

## Novel Insights into the Development of the Avian Nasal Cavity

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**Running Title:** Nasal plug in chicken embryo

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as doi: [10.1002/ar.24349](https://doi.org/10.1002/ar.24349)

## Abstract

In embryonic amniotes, patterning of the oral and nasal cavities requires bilateral fusion between craniofacial prominences, ensuring an intact primary palate and upper jaw. After fusion has taken place, the embryonic nasal cavities open anteriorly through paired external nares positioned directly above the fusion zones, and bordered by the medial nasal and lateral nasal prominences. In this study, we show that in the chicken embryo, the external nares initially form as patent openings, but only remain so for a short period of time. Soon after the nasal cavities form, the medial nasal and lateral nasal prominences fuse together in stage 29 embryos, entirely closing off the external nares for a substantial portion of embryonic and fetal development. The epithelium between the fused prominences is then retained, and eventually develops into a nasal plug that obstructs the nasal vestibule through majority of the fetal period. At stage 40, the nasal plug begins to break down through a combination of cellular remodeling, apoptosis, as well as non-apoptotic necrosis, leading to completely patent nasal cavities at hatching. These findings place chickens in a category with several species of nonavian reptiles and mammals (including humans) that have been found to develop a transient embryonic nasal plug. Our findings are discussed in the context of previously reported cases of nasal plugs as part of normal embryonic development, and provide novel insight into the craniofacial development of a key model organism in developmental biology.

**Key words:** Chicken, Nasal cavity, Nasal plug, Prominences, Craniofacial

## INTRODUCTION

The components of embryonic craniofacial development are remarkably conserved across amniotes (Abramyan and Richman, 2015; Abramyan et al., 2015). The face is assembled from a set of craniofacial prominences that grow out bilaterally and fuse together towards the front of the face. The lower jaw forms from paired mandibular prominences (Wedden, 1987; Richman and Tickle, 1989). The upper jaw arises from a frontonasal mass (or frontonasal prominence), paired maxillary prominences, paired lateral nasal prominences, and paired medial nasal prominences (which arise from the frontonasal mass) (Fig. 1) (Yee and Abbott, 1978; Will and Meller, 1981; Abramyan et al., 2015). In mammals the maxillary prominences fuse with the medial nasal prominences to form an intact upper jaw and nasal cavities (Jiang et al., 2006). In avian embryos, the medial nasal prominences are often poorly defined as distinct structures since they do not develop a deep midsagittal groove between them. Therefore, the avian maxillary prominence is typically described as fusing with the globular process of the frontonasal mass, caudal to the medial nasal prominence proper (Tamarin et al., 1984) (Fig.1). Fusion between the globular processes and the maxillary prominences completes patterning of the primary palate (anterior-most region of the palate), upper jaw, as well as upper lip and nasal cavities (described in detail by Abramyan and Richman, 2015 and Abramyan et al., 2015). Despite minor differences, the extraordinary conservation of embryonic craniofacial patterning allows for extensive use of both mouse and chicken in characterization of human craniofacial development and pathology (Bedell et al., 1997a; Bedell et al., 1997b; Dodgson and Romanov, 2004; Abramyan and Richman, 2018).

As the upper jaw forms through fusion of the prominences, the nasal and oral cavities separate from each other and become distinct structures. Externally, the openings to the nasal cavities are visible as paired external nares above the mouth in mammals and sauropsids, the clade that includes extant reptiles and birds. Internally, the nasal cavities connect with the oral cavity and the pharynx through paired internal openings called choanae (Kim et al., 2004; Jankowski, 2011). The nasal cavities themselves may be divided into three anatomical compartments arranged anterior to posterior: the rostral nasal vestibule, the nasal cavity proper, and the nasopharyngeal duct (Parsons, 1959; Parsons, 1970; Witmer, 1995). In birds, the vestibule is particularly expanded, while the nasopharyngeal duct is highly reduced or often absent (Witmer, 1995). Within the aforementioned compartments, amniotes develop nasal conchae (turbinals), which can range in number from one in squamates to a row of conchae of variable number in mammals and birds (Hillenius, 1994; Van Valkenburgh et al., 2014). Birds develop three sets of conchae. The rostral nasal conchae (anterior turbinals) develop in the vestibule, while the middle and caudal conchae (middle and posterior turbinals) form in the respiratory and olfactory portions of the nasal cavity proper (Witmer, 1995).

