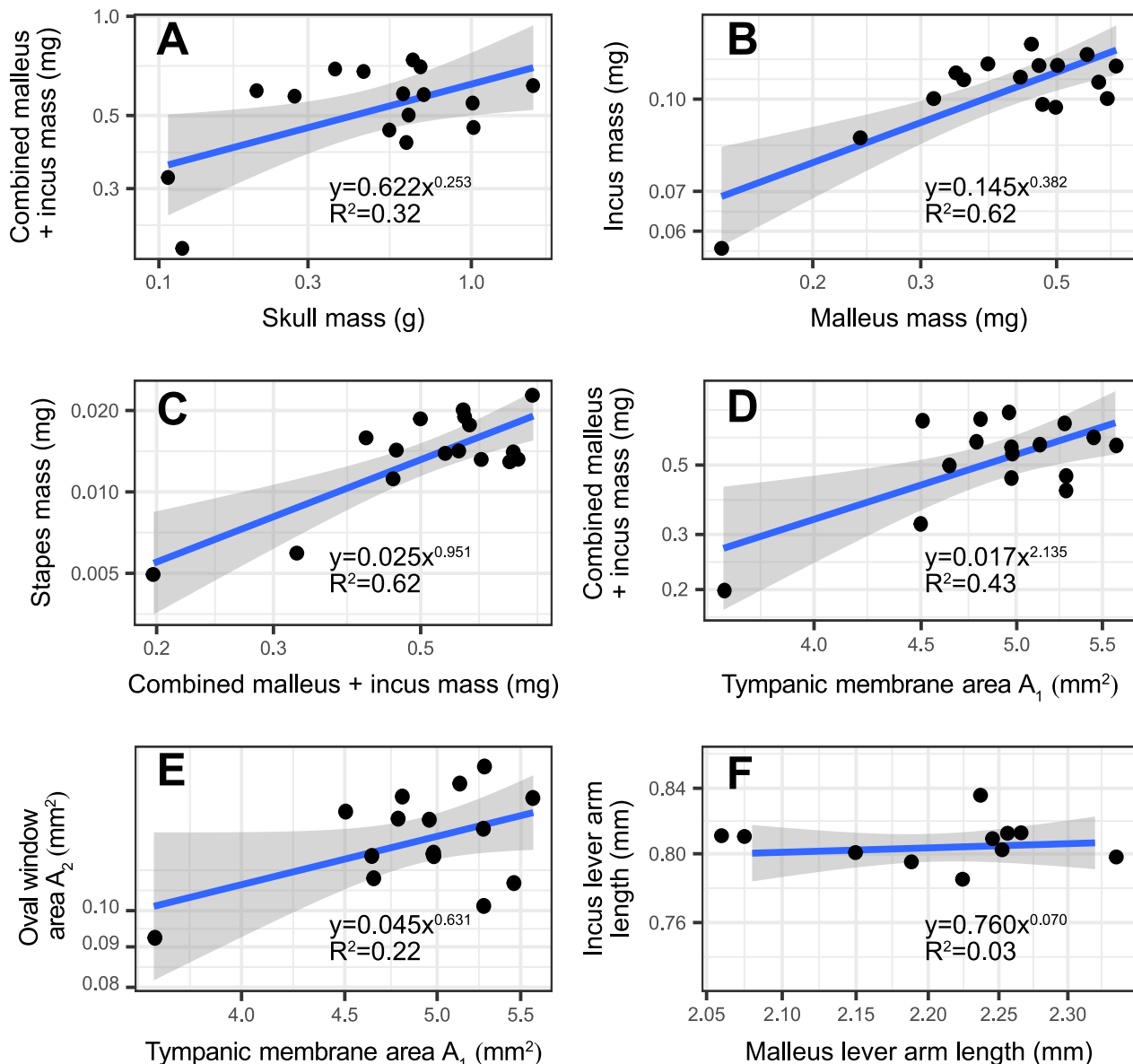
Figure 2. Skull mass and middle ear parameters plotted on log-log axes. The LS regression analyses were performed, and the best-fit slopes and confidence limits are shown, together with the regression equations for each plot.

malleus and incus and to the type of connection the malleus makes with the ectotympanic. Ossicle proportions and degree of connections are generated in growth and have changed in evolution, surely resulting in changing modularity patterns. Fleischer (1978) regarded marsupials as having mostly an ancestral middle ear type, although in his detailed descriptions (Fleischer 1973) on four marsupial species (*Metachirops* sp., *Notoryctes typhlops*, *Petaurus breviceps*, and *Thylogale brunnii*) he mentioned clear differences in these species, e.g., regarding size relations between malleus and incus, and the connection between the gonial of malleus and the ectotympanic. Following Fleischer (1973, 1978) classification, *Monodelphis domestica* exhibits the ancestral middle ear type.

A quantitative examination of middle ear structures of mammals at perinatal ages was conducted by Golden (1997). He produced three-dimensional reconstructions of volumes of middle ear ossicles using histological sections in three rodents: the fat-tailed gerbil *Pachyuromys*

duprasi, the house mouse *Mus musculus*, and the common rat *Rattus norvegicus*. The altriciality of these placentals is not comparable with the extreme one of marsupials such as *Monodelphis*, but this work surely serves to document changing proportions of the ossicles, even from unossified precursors, including prenatal specimens in the case of the mouse and specimens from the age of birth in the other two species. Golden (1997) reported how the growth trajectory during the first 16 days of postnatal growth – as measured in volume – of the incus and the stapes is quite similar and contrasts with a diverging one of the malleus. Curiously, this author uses this pattern, similar to the one we report for *Monodelphis*, to suggest a second pharyngeal arch origin for the incus and to question the paradigm of middle ear transformations based on palaeontological and embryological data (Maier and Ruf 2016a).

