nature ecology & evolution

ARTICLES

https://doi.org/10.1038/s41559-018-0713-1

Dinosaur ossification centres in embryonic birds uncover developmental evolution of the skull

Daniel Smith-Paredes[1,3*], Daniel Núñez-León[1,4], Sergio Soto-Acuña[1], Jingmai O'Connor[2], João Francisco Botelho[1] and Alexander O. Vargas[1*]

Radical transformation of the skull characterizes bird evolution. An increase in the relative size of the brain and eyes was presumably related to the loss of two bones surrounding the eye, the prefrontal and postorbital. We report that ossification centres of the prefrontal and postorbital are still formed in bird embryos, which then fuse seamlessly to the developing nasal and frontal bones, respectively, becoming undetectable in the adult. The presence of a dinosaur-like ossification pattern in bird embryos is more than a trace of their evolutionary past: we show how persistent modularity of ossification centres has allowed for evolutionary re-organization of skull architecture in evolution. Our findings also demonstrate that enigmatic mesodermal cells forming the posterior region of the avian frontal correspond to the ossification centre of the postorbital, not the parietal, and link its failure to develop into an adult bone to its incorporation into the expanded braincase of birds.

The adult skull of early tetrapods was composed of numerous independent bones, several of which are absent in modern lineages[1,2]. Some of these bones have ceased to form completely; however, single bones in the adult skull may develop from more than one embryonic ossification centre, fusing without leaving any trace during development. These ossification centres may correspond to 'lost' elements that have ceased to develop as independent bones. A recent example is the discovery in several species of mammals of two previously overlooked ossification centres that fuse to form the intertemporal bone, and which once developed into the independent postparietal and tabular bones of remote ancestors[3].

The toothless skull of modern birds is notable for its radical evolutionary transformation. Since their ancestors among early theropod dinosaurs, there was a marked increase in the relative size of the brain and eye[4], which especially affected the circumorbital bones (those surrounding the eye). The frontal bone expanded towards posterior[4], and the prefrontal and postorbital bones are presumed to have been lost. Erdmann described in 1940 the presence of early circumorbital ossification centres in chicken embryos, which then fuse seamlessly to other developing bones[5]. An obvious question is whether these could correspond to the prefrontal or postorbital once present in the skull of ancient theropods. Comparisons of relative position and shape could support this hypothesis, but no photographic documentation is currently available. Additionally, their presence in other avian taxa remains uncertain. For instance, Erdmann reported that the frontal bone in the chicken has an additional posterior ossification centre[5], but developmental sequences of species from different orders have failed to detect it, including Palaeognathae[6–10], Anseriformes[11,12], Galliformes[13–16] and Charadriiformes[12,17]. Likewise, a lacrimal bone forming from two ossification centres was reported in the chicken, but, other than this species, two lacrimal ossification centres have only been detected in the emu (*Dromaius novaehollandiae*)[6]. A reasonable concern is that the stages in which these ossification centres are observable as separate elements could have been missed in many species due to insufficient stage sampling, especially in wild taxa where embryo harvesting is often limited.

To re-assess the presence of circumorbital ossification centres in avian embryos, we obtained developmental series from a broad phylogenetic sample comprising six different orders of Neognathae, as well as the Chilean tinamou *Nothoprocta perdicaria*, which belongs to the Palaeognathae, the other main lineage of modern birds. We also observed circumorbital ossification centres in embryos of *Alligator mississippiensis*, representing birds' closest living relatives and presenting the entire set of circumorbital bones once present in ancient theropods. Our analysis also includes an updated review on the evolution of circumorbital bones along the dinosaur–bird transition, including first-hand examination of key fossil specimens. The combination of embryological and fossil evidence demonstrates that the ossification centres of the prefrontal and postorbital are still present in bird embryos, but, instead of becoming independent bones, they fuse quickly to the developing nasal and frontal, respectively, clarifying homologies and uncovering unexpected evolution of the avian skull.

Results

Development of circumorbital ossification centres. Our developmental sequences of embryos uncovered unprecedented information on the early skull ossification centres that are present. As in previous studies of skull ossification in birds[6,11,13,17–19] we found no evidence for intraspecific variation in the number of ossification centres, or of developmental instability, such as the asymmetric presence of an ossification centre at only one side of the skull. Ossification sequences for all species are summarized in Supplementary Table 1.

The lacrimal bone of birds (referred to in some older works as the prefrontal[15]) is found at its typical position at the anterior margin of the orbit[20]. In almost all birds studied, we found a developmental stage where two independent ossification centres of the lacrimal are present, before fusing to form a single ossification centre, which becomes the adult lacrimal bone. The size and shape of the

[1]Laboratorio de Ontogenia y Filogenia, Departamento de Biología, Facultad de Ciencias de la Universidad de Chile, Santiago, Chile. [2]Institute of Vertebrate Paleontology and Paleoanthropology, Beijing, China. [3]Present address: Department of Geology and Geophysics, Yale University, New Haven, CT, USA. [4]Present address: Paläontologisches Institut und Museum, Universität Zürich, Zürich, Switzerland. *e-mail: dsmithparedes@yale.edu; alexvargas@uchile.cl