In neonatal amniotes, the nasal cavities have to be patent since they are utilized for a variety of postnatal functions including respiration, olfaction, vocalization, air filtration, heat dissipation, as well as warming of inhaled air (Hillenius, 1994; Dahl and Mygind, 1998). However, during embryonic development, studies have found that nasal cavities are often transiently obstructed by

tissue, and must be recanalized prior to hatching or birth. Obstruction of the nasal passages can occur via blockage of the choanae, external nares, or both (Diewert and Shiota, 1990; Buchtova et al., 2007). The closure and subsequent reopening of the mammalian choana is well studied due to its association with choanal atresia, a condition that co-occurs with a number of craniofacial syndromes, where the choanae fail to reopen and thus nasal respiration is obstructed (Kurosaka, 2019). Obstruction of the external nares however, has received relatively less attention and is thought to occur through the formation of transient epithelial nasal plugs, both in reptiles (Howes and Swinnerton, 1901; Buchtova et al., 2007) and mammals (Bollert and Hendrickx, 1971; Nishimura, 1993; Wassif et al., 2001; Kim et al., 2004; Alomaisi et al., 2018), with no recorded cases in avian embryos.

The purpose of this study was to closely examine the formation of the avian nasal cavity, with the use of the chicken embryo as a model. We were specifically interested in determining whether a nasal plug forms in this group at any point during embryonic and fetal stages of development. Surprisingly, this aspect of development in the chicken embryo appears to have gone unobserved, despite the fact that they have been used extensively as a model of developmental biology and embryology for over a century (Kain et al., 2014; Abramyan and Richman, 2018). In this study, we applied a combination of methods including X-ray micro computed tomography (microCT) (Rowe et al., 2005; Rossie, 2006; Craven et al., 2007), as well as traditional paraffin histology and tests for cellular apoptosis, to characterize the shape and structure of developing nasal cavities in the chicken embryo. We expect our findings to further the potential of the chicken embryo as a

model organism for the study of human craniofacial development and pathology.

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## **MATERIALS AND METHODS**

### **Embryo Acquisition and Staging**

Animal work was performed according guidelines from the University of Michigan Animal Care and Use program. Fertile chicken (*Gallus gallus*) eggs were obtained from the Michigan State University Poultry Teaching & Research Center, incubated in our laboratory at 38.0 °C, and staged according to Hamburger and Hamilton, (1951) (Table 1). At day 2.5 of incubation (stage 15), eggs were windowed using standard procedures. Upon reaching stages required for various analyses, specimens were harvested and placed in cold 1x phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA)/PBS overnight, followed by dehydration in ethanol (EtOH) concentration series up to 70% for long term storage. Animals at stage 34 and above were first decapitated in ovo before harvesting.

### **MicroCT scanning conditions**

There are a number of contrasting agents currently used to stain soft tissue for microCT (Gignac et al., 2016). In testing both phosphomolybdic acid (PMA) and iodine potassium iodide (IKI or Lugol's solution) in embryos of various stages, we found that PMA provided the best contrast, highest penetrance, and reduced side-effects (e.g. tissue shrinkage) in embryos younger than stage 30, while IKI was a better stain for stage 34 and stage 38 specimens. Fixed specimens in 70% ethanol were transferred to IKI or PMA for staining and subsequent scanning. Stage 28 - 29 specimens were stained with 1% PMA for 4 days. Afterward, they were embedded in .75% agarose

for scanning. Stage 34 specimens were stained with 5% IKI for 4 days with one replacement at day 2, and scanned in 75% ethanol. Specimens were scanned in a 19 mm diameter specimen holder, over the entirety of the head using a  $\mu$ CT100 Scanco Medical microCT system (Bassersdorf, Switzerland). Scan settings were: voxel size 10  $\mu$ m, 70 kVp, 114  $\mu$ A, 0.5 mm AL filter, integration time of 500 ms, and 1500 projections. Stage 38 specimens were stained with 10% IKI for 6 days, with replacement every 2 days, and scanned in 25% ethanol. Specimens were then placed in a 34 mm diameter specimen holder and scanned over the entirety of the head using a  $\mu$ CT100 Scanco Medical microCT system (Bassersdorf, Switzerland). Scan settings were: voxel size 12  $\mu$ m, 70 kVp, 114  $\mu$ A, 0.5 mm AL filter, integration time of 500 ms, and 1500 projections.