A more detailed study aiming at contrasting the developmental versus the adult and functional structural

units in the middle ear would have to discriminate different parts of the ossicles. In general, one speaks of three middle ear ossicles in mammals, but in fact the anterior process of the mammalian malleus, the gonial (homologous with the prearticular of early synapsids; Maier and Ruf 2016a) is a different element, distinguishable in development in view of its position and mode of ossification from the articular-derived portion of the malleus (Sánchez-Villagra et al. 2002; Maier and Ruf 2016b; Maier et al. 2018). Likewise, other embryological distinctions could be made: the footplate of the stapes is mesoderm derived as opposed to the rest, derived from the neural crest (Tucker 2017). It would seem that there is much evolvability in the modularity of middle ear structures in mammals throughout development and that adult structures of different developmental origins may operate as modules. Anatomical modules had been identified in the synapsid evolution leading to the DMME (Navarro-Díaz et al. 2019; Mao and Meng 2020). Modularity, not surprisingly, exists in both the phylogeny and ontogeny of the jaw/middle ear structures of mammals. Hearing function is another subject of fundamental importance when discussing these structures.

Significant events in the ontogeny of hearing occur similarly in both marsupials and eutherians. The onset of hearing and in particular attainment of adult hearing being comparable between the house mouse and *Monodelphis*, thus, there is no apparent retardation nor acceleration in the development of auditory function in the marsupial, which could be related to its early birth (Reimer 1996).

Our results shed some light on the postnatal development of *Monodelphis* hearing. In *Monodelphis* there is an air-filled space by PND 26, the middle ear cavity, relevant for hearing (Aitkin et al. 1997). Reimer (1996) provided data on the development of the sensitivity and frequency range of hearing of *Monodelphis domestica* through her measurements on the brainstem auditory evoked potentials (BAEPs). She showed that the onset of hearing in *Monodelphis* occurs at 29 PND, and the ear canals are already open at this time, and within roughly 10 days, by about 40 PND, the maturational changes in hearing are basically completed. Our data on middle ear ossicles does not provide any information on morphological change that could be related to this functional one.

The relation of stapes mass against the combined mass of malleus and incus is isometric. This follows the interspecific pattern found both for adult mammals in general, both for placentals (Nummela 1995) and marsupials (Nummela and Sánchez-Villagra 2006), although the R^2 value is much lower from the one found in the interspecific analyses for adults.

From the functional point of view, the S/(M+I) isometry here in *Monodelphis domestica* (Fig. 2C) is interesting, as it suggests that the ossicular chain is an entity where M+I together function as one unit even in these non-adults, although this contradicts our observations on growth trajectories. The slope of 0.951 indicates that even in the ontogenetic pattern, the stapes grows in the same pace with the M+I. However, these animals might use bone-conduction hearing at this stage, together with

sound being transmitted by the middle ear. A massive malleus may be advantageous, as bone conduction hearing is profited by extra mass, as that changes the rotation axis of the ossicular chain, and this helps bone-conducted sounds to create a phase difference between the tympanic membrane and the inner ear fluid (Nummela 1995; Stenfelt et al. 2002). This might explain the positive allometry of the *Monodelphis* ossicles in relation to the tympanic membrane area (Fig. 2D).

While it is unclear how much *Monodelphis domestica* uses air-conducted hearing at this stage, it is more probable that they use bone-conducted hearing while being attached to the nipple of their mother (Sánchez-Villagra and Smith 1997). In early postnatal stages the primary jaw joint is still in function in marsupials while the secondary jaw joint is not fully developed yet (Maier 1987, 1990; Sánchez-Villagra et al. 2002). Although the maturation of the auditory sensitivity in *Monodelphis* during this early postnatal stage may be partly due to a functional middle ear mechanics to some degree, at least in the low-frequency part of the hearing range, we suggest that the inner ear maturational changes are largely responsible for the development of hearing in these young animals.

The model of Hemilä et al. (1995) predicting the high-frequency hearing limit at 60 dB SPL was based on internal isometry discovered between the middle ear ossicular masses and lengths, and tympanic and oval window areas among adult placentals (Hemilä et al. 1995; Nummela 1995), showing that the high-frequency hearing limit of a mammal can be predicted on the basis of its middle ear ossicular mass. This model was successfully applied also to adult marsupials (Nummela and Sánchez-Villagra 2006). However, the model is not applicable to our material. First, the middle ear structures in our postnatal specimens do not show such isometry as is required for using the model of Hemilä et al. (1995); although the relation of S/(M+I) is isometric, the relation of A_2/A_1 is not (Fig. 2E), neither is the relation of L_2/L_1 (Fig. 2F), and hence the foundations of the model are not fulfilled by our material. Second, in the BAEPs of Reimer (1996), the high-frequency hearing limit increased with age, while at the same time the ossicular mass grows, according to our results. This is contradictory to the model, according to which the high-frequency hearing limit should decrease with an increasing ossicular mass.

Acknowledgements

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