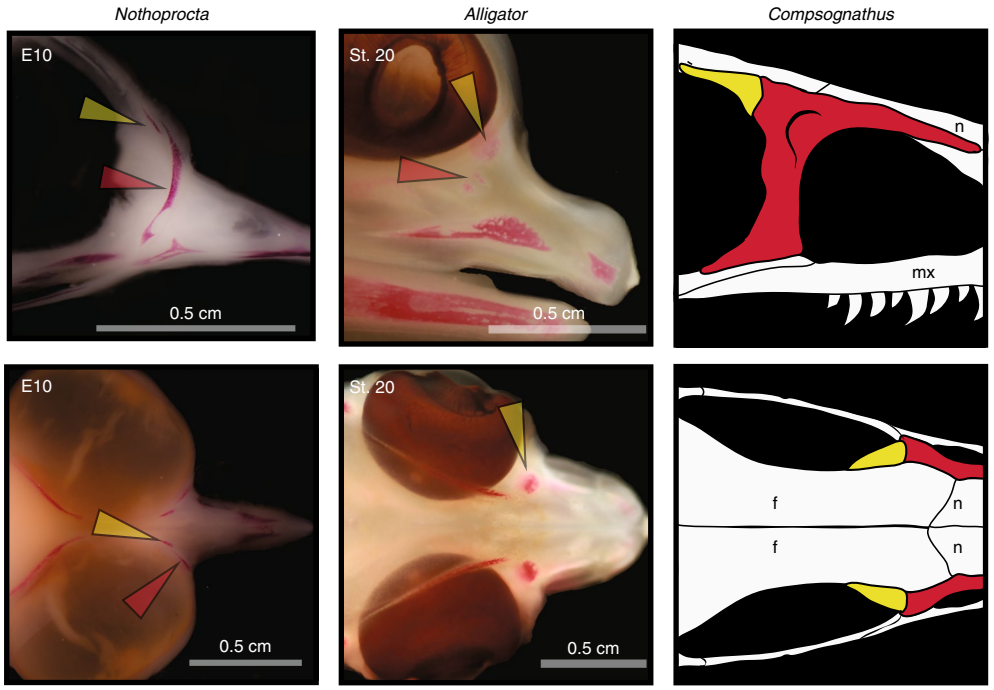

Fig. 1 | Early formation of a prefrontal ossification centre in a palaeognathous bird. In addition to the lacrimal (red arrowhead), a previously unknown ossification (yellow arrowhead) is found at the dorsal-anterior margin of the eye in the Chilean tinamou (*Nothoprocta*) embryo at the 10th day (E10) of incubation (above, lateral view; below, dorsal view). It then quickly fuses to the nasal, becoming undetectable in the adult. This ossification is in a position comparable to the embryonic ossification centre of the prefrontal of a crocodylian (*Alligator*) *at stage 20 (St. 20) of development*. An adult prefrontal in comparable position was also present in theropod dinosaurs (exemplified by the coelurosaur *Compsognathus*), which are direct ancestors of birds. n, nasal bone; mx, maxillary bone; f, frontal bone.

lacrimal ossification centres can vary considerably among bird species (Supplementary Fig. 1). Only in the pigeon, we did not recover evidence for more than a single ossification centre. However, the apparent absence of an ossification centre is always regarded with caution in studies characterizing skull development[3,6]: agenesis is uncertain because an ossification centre may have not formed yet, or may have already fused to another developing bone. Also, it remains possible that some skeletal elements do not progress beyond the pre-osteogenic condensation, before fusing to others. Importantly, we found that the lacrimal of the non-avian archosaur *A. mississippiensis* also develops from two ossification centres, as observed at stages 19–20 (Supplementary Fig. 1). Published ontogenetic sequences of alligator embryos show a single embryonic lacrimal at stage 21 (ref. [21]), indicating that they have already fused by then.

Our developmental sequences for Chilean tinamou document a previously undescribed ossification centre towards the anterior margin of the eye, dorsally positioned with respect to the lacrimal, which then fuses to the developing nasal bone (Fig.1 and Supplementary Figs. 2 and 3). Importantly, a corresponding ossification centre is found at the same position in the embryonic skulls of *A. mississippiensis*, which gives rise to the prefrontal bone (Fig. 1). In the Chilean tinamou, fusion of this prefrontal ossification centre to the nasal gives rise to a posteriorly projected process of the nasal, conferring an elongated 'V' shape to this bone (Supplementary Fig. 2). The nasal in several Palaeognathae also shows this posteriorly projected process, suggesting that this ossification centre may be present in other members of this clade.

The eye of birds is surrounded in its dorsal and postero-dorsal region by the large frontal bone. We confirmed the previous report by Erdmann[5] that in the chicken, two separate ossification centres give rise to the frontal (Supplementary Fig. 4). We also found that two ossification centres fuse to become the frontal in the domestic duck *Anas platyrhynchos*, and the Chilean lapwing *Vanellus chilensis* (Supplementary Fig. 5). In the chicken, the posterior ossification centre is considerably larger and more antero-posteriorly expanded than in duck or lapwing, a reminder that this species is often not representative of other birds. Comparison to alligator embryos reveals a corresponding ossification centre at the same position as the posterior ossification centre of birds: at the posterior margin of the eye, behind the thin and elongated embryonic frontal (Fig. 2). This ossification centre develops into the independent postorbital bone of the adult alligator[21].

Evolution of circumorbital bones in the fossil record. The fossil record provides a unique and irreplaceable source of information on the evolution of circumorbital bones in the lineage leading to birds. As shown above, developmental sequences of the Tinamou reveal the presence of an embryonic ossification centre that is comparable to that of the prefrontal in alligator embryos. As in the alligator, theropods present an independent adult prefrontal bone at the same position[20]. No other bone was present in this antero-dorsal region that may represent a potentially alternative homologue to the ossification centre found in embryos of the Chilean tinamou. The evolution of the prefrontal bone is summarized in Supplementary Fig. 6. Compared with basal Triassic forms such as *Herrerasaurus*[22] and *Coelophysis*[23] or *Tawa*[24], the adult prefrontal became reduced in Coelurosauria (Fig. 3). In forms such as Tyrannosauridae, suture lines reveal that the prefrontal could fuse to other bones: it is fused to the frontal in a specimen of *Gorgosaurus* (*Albertosaurus*) UA 10, and to the lacrimal in specimens of *Gorgosaurus libratus* (TMP 94.12.602)[25] and *Tyrannosaurus rex* (FMNH 2081)[26]. An independent prefrontal is still observable in taxa closer to birds, as reported for *Compsognathus*[27], the therizinosaur *Erlikosaurus andrewsi*[28] and the alvarezsaurs *Haplocheirus sollers*[29] and *Shuvuuia deserti*[30]. Oviraptorosauria and Paraves (which arguably form a clade, Pennaraptora[31,32]) typically do not show any evidence of an independent adult prefrontal. Thus, loss