### **Volume Rendering and Segmentation**

3D volume rendering was performed in MicroView (open-source software, Parallax Innovations Inc., Ilderton, ON, Canada). MicroCT scan .vff files were uploaded to MicroView, reoriented and cropped as needed. Files were then saved as .vtk files and uploaded to 3D Slicer version 4.2.2 (open-source software, <http://www.slicer.org>) for segmentation. Figures were compiled in Adobe Photoshop and Adobe Illustrator (Adobe Systems Inc., San Jose, CA).

### **Histology**

Different samples were used for histology and microCT in order to avoid unintended effects on tissue morphology such as shrinkage due to PMA or IKI staining. Embryos were harvested and



fixed in 4% PFA overnight, followed by dehydration in an ethanol concentration series up to 70%. Stage 32 and above specimens were decalcified in Morse's solution for up to 1 week. Specimens were then embedded in paraffin and sectioned into 7 $\mu$ m slices and mounted on slides. Sections were stained with picosirius red to highlight skeletal and soft tissue, and alcian blue to highlight cartilage (Buchtova et al., 2007). Bright field images were captured using a Nikon Eclipse E800 microscope equipped with a CoolSNAP-EZ CCD camera (Photometrics, Tucson, AZ, USA), and NIS-Elements BR v. 4.12.01 software (Nikon, Melville, NY, USA).

### **DAPI Staining and TUNEL Assay**

Sections from specimens prepared for histological analysis were also utilized for 4' 6-diamidino-2-phenylindole (DAPI) staining, as well as terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) analysis. TUNEL was performed with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (EMD Millipore, Billerica, MA - S7110). Nuclei were stained with DAPI using Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA). Fluorescence images were captured using a Nikon Eclipse Ts2R inverted microscope (Nikon, Melville, NY, USA) equipped with a CoolSNAP Dyno monochrome CCD camera (Photometrics, Tucson, AZ, USA), and NIS-Elements BR v. 5.02 software (Nikon, Melville, NY, USA).

## RESULTS

According to our findings, nasal cavity formation in the avian embryo may be divided into three phases. Phase 1 describes the period immediately after the fusion of the primary palate, where we observed a second fusion event between the medial nasal and lateral nasal prominences. Phase 2 involves the establishment of a nasal plug in the nasal vestibule and the formation of nasal conchae. Phase 3 focuses on the cellular processes involved in recanalization of the nasal vestibule, resulting in patent nasal cavities prior to hatching. These three sequential phases describe a developmental arc consisting of formation, establishment, and breakdown of nasal plugs in the chicken embryo.

### **Phase 1: Fusion of External Nares (stages 28-29)**

Isosurface renderings of stage 28 chicken embryos reveal the establishment of open external nares after initial fusion between the maxillary prominence and the globular process of the frontonasal mass (Fig. 2A,A' - black arrowheads). With transition to stage 29, the fusion zone expands (Fig. 2B,B' - black arrowheads), the external nares become reduced in size to a small opening (Fig. 2B,B' - red arrow), and complete closure occurs shortly thereafter. In order to better understand the epithelial-mesenchymal interaction during this process, we compared the newly identified nasal cavity fusion zone to the well-studied fusion of the primary palate/upper jaw in stage 29 animals (Fig. 3). Cross sections in the transverse plane show retained epithelium between the medial nasal and lateral nasal prominences after closure of the nasal cavity (Fig. 3A-B' – red arrowhead). In contrast, the fusion zone between the maxillary prominence and the frontonasal mass (primary

palate fusion) exhibited continuous mesenchyme, which indicates breakdown of the intervening epithelium (commonly referred to as the nasal fin) (Fig. 3C-D').

### **Phase 2: Extent of Nasal Plug and Interaction With the Nasal Conchae (stages 34-38)**

At stage 34 (Fig. 4A), a coronal section of the beak reveals obstruction of the nasal vestibule at the level of the external nares (Fig. 4B). In the section immediately posterior to the nares, the developing rostral concha is visible as a delineation within the tissue comprising the nasal plug (Fig. 4C). Further posterior, the middle nasal and caudal nasal conchae are also apparent at this stage, but in the form of outgrowths from the lateral nasal walls into the respiratory and olfactory portions of the nasal cavity, posterior to the nasal vestibule (Fig. 4D). Stage 38 specimens were also analyzed in a similar manner (Fig. 4E), and reveal more extensive rostral conchae within the nasal plug (Fig. 4F). A transverse view of a stage 38 embryo reveals that the nasal plug is limited to the nasal vestibule, while the posterior nasal cavity remains free of obstruction, as evidenced by the darker color of the patent cavity space (Fig. 4G' - white arrowhead). Furthermore, the relative positions of the nasal conchae are confirmed in the transverse view, with the rostral conchae developing within the plug tissue in the anterior vestibule and the middle and caudal conchae developing within the patent posterior nasal cavity (Fig. 4G,G').

### **Phase 3: Recanalization of the Nasal Cavity (stages 39-44)**

Histological analysis of stage 39-44 embryos revealed progressive breakdown of the cells encapsulated within the nasal vestibule. At stages 39 and 40, the nasal cavities exhibit uniform concentration of epithelial cells continuous with the epithelium of the nasal wall and alinasal folds (Fig. 5A-B'). Beginning with stage 41, the cells at the center of the epithelial plug appear to expand, as evidenced by larger cytoplasmic area and lighter staining (Fig. 5C,C'). Subsequent stages reveal progressive expansion of the central cells, eventually leading to their rupture and complete canalization by stage 44 in all embryos examined (Fig. 5D-F'). Some embryos did exhibit partial canalization as early as stage 42 but not earlier (data not shown). Closer inspection of the cells at the putative opening of the external naris revealed stretching of the cells across the nasal cavity (Fig. 5G), eventually tearing apart during the recanalization process (Fig. 5H).

Since picosirius red staining did not sufficiently resolve the position of cells along the edges of the nasal plug, we decided to apply DAPI nuclear staining to sections equivalent to those of the histological preparations (illustrated in Fig. 6A). DAPI staining confirmed widening of the nasal vestibule, with the center exhibiting less nuclear signal as fewer, more expanded cells occupy that space. The cells closer to the edges of the nasal vestibule intercalate and stack into organized columns along the walls. This behavior may be observed as early as stages 39-40 in some cells (Fig. 6B,C), continuing into stage 41, and reaching its most-organized state at stage 42 (Fig. 6D,E). Columns then begin breaking down by stage 43 (Fig. 6F). Due to the apparent breakdown of cell columns at stage 43, stage 41-43 specimens were tested for apoptotic signal using a TUNEL assay.

Stage 41 specimens exhibited no TUNEL signal (Fig. 7A), while stage 42 specimens revealed signal along the walls of the nasal vestibule (Fig. 7B). By Stage 43, the signal is once again lost (Fig. 7C). A magnified view of stage 42 revealed a very strong and specific signal within the intercalated cellular columns lining the walls of the primitive nasal cavity (Fig. 7D-F).

## DISCUSSION

Among the reptilian relatives of birds, the formation of a cellular nasal plug has been described in the tuatara embryo (*Sphenodon punctatus*) (Howes and Swinnerton, 1901), as well as the African rock python (*Python sebae*) (Buchtova et al., 2007). While Howes and Swinnerton, (1901) do not describe the exact stages of nasal plug formation and breakdown in tuatara, the python appears to exhibit a similar period of nasal vestibule occlusion when compared to chicken (Fig. 8). Additionally, Abramyan et al., (2015) have previously described fusion between the lateral nasal and medial nasal prominences, similar to chicken, in embryonic stages of three lizard species: *Aspidoscelis uniparens* (Teiidae) at stage 12, *Pogona vitticeps* (Agamidae) stage 31, and *Chamaeleo calytratus* (Chamaeleonidae) at stage 34 (see Abramyan et al., 2015 for staging methods used). The fact that this process was identified across such distantly related species suggests that it is part of normal lacertilian embryonic development. Unfortunately, limited sample availability precluded study of later stages to detect whether the epithelial seams between the fused prominences go on to form a more substantial nasal plug in lizards as they do in chicken and snake. While crocodylian and testudine embryos remain to be studied, the studies cited above, in conjunction with the work presented in this manuscript, suggest that nasal plug formation may be a shared developmental characteristic across sauropsids.