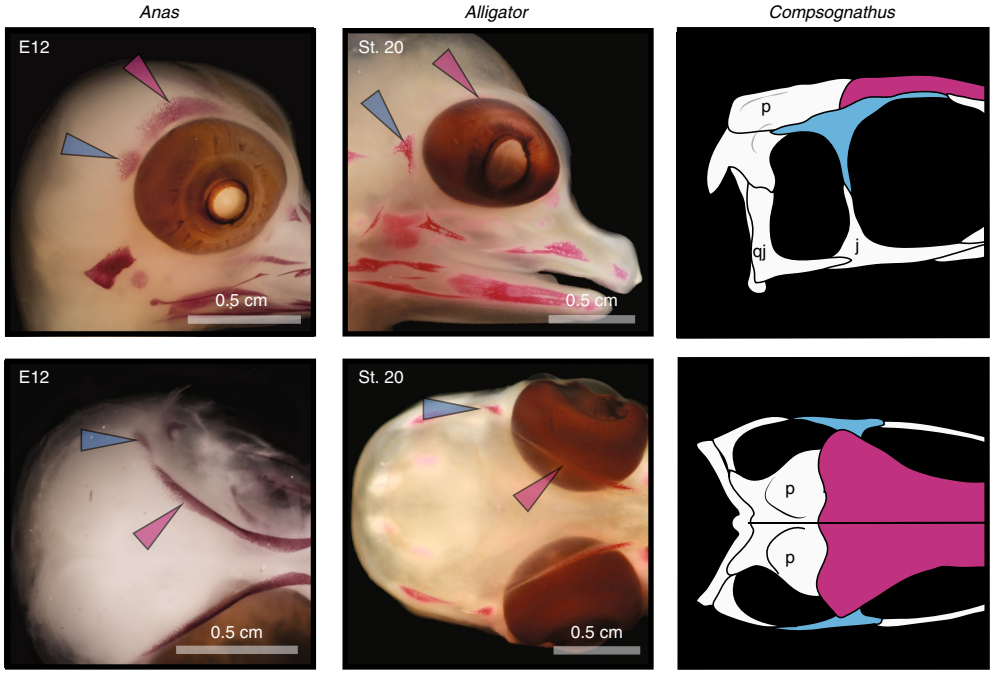

Fig. 2 | Early formation of a postorbital ossification centre in neognathous birds. At the 12th day (E12) of incubation in the domestic duck (*Anas*), two ossification centres, one anterior (magenta arrowhead) and another posterior (blue arrowhead), can be seen that give rise to the bone traditionally identified as the frontal. The posterior ossification was also detected in chicken and Chilean lapwing. In both lateral view (above) and dorsal view (below), those centres are comparable to the ossification centres that in *Alligator* give rise to frontal (magenta arrowhead) and postorbital (blue arrowhead) bones, respectively, at stage 20 (St. 20) of embryonic development. The adult postorbital of the alligator develops the same tri-radiate shape as in the theropod ancestors of birds, as exemplified by the coelurosaur *Compsognathus*. p, parietal bone; qj, quadratojugal bone; j, jugal bone.

of the adult prefrontal is often considered to have already occurred near the origin of these taxa. Their lacrimal is distinctively T-shaped, possessing a postero-dorsal process that makes it distinct from the 'inverted-L' shape of other theropods[33–40] (Fig. 3, see the T-shaped lacrimal of *Velociraptor*). This postero-dorsal process is in the position previously occupied by the prefrontal bone, which has led to the suggestion that it could actually correspond to a prefrontal that has fused seamlessly to the lacrimal during ontogeny[41,42] (hypothesis illustrated for *Velociraptor* in Fig. 3). Importantly, in at least some Pennaraptora, a separate prefrontal has been mentioned, as in specimen MOR747 of the dromaeosaurid *Deinonychus antirrhopus*[43]. Our first-hand observations of this fossil discard the possibility of a crack or artefactual separation, thus confirming the presence of an unfused prefrontal, separated from the L-shaped lacrimal by a flat, partially overlapping contact surface, as is common among cranial bones (Fig. 4a,b and Supplementary Fig. 7). Other Pennaraptora in which a separate prefrontal has also been mentioned in recent publications include the dromaeosaurid *Sinornithosaurus millenii*[31], and the oviraptorosaurs *Huanansaurus ganzhouensis*[44] and *Nemegtomaia barsboldi*[45]. In the emblematic taxon *Archaeopteryx lithographica*, the condition has been reconstructed as presenting a typically T-shaped lacrimal and no prefrontal[46–48]. However, a prefrontal has been described by some authors in the fifth (Eichstätt) specimen of *Archaeopteryx*[49,50]. Our first-hand examination of this specimen confirms the presence of a separate element in the appropriate position (Fig. 4c,d and Supplementary Fig. 8a,b). Published photographs of the 10th (Thermopolis) specimen also suggest an inverted-L-shaped lacrimal, with a possible independent prefrontal[51], and the 12th specimen has recently been described as presenting a separate prefrontal[52].

In summary, our updated review of prefrontal evolution reveals that it was not lost in the adult of all Pennaraptora; rather, several specimens show a separate prefrontal with the inverted-L lacrimal. Considering the continued presence of a prefrontal ossification centre in embryos of the Chilean tinamou (a modern Pennaraptora), the best inference is that an independent ossification centre of the prefrontal was also formed in embryos of basal Pennaraptora, fusing seamlessly to the lacrimal in most cases (where it became T shaped), but developing as an independent bone in some species or individuals. Because these specimens are nested phylogenetically among taxa with no adult prefrontal and a T-shaped lacrimal, they probably represent independent reversals to non-fusion. In the Ornithothoraces (which include the ancestors of modern birds), no adult specimen has ever been reported to present an independent prefrontal. Basal Enantiornithes often show adult specimens with a typically T-shaped lacrimal as in early Pennaraptora. However, we present new evidence that confirms that an independent prefrontal ossification centre also continued to form in these Mesozoic birds: the fossil of an enantiornithine hatchling (IVPPV15564A) shows a separate prefrontal, and a lacrimal lacking a postero-dorsal process (Fig. 4e,f and Supplementary Fig. 8c,d).