Description of nasal plugs in mammalian embryos go back to studies on humans published in the early 1900's (Schaeffer, 1910). Since then very few studies have described the mammalian nasal

plugs in non-human embryos, with available information limited to studies of rabbit (Alomaisi et al., 2018), baboon (Bollert and Hendrickx, 1971), and mouse (Rugh, 1968) (Fig. 8). Recently, more detailed studies of human embryos have described the plug as developing between the 8<sup>th</sup> week of gestation (Carnegie stage 20) (Yoon et al., 2000; Kim et al., 2004) and the 10<sup>th</sup> week (Nishimura, 1993). Recanalization is then through to occur between the 13<sup>th</sup> and 17<sup>th</sup> weeks of gestation (Kim et al., 2016), with some studies suggesting retention of the plug till the 22<sup>nd</sup> week (Diewert and Shiota, 1990) or even 24<sup>th</sup> week (Kumoi et al., 1993; Sarnat and Yu, 2016) (Fig. 8). Taken from the limited data that is available, comparative analysis of nasal plug formation reveals an earlier loss of nasal plugs in mammalian embryos in comparison to sauropsid lineages (Fig. 8). However, further studies are required to establish more definite stages of nasal plug formation and breakdown, both in humans as well as other mammals.

Additionally, the nasal plugs in the human embryo are described as occurring in the “anterior” region of the nasal cavity (Diewert and Shiota, 1990; Kim et al., 2004; Kim et al., 2016), similar to the chicken nasal plug being limited to the nasal vestibule. This similarity may inform us about the role of the plugs in the respective species, as well as furthering our understanding of nasal cavity compartmentalization and evolution in amniotes. That said, there do seem to be differences between human nasal plugs and the structure we describe in chicken. Photomicrographs of human nasal plugs in several independent studies appear to show discontinuity between the epithelial plug and the epithelium lining the nasal vestibule (Schaeffer, 1910; Humphrey, 1969; Diewert and

Shiota, 1990), while in the chicken, we show that the epithelial cells of the nasal plug are continuous with those of the nasal vestibule and even the craniofacial prominences. Therefore, these structures may be convergent in humans and birds and may warrant different nomenclature.

### **Recanalization of the Nasal Vestibule**

Very few studies have addressed the cellular mechanism underlying the recanalization of the nasal vestibule (Masumoto et al., 2010). Schaeffer, (1910) initially suggested that human nasal plugs are physically shed rather than broken down. Indeed since the nasal plugs appear to be physically disconnected from the craniofacial epithelium, as noted earlier, this may be the case in humans, but is not so in chicken. Furthermore, in a study of human fetus at 15 weeks, Masumoto et al., (2010) state that the nasal cavity is negative for TUNEL immunoreactivity. However, close inspection of the image presented in their study does appear to show some signal in the walls of the nasal plug, albeit less organized than what we observed in chicken. Therefore, further investigation of apoptosis in the human nasal plug is certainly warranted, especially given our results.

### **Nasal Concha Formation Within the Nasal plug**

The avian rostral conchae are morphologically and functionally analogous to the mammalian maxilloturbinals, although they are thought to have arisen independently in each group during their transition into endothermy (Parsons, 1967; Hillenius, 1992; Witmer, 1995; Ruben et al., 1998;



Hillenius and Ruben, 2004). Our results show that in the avian embryo, the rostral concha grows within the tissue comprising the nasal plug, which then breaks down around it. In mammals, this process is less well understood. At first glance, it would appear that mammals experience direct outgrowth of conchae from the nasal wall into a patent nasal vestibule (Smith and Rossie, 2008; Van Valkenburgh et al., 2014), including in humans (Bingham et al., 1991; Wake et al., 1994; Wang and Jiang, 1997). However, the human literature (which is the most extensive) on nasal plug and nasal turbinal development reveals an overlap in the timing of development of these two structures. In describing the developing human maxilloturbinals, several studies describe the nasal turbinals in 8-week-old embryos, with no mention of a nasal plug (Bingham et al., 1991; Wake et al., 1994; Wang and Jiang, 1997; Müller and O'Rahilly, 2004; Neskey et al., 2009). On the other hand, studies focusing on nasal plugs describe them as forming at the same developmental time points (Carnegie stage 20, ~8 weeks of gestation) with no mention of the nasal turbinals (Diewert and Shiota, 1990; Yoon et al., 2000; Kim et al., 2004). This disparity may be due to misstaging of embryos, or positional differences between histological sections. Therefore, it remains unknown whether mammalian maxilloturbinals also develop within the nasal plug or not.

### **Conclusions:**

In this study, we describe a developmental process that has gone unnoticed in the chicken embryo: the development of a nasal plug. The obstruction and subsequent recanalization of embryonic nasal

cavities may at first seem like a counterintuitive developmental step, and yet is observed repeatedly across diverse amniote lineages. Yet, despite the seeming ubiquity of epithelial obstructions in embryonic cavities, there is little in the way of hypotheses in the current literature for their existence. Furthermore, while the nasal plugs have been recognized in embryological studies, few studies describe the cellular processes involved in their development and subsequent breakdown, with some conflicting ideas of whether they are extruded or undergo cellular breakdown within the cavity. In working with the chicken embryo, we have advanced the knowledge of these structures further by not only describing their formation, but also the cellular breakdown involved in recanalization. Through this study, we hope to pave the way for further research of these tissues across taxa, thereby filling this gap in the knowledge of amniote developmental biology.

### **Acknowledgements**

We would like to thank Michelle Lynch for assistance with microCT scan optimization. Specimens were scanned at the University of Michigan School of Dentistry MicroCT Core funded in part by NIH/NCRR S10RR026475-01. J.A. is a member of the Michigan Integrative Musculoskeletal Health Core Center (MiMHC) and as such, research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number P30 AR069620. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Funding was also provided by Undergraduate Student Independent Research Grant from the University of Michigan-Dearborn to Z.A. and startup funds from the University of Michigan-Dearborn to J.A. Lastly, we would like to thank the reviewers whose suggestions greatly improved our manuscript.

### **Conflict of Interest:**

The authors declare no conflict of interest.

### **Author Contributions:**

Zahra Albawaneh and Raana Ali contributed equally to this work. Zahra Albawaneh was responsible for microCT segmentation, histology, and drafting of the manuscript. Raana Ali was responsible for data interpretation, histology, and constructive review of manuscript. John Abramyan was responsible for project design, figure preparation, and drafting of the manuscript.

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## Figure Legends

**Fig. 1.** Depiction of a stage 28 chicken embryo with red shading highlighting the regions of fusion between the maxillary prominences and the globular processes of the frontonasal mass. Asterisk indicates open nasal cavity. fnm, frontonasal mass; gb, globular process; lnp, lateral nasal prominence; md, mandibular prominence; mnp, medial nasal prominence; mxp, maxillary prominence; s, stomodeum.

**Fig. 2.** MicroCT reconstructions of stage 28 and stage 29 chicken embryos exhibit differences in the degrees of external naris opening. Stage 28 specimens reveal open external naris (asterisk) and a relatively smaller region of fusion (black arrowheads) when the globular process and maxillary prominence first fuse (**A, A'**). Stage 29 specimens reveal an expanded region of fusion (black arrowheads) (**B, B'**), with a small opening to the nasal cavity visible at the cranial end (red arrow) (**B'**). e, eye; fnm, frontonasal mass; gp, globular process of the frontonasal mass; lnp, lateral nasal prominence; md, mandibular prominence; mnp, medial nasal prominence; mxp, maxillary prominence; s, stomodeum.

**Fig. 3.** Corresponding microCT slices and paraffin histological sections in the transverse plane of stage 29 embryo comparing fusion of the external naris and primary palate. Fused nasal prominences reveal retained epithelial seam between the lateral and medial nasal prominences (**A-B'**-red arrowhead). Sections through the newly fused primary palate reveal continuous mesenchyme between the maxillary prominence and the frontonasal mass (**C-D'**). ant, anterior; e, eye; fnm, frontonasal mass; lat, lateral; lnp, lateral nasal prominence; med, medial; mnp; medial nasal prominence; mxp, maxillary prominence; nc, nasal cavity; pos, posterior; s, stomodeum.