Our developmental sequences of chicken, duck and lapwing have also documented an embryonic ossification centre present in the posterior region of the frontal, which compares well with the postorbital ossification centre of alligator embryos. As in alligator, an adult postorbital with the same position and tri-radiate shape was also present in ancient theropod ancestors of birds (Figs. 2 and 3). There are no other bones in ancient theropods that could represent alternatives, such as the postparietal or the postfrontal, which are absent in dinosaurs in general and which are mostly found only in distant relatives of birds (such as squamates). The fossil evidence on the evolutionary history of the postorbital is summarized in Supplementary Fig. 9. The postorbital was present in theropods and early Avialae as a triradiate element separating the temporal

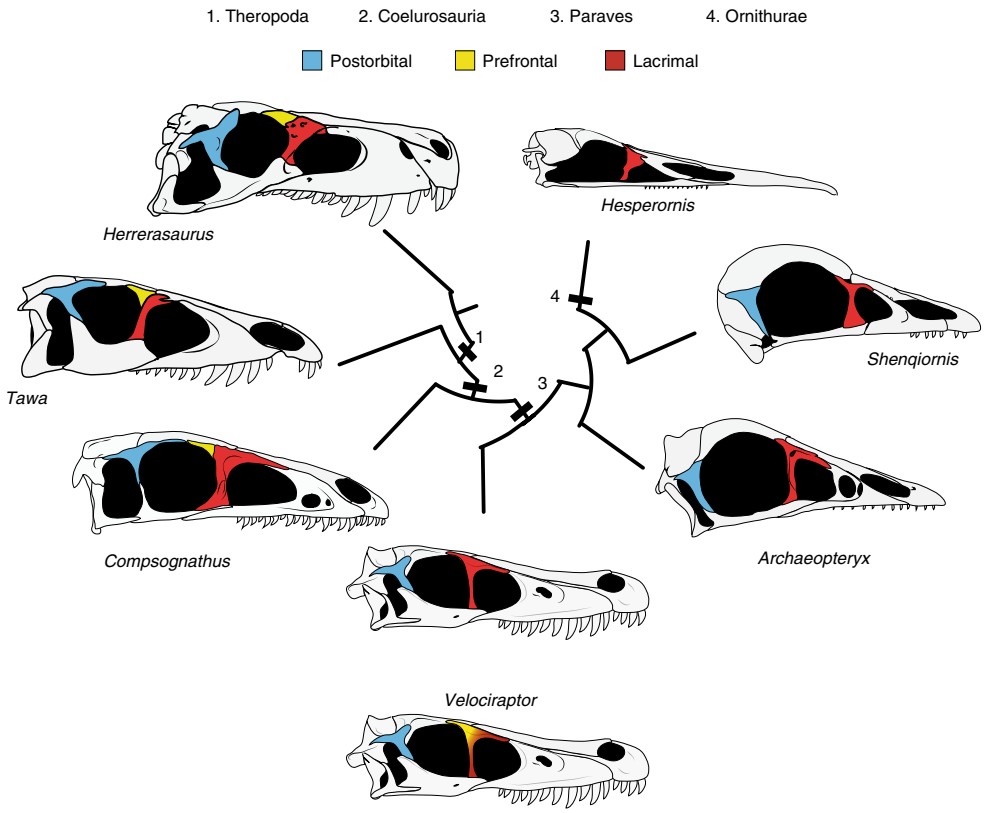

Fig. 3 | Evolution of adult circumorbital bones along the dinosaur–bird transition. In early dinosaurs such as *Herrerasaurus*, the anterior border of the orbit comprises the lacrimal and the more dorsally located prefrontal; its dorsal border is formed by the frontal and, towards posterior, the tri-radiate postorbital bone. In coelurosaurs such as *Compsognathus*, the lacrimal bone presents an inverted-L shape and is larger relative to the smaller prefrontal. Within Paraves such as *Velociraptor*, the prefrontal bone is typically absent, along with a T-shaped lacrimal, which has a posterior process in the position where the prefrontal used to be. A possible interpretation is that the T-shaped lacrimal of maniraptorans is a composite of the lacrimal and the prefrontal bone, fused into one single element, so the prefrontal bone corresponds to the posterior process of the lacrimal (*Velociraptor*, bottom image). Closer to modern birds, the postorbital bone was lost within Euornithes such as *Hesperornis*[74]. Although it was also reduced or absent in some Enantiornithes, it is a large bone in other members of this clade, such as *Shenqiornis*[56].

fenestrae and the orbit (Fig. 3). It may have been reduced in some *Archaeopteryx* specimens: it is not clearly preserved in most specimens[53], but has been commonly accepted as present, based on a groove on the postorbital process of the jugal[48]. This was recently confirmed by the new 12th specimen where the Y-shaped postorbital is present and contacts the jugal, closing the postorbital bar[52]. Other forms closer to modern birds than *Archaeopteryx*, such as the Jeholornithiformes and the basal pygostylian lineages, have preserved a robust T-shaped postorbital that fully separated the infratemporal fenestra from the orbit. In the lineage leading to modern birds, an independent postorbital was lost at some point between the origin of Euornithes and the origin of Ornithurae; this cannot be established more precisely because preservation of basal Euornithes does not allow recognition of the presence or absence of a postorbital (Supplementary Fig. 9, orange). Importantly, the loss of the postorbital may have coincided with enlargement of the frontal bone and the expansion of relative brain and eye size to approximately modern proportions, which occurred near the origin of Euornithes[54]. *Ichthyornis*, an extinct close relative of the crown group, possessed an extensive adductor chamber enclosed by bone and margined anteriorly by a posteriorly pointing postorbital process[55]. Although this projection is not separated as an independent element, the overall configuration resembles more the condition of non-avian dromaeosaurids, suggesting fusion of the postorbital to the rest of the skull roof[55]. This interpretation is strongly supported by our embryological data, which show fusion of a postorbital ossification centre to the frontal in crown birds. The postorbital may have also been lost independently in another lineage of Mesozoic birds, the Enantiornithes. Within this clade, members of the Bohaiornithidae are documented with a robust T-shaped postorbital, also considered to rostrally delimit the infratemporal fenestra. However, at least one basal enantiornithine, *Pengornis houi*, clearly has a reduced postorbital, which does not fully separate the orbit from the infratemporal fenestra, and, in at least one Late Cretaceous specimen, a free bone is no longer present[56]. The adult postorbital may have therefore also become reduced and lost in enantiornithines, independently from modern birds; alternatively, the Bohaiornithidae may represent a reversion.

Discussion

Developmental evolution of the lacrimal. Our data reveal that the lacrimal of Archosauria develops from fusion of two ossification centres, as previously reported only in the chicken[5]. These ossification centres have been previously proposed to correspond to a prefrontal and a lacrimal[5]. This interpretation may appear consistent with current fossil evidence discussed above, that the prefrontal once fused to the lacrimal in ancient Pennaraptora. However, our data discard this possibility since none of these lacrimal ossification centres are in a dorsal position comparable to a prefrontal, and the alligator lacrimal also develops from two ossification centres, while at the same time presenting an independent, dorsally positioned prefrontal. Importantly, a condition similar to the alligator was

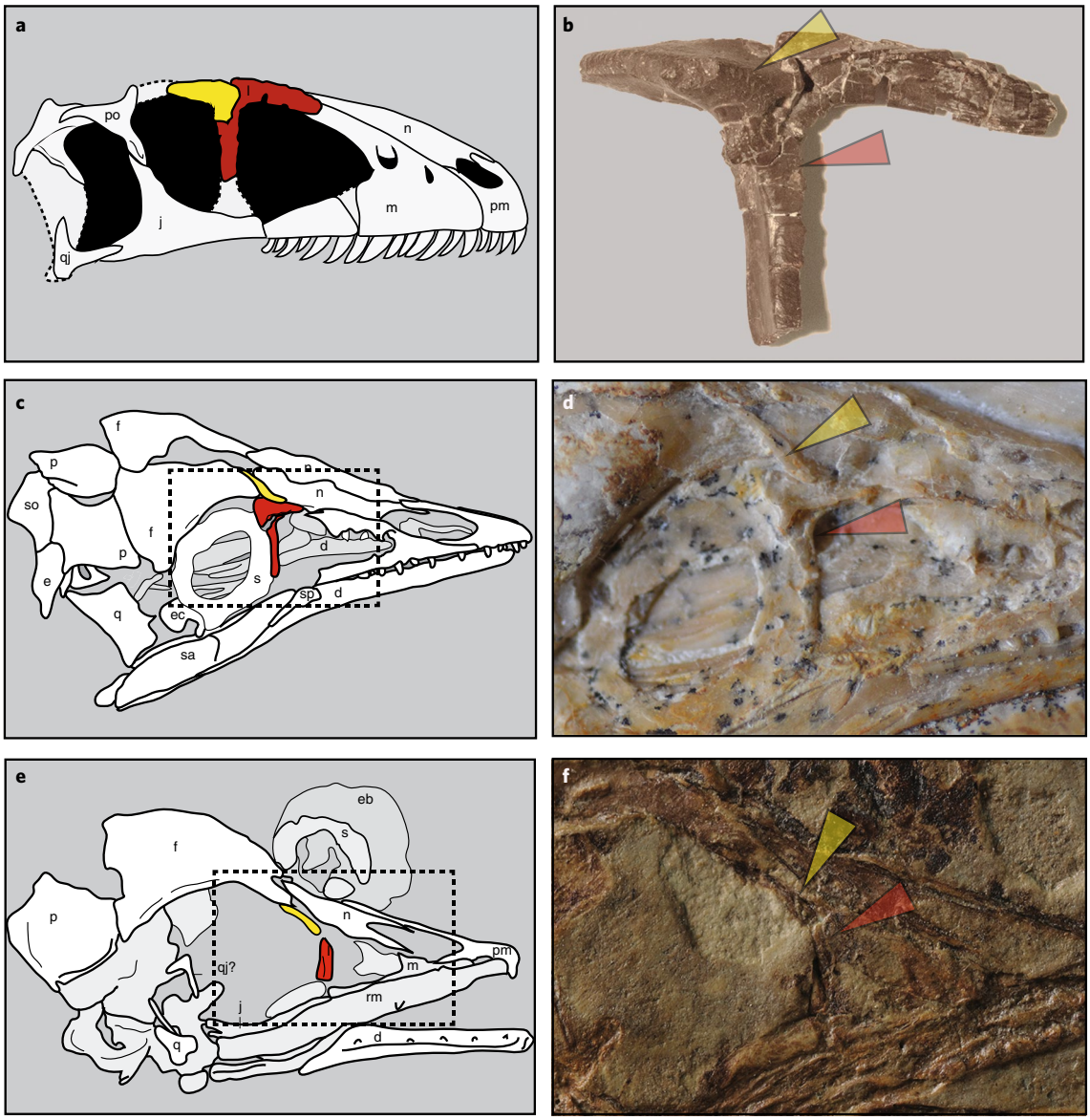

Fig. 4 | Formation of the prefrontal as a separate ossification centre in fossil Paraves. Most Paraves show a T-shaped lacrimal in absence of a prefrontal. It has been suggested that the prefrontal did form separately in Paraves, but fused completely to the lacrimal, becoming its posterior process. **a**–**f**, Consistent with this hypothesis, we found evidence for separate formation of the prefrontal (yellow arrowhead) in key Paravian taxa, such as *Deinonychus* MOR747 (**a** and **b**), the fifth *Archaeopteryx* specimen, from Eichstätt (JM2257) (**c** and **d**) and an unnamed juvenile enantiornithine specimen IVPP V1556 (**e** and **f**). These taxa are successively closer to birds but they are also more closely related to forms with T-shaped lacrimals than they are to each other. This discontinuous phylogenetic pattern suggests that a prefrontal continued to form separately in Paraves, and then fused to the lacrimal in most taxa, producing the T-shaped lacrimal. Red arrowhead, lacrimal; d, dentary; ec, ectopterygoid; l, lacrimal; m, maxilla; pm, premaxilla; po, postorbital; q, quadrate; qj, quadratojugal; rm, right maxilla; sa, surangular; so, supraoccipital; sp, splenial; f, frontal; e, exoccipital; s, sclerotic ring; eb, eye ball.

observed in the Chilean tinamou: the lacrimal develops from two ossification centres, while a separate ossification centre above the lacrimal corresponds to that giving rise to the prefrontal in alligator. The best inference is that the primitive condition for archosaurs is to present an independent, dorsally positioned prefrontal, and a lacrimal that develops from two ossification centres.

Developmental evolution of the prefrontal. In the Chilean tinamou, the fact that the prefrontal ossification centre continues to form independently is consistent with fossil evidence that it also formed independently in non-avian Pennaraptora and Mesozoic birds, against the common assumption that these taxa formed no prefrontal, and had a merely T-shaped lacrimal. Importantly, while the prefrontal fused to the lacrimal in these taxa, its ossification centre fuses to the nasal in the Chilean tinamou. This evolutionary change can only be recognized through the combined input of palaeontological and embryological data, and highlights evolutionary possibilities that are allowed by the persistence of developmental modularity in the formation of independent ossification centres. A prefrontal ossification centre that forms separately in the embryos of Archosauria allows for different developmental trajectories (Fig. 5): (1) to form an independent skull bone in the adult, as in modern crocodilians and most dinosaurs; (2) to fuse to the lacrimal, as in early pennaraptoran dinosaurs; or (3) to fuse to the nasal, as in the Chilean tinamou. Other developmental-phylogenetic outcomes are possible that are not shown in Fig. 5, such as fusion of the

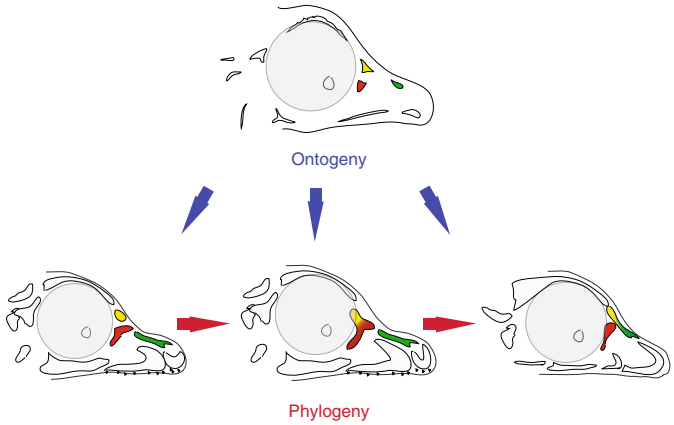

Fig. 5 | Evolutionary consequences of embryonic modularity. From a common embryonic pattern, different developmental outcomes might develop (blue arrows) during the evolutionary history of a lineage (red arrows). As exemplified here, the separate ossification centres of the prefrontal (yellow), lacrimal (red) and nasal (green) might continue to develop separately, as in basal theropods. Another alternative outcome could be the fusion of the prefrontal to the lacrimal (as in the majority of maniraptoran dinosaurs), which may then switch to fusing to the nasal instead (as in the Chilean tinamou). Credit: embryo outlines modified from ref. [21], Oxford University Press.

prefrontal to the frontal bone, as in some tyrannosaurids[26]. Therefore, an independent ossification centre of the prefrontal has allowed to reinforce different aspects of skull architecture along evolution. The formation of an independent ossification centre can also explain how some members of Pennaraptora reversed to developing an independent adult prefrontal, and also the possible re-appearance of an independent postorbital in Bohaiornithidae: as long as a separate ossification centre is formed, reversal to an independent bone remains conceivable. The persistence of ancestor-like patterning in embryonic development has been argued to allow for morphological innovation, as exemplified by experimental atavisms[57]. The evolution of the avian skull shows how persistence of ancient embryonic modules also allows for deconstruction and reassembly of composite structures, with the potential of becoming new adaptations. This is supported by other examples such as the intermedium in the embryonic ankle of neognathous birds, which has switched from fusing to the tibiale to fusing to the calcaneum[58], a modification that also occurred in chameleons, where it is considered adaptive for climbing[59]. Another case of a switch in fusion patterns may have occurred with an ancient centrale ('element m') in the ankle of tree-climbing salamanders[60].

Developmental evolution of the postorbital. Our study also confirmed that two ossification centres give rise to the frontal in the chicken, which we also documented in duck and lapwing embryos. The possible identity of the posterior ossification centre in chicken has been the subject of previous discussion: fate-mapping studies in this species show that the anterior frontal is derived from ectodermal neural crest, whereas the posterior portion is derived from mesoderm[61–63]. In mouse, the frontal is derived entirely from neural crest cells[64], whereas the parietal bone (immediately posterior to the frontal) is derived from mesoderm; this is also the case for the salamander *Ambystoma*[65]. This ectodermal–mesodermal boundary has led to the proposal that the posterior ossification centre of the chicken frontal corresponds to the parietal[66,67], including the suggestion that the adult bone found posterior to the frontal, traditionally identified as the avian parietal, is in fact another bone: the postparietal. This proposal has been formally challenged by a recent study that considers quantitative three-dimensional geometric morphometrics, muscle insertion sites and relative position of embryonic brain regions, which support the traditional identification of the parietal across reptiles, dinosaurs and birds[68]. In this regard, it is noteworthy that our own independent line of evidence supports identification of the posterior ossification as the postorbital, rather than the parietal. Our data are consistent with the morphological evidence, and do not contradict fate-mapping studies either, since the germ layer giving rise to the postorbital bone is currently unknown for any tetrapod: salamanders and mammals do not form a postorbital. By combining fate maps of birds with our own data, we predict that the adult postorbital bone of reptiles (such as the alligator) is probably of mesodermal origin.

The fact that the postorbital ossification centre has been incorporated to the avian skull roof is linked to its failure to develop into an independent bone. Importantly, the postorbital ossification centre of birds no longer carries intrinsic morphological information: on quickly fusing to the frontal, it develops as a mere continuation of this embryonic bone. Fate mapping of this region shows that the mesodermal cells that correspond to the left and right postorbital ossification centres grow medially and contact each other at the midline, becoming part of the braincase[64], an unprecedented case within Tetrapoda. Importantly, loss of the postorbital as an independent bone occurred somewhere near the origin of Ornithurae, which also coincides with the attainment of modern-like proportions of the braincase of taxa such as *Ichthyornis*. Therefore, incorporation of the postorbital into the frontal may have played a key role in accommodating a larger brain in the evolution of birds[54].

Methods

Animal collection and staging. All procedures were formally approved by the ethics committee of the Facultad de Ciencias, Universidad de Chile. None of the wild species used are listed in any conservation category of concern. Chilean tinamou (*N. perdicaria*), chicken (*Gallus gallus*) and mallard duck (*A. platyrhynchos*) eggs were purchased from local farms: Tinamou Chile (www.perdiz.cl), Chorombo S.A and Avícola Metrenco, respectively. Rock dove (*Columba livia*) and budgerigar (*Melopsittacus undulatus*) eggs were obtained from birds bred at facilities of the Facultad de Ciencias, Universidad de Chile. Eggs from wild Chilean lapwing (*V. chilensis*) and common coot (*Fulica armillata*) were collected at the beginning of the breeding season with permission from Servicio Agrícola Ganadero (Government of Chile).

All eggs were incubated at 37.5 °C and 70% humidity in an incubator with automatic rotation. Developmental stages for all species were determined according to Hamburger and Hamilton (HH) stages for chicken[69]. Regardless of species, we only considered embryos during a developmental interval comparable to stages HH34–HH37 of chicken, when ossification and other key events of skull development occur.

The number of embryos harvested per embryonic day depended on the availability of eggs; in the case of the farm-purchased Chilean tinamou, domestic duck eggs, and the laboratory-bred budgerigars, five embryos per day were collected for each species. Staging of lapwing and coot embryos before opening the eggs was more difficult, but age was estimated following the Hays and LeCroy method[70], obtaining between one and two embryos per HH-comparable stage. Nine fertile pigeon eggs were also obtained, from which embryos at six different stages were collected after candling the eggs. In the case of the more accessible chicken eggs, 5 embryos were collected every 2 h during embryonic days 9 and 10, and 10 embryos were collected each day thereafter until embryonic day 14.

Obtaining sequences of rapid skull development in birds. In all taxa, we aimed to obtain closely spaced stages to document sequences of skull ossification as continuously as possible. Pre-osteogenic condensations can be informative about skull development and evolution, but molecular markers have only allowed their visualization on histological sections[71,72] and no reports of their whole-mount visualization have yet been published. We therefore focused only on ossification centres, since virtually all comparative data (relative position, number and shape) are only available from this stage onwards. For species whose fertilized eggs were easily available (duck, chicken and tinamou), our initial strategy was to harvest eggs at daily intervals. Classic staging criteria, such as incubation time and the progress of external traits proposed by Hamburger and Hamilton[69], were effective for comparing general embryonic development and age, but ineffective for a more detailed study of skull ossification sequences: radical differences in skull development would be observed in embryos that would all correspond to stage HH36 (in chicken, within a single incubation day) according to classic criteria

of external appearance. Progress, but not ossification sequence, varied greatly among individuals with identical time and conditions of incubation, presumably because of slight differences in other variables that affect the speed of development, such as egg size, genetic variation or time from fertilization until collecting and cooling of the newly fertilized egg at the farm. However, individual specimens were easily ordered into developmental series on the basis of intermediate traits, bridging morphological gaps between separated stages. These traits include first appearance of ossification centres, their shape and relative size. This approach also proved effective to order embryos from species in which time of incubation was only estimated (those collected from the wild, or incubated by the mother for an unknown period, before being transferred to the incubator). During the period of fast skull development, extensive harvesting remains the best strategy to randomly obtain embryos that document the presence of ossification centres that then quickly fuse to other elements. Especially, intense harvesting was required for the chicken, in which skull development progresses faster than in duck and tinamou.

Skeletal staining. Most studies have used double skeletal staining of alizarin red for bone and alcian blue for cartilage. However, alcian blue solution is acidic and can decalcify skeletal elements[73], such that early ossification centres may be dissolved completely. Double staining is further uninformative, since circumorbital bones develop directly, with no cartilaginous precursor. We therefore only used alizarin red staining for this study. Eggs were cooled with ice before harvesting; embryos were fixed in 100% methanol for 3 days and postfixed in 10% buffered formalin for 20 min before staining with 0.03% alizarin red (Sigma-Aldrich) in 0.5% KOH for 1 h at room temperature. Embryos were washed in distilled water and then cleared in a sequence of 2% KOH, 20% glycerol, 50% glycerol and 85% glycerol. Thirty-three *Nothoprocta*, 98 *Gallus*, 32 *Anas*, 9 *Columba*, 17 *Vanellus*, 14 *Fulica* and 25 *Melopsittacus* embryos with morphologies allowing for equating them to chicken HH stages 32–39 were stained in this way (results summarized in Supplementary Table 1). Five *A. mississippiensis* embryos stained in this same way were kindly provided to us for observation by Bhart-Anjan Bhullar at Yale University.

Museum specimens. The following specimens were analysed from first-hand observations: *T. rex* (FMNH 2081) at the Field Museum of Natural History in Chicago; *D. antirrhopus* (MOR747) partial skull at the Museum of the Rockies, Bozeman; Enantiornithine juvenile specimen (IVPPV15564A) at the Institute of Vertebrate Paleontology and Paleoanthropology, Beijing, China; and *A. lithographica* (JM2257) at the Jura Museum in Eichstätt, Germany.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

High-resolution photographs of the specimens are available in the figures and also can be provided by request.

Received: 26 June 2018; Accepted: 5 October 2018;
Published online: 19 November 2018

References

1. Sidor, C. A. Simplification as a trend in synapsid cranial evolution. *Evolution* **55**, 1419–1442 (2001).
2. Gregory, W. K. 'Williston's law'relating to the evolution of skull bones in the vertebrates. *Am. J. Phys. Anthropol.* **20**, 123–152 (1935).
3. Koyabu, D., Maier, W. & Sanchez-Villagra, M. R. Paleontological and developmental evidence resolve the homology and dual embryonic origin of a mammalian skull bone, the interparietal. *Proc. Natl Acad. Sci. USA* **109**, 14075–14080 (2012).
4. Bhullar, B.-A. S. et al. How to make a bird skull: major transitions in the evolution of the avian cranium, paedomorphosis, and the beak as a surrogate hand. *Integr. Comp. Biol.* **56**, 389–403 (2016).
5. Erdmann, K. Zur entwicklungsgeschichte der knochen im schädel des huhnes bis zum zeitpunkt des ausschlüpfens aus dem ei. *Zoomorphology* **36**, 315–400 (1940).
6. Maxwell, E. E. & Larsson, H. C. E. Comparative ossification sequence and skeletal development of the postcranium of palaeognathous birds (Aves: Palaeognathae). *Zool. J. Linn. Soc.* **157**, 169–196 (2009).
7. Webb, M. The ontogeny of the cranial bones, cranial peripheral and cranial parasympathetic nerves, together with a study of the visceral muscles of struthio. *Acta Zool.* **38**, 81–203 (1957).
8. Parker, T. J. Observations on the anatomy and development of Apteryx. *Phil. Trans. R. Soc. B* **182**, 25–134 (1889).
9. Parker, T. J. Additional observations on the development of *Apteryx*. *Phil. Trans. R. Soc. B* **183**, 73–84 (1892).
10. Parker, W. K. On the structure and development of the skull in the ostrich tribe. *Phil. Trans. R. Soc. B* **156**, 113–183 (1866).
11. Maxwell, E. E. Ossification sequence of the avian order Anseriformes, with comparison to other precocial birds. *J. Morphol.* **269**, 1095–1113 (2008).
12. Starck, J. M. in *Current Ornithology* Vol. 10 (ed. Power, M.) Ch. 6 (Springer, Boston, 1993).
13. Maxwell, E. E. Comparative embryonic development of the skeleton of the domestic turkey (*Meleagris gallopavo*) and other galliform birds. *Zoology* **111**, 242–257 (2008).
14. Parker, W. K. On the structure and development of the skull of the common fowl (*Gallus domesticus*). *Phil. Trans. R. Soc. B* **159**, 755–807 (1869).
15. Jollie, M. T. The head skeleton of the chicken and remarks on the anatomy of this region in other birds. *J. Morphol.* **100**, 389–436 (1957).
16. Nakane, Y. & Tsudzuki, M. Development of the skeleton in Japanese quail embryos. *Dev. Growth Differ.* **41**, 523–534 (1999).
17. Maxwell, E. E. & Harrison, L. B. Ossification sequence of the common tern (*Sterna hirundo*) and its implications for the interrelationships of the Lari (Aves, Charadriiformes). *J. Morphol.* **269**, 1056–1072 (2008).
18. Maxwell, E. E., Harrison, L. B. & Larsson, H. C. Assessing the phylogenetic utility of sequence heterochrony: evolution of avian ossification sequences as a case study. *Zoology* **113**, 57–66 (2010).
19. Maxwell, E. E. *Evolution and Avian Ossification Sequences*. PhD thesis, McGill Univ. (2008).
20. Baumel, J. & Witmer, L. in *Handbook of Avian Anatomy: Nomina Anatomica Avium* (ed. Baumel, J.) 45–132 (Publications of the Nuttall Ornithological Club, Cambridge, 1993).
21. Rieppel, O. Studies on skeleton formation in reptiles. v. Patterns of ossification in the skeleton of *Alligator mississippiensis* Daudin (Reptilia, Crocodylia). *Zool. J. Linn. Soc.* **109**, 301–325 (1993).
22. Sereno, P. C. & Novas, F. E. The skull and neck of the basal theropod *Herrerasaurus ischigualastensis*. *J. Vertebr. Paleontol.* **13**, 451–476 (1994).
23. Colbert, E. H. *The Triassic Dinosaur Coelophysis* (Museum of Northern Arizona Press, Northern Arizona Society of Science and Art, Flagstaff, 1989).
24. Nesbitt, S. J. et al. A complete skeleton of a Late Triassic saurischian and the early evolution of dinosaurs. *Science* **326**, 1530–1533 (2009).
25. Currie, P. J. Cranial anatomy of tyrannosaurid dinosaurs from the Late Cretaceous of Alberta, Canada. *Acta Palaeontol. Pol.* **48**, 191–226 (2003).
26. Brochu, C. A. Osteology of *Tyrannosaurus rex*: insights from a nearly complete skeleton and high-resolution computed tomographic analysis of the skull. *J. Vertebr. Paleontol.* **22**, 1–138 (2003).
27. Peyer, K. A reconsideration of *Compsognathus* from the Upper Tithonian of Canjuers, southeastern France. *J. Vertebr. Paleontol.* **26**, 879–896 (2006).
28. Lautenschlager, S., Witmer, L. M., Altangerel, P., Zanno, L. E. & Rayfield, E. J. Cranial anatomy of *Erlikosaurus andrewsi* (Dinosauria, Therizinosauria): new insights based on digital reconstruction. *J. Vertebr. Paleontol.* **34**, 1263–1291 (2014).
29. Choiniere, J. N., Clark, J. M., Norell, M. A. & Xu, X. Cranial osteology of *Haplocheirus sollers* Choiniere et al., 2010 (Theropoda: Alvarezsauroidea). *Am. Mus. Novit.* **51**, 1–44 (2014).
30. Chiappe, L. M., Norell, M. A. & Clark, J. M. The skull of a relative of the stem-group bird *Mononykus*. *Nature* **392**, 275–278 (1998).
31. Turner, A. H., Makovicky, P. J. & Norell, M. A. A review of dromaeosaurid systematics and paravian phylogeny. *B. Am. Mus. Nat. Hist.* **371**, 1–206 (2012).
32. Foth, C., Tischlinger, H. & Rauhut, O. W. New specimen of *Archaeopteryx* provides insights into the evolution of pennaceous feathers. *Nature* **511**, 79–82 (2014).
33. Sues, H.-D. The skull of *Velociraptor mongoliensis*, a small Cretaceous theropod dinosaur from Mongolia. *Paläont. Z.* **51**, 173–184 (1977).
34. Gao, C., Morschhauser, E. M., Varricchio, D. J., Liu, J. & Zhao, B. A second soundly sleeping dragon: new anatomical details of the Chinese troodontid Mei long with implications for phylogeny and taphonomy. *PLoS ONE* **7**, e45203 (2012).
35. Makovicky, P. J., Norell, M. A., Clark, J. M. & Rowe, T. Osteology and relationships of *Byronosaurus jaffei* (Theropoda: Troodontidae). *Am. Mus. Novit.* **3402**, 1–32 (2003).
36. Bever, G. S. & Norell, M. A. The perinate skull of *Byronosaurus* (Troodontidae) with observations on the cranial ontogeny of paravian theropods. *Am. Mus. Novit.* **3657**, 1–52 (2009).
37. Xu, X., Wang, X.-L. & Wu, X.-C. A dromaeosaurid dinosaur with a filamentous integument from the Yixian Formation of China. *Nature* **401**, 262–266 (1999).
38. Burnham