**Fig. 4.** MicroCT slices through the beaks of stage 34 and stage 38 embryos reveals persistent occlusion of the nasal vestibule. Coronal slices through stage 34 embryo (**A**) reveals occlusion of the external naris (**B**), and the beginning of rostral concha formation (**C**). Further posterior slice reveals outgrowth of the middle and caudal conchae into the nasal cavity (**D**), which looks patent based on darker shading. A stage 38 specimen (**E**) reveals more advanced rostral conchae (**F**). Section in the transverse plane confirms that the nasal plug is restricted to the nasal vestibule, whereas the nasal cavity proper appears patent (**G,G'**-white arrowhead). cc, caudal nasal conchae; en, external naris; mc, medial nasal conchae; rc, rostral nasal conchae.

**Fig. 5.** Recanalization of the nasal vestibule observed through histological sections in the coronal plane. In specimens ranging from stage 39-43, the external naris is obstructed by cells of the nasal plug, which are continuous with the epithelium covering the nasal wall and alinasal fold (arrow)



(A-E'). Beginning from stage 41, specimens exhibit expansion of the cells in the center of the nasal plug (asterisk) (C-E'). Complete recanalization of the nasal vestibule was observed in all stage 44 specimens (F,F'). Magnified views of stage 42 and 43 specimens reveal stretched appearance of cells at the opening of the external naris (G), before they rupture during the recanalization process (H). af, alinasal fold; nc, nasal cavity; ns, nasal septum; nw, nasal wall; rc, rostral nasal concha.

**Fig. 6.** DAPI staining was performed on histological sections of the embryonic beak in the coronal plane, as illustrated in panel (A). Stained sections reveal progressive loss of nuclear signal through stages 39 – 43. At stage 39, the nasal plug is intact and reveals relatively uniform signal in the center of the nasal vestibule (B). By stage 40, signal is slightly reduced in the center and the cells along the edges exhibit some intercalation and stacking (C). Around stages 41-42, signal in the center is further reduced, while cells along the edges become highly organized into columns (D,E). At stage 43, columns appear to lose their organization (F). af, alinasal fold; af, alinasal fold; nc, nasal cavity; ns, nasal septum; nw, nasal wall; rc, rostral nasal concha.

**Fig. 7.** TUNEL analysis for apoptotic activity applied to stage 41 – 43 specimens. Stage 41 exhibits little to no TUNEL signal (green) as compared to background DAPI stained cells (blue) at stage 41 (A). At stage 42, TUNEL-positive cells become apparent along the walls of the nasal vestibule (B), with signal disappearing by stage 43 (C). Closer examination of stage 42 specimen TUNEL-

positive region **(D)**, in comparison to DAPI staining **(E)**, reveals localization of TUNEL signal to cells that make up the columns lining the walls of the primitive nasal vestibule **(F)**.

**Fig. 8.** Scaled comparison of nasal plug formation and loss across amniote taxa relative to their respective total incubation periods. Chicken (*Gallus gallus*) (Hamburger and Hamilton, 1951) and python (*Python sebae*) (Boughner et al., 2007; Buchtova et al., 2007) exhibit similar period of nasal plug retention **(A,B)**. In the tuatara (*Sphenodon punctatus*), Howes and Swinnerton, (1901) do not describe timing of nasal plug formation, but simply state that they are resorbed shortly before hatching (at 259 days - Thompson, 1990) **(C)**. In the mouse (*Mus musculus*), the nasal plug is observed at E16, but loss is described as being prior to birth (Rugh, 1968) **(D)**. In the rabbit (*Oryctolagus cuniculus*), nasal plugs form at 12 days and breaks down at 20-22 days (Alomaisi et al., 2018) **(E)**. In baboon (*Papio cynocephalus*), nasal plugs are observed at 40 days (Stage XIX - 16.3 mm), but loss prior birth is not recorded (Bollert and Hendrickx, 1971) (173 day gestation period - Albrecht and Townsley, 1978) **(F)**. In humans, formation of the nasal plug occurs at 56-70 days (8-10 weeks) (Nishimura, 1993; Yoon et al., 2000; Kim et al., 2004), while various dates are recorded for the period of nasal plug loss, ranging from 91-119 days (Kim et al., 2016), to 154 days (Diewert and Shiota, 1990) and 168 days (Kumoi et al., 1993; Sarnat and Yu, 2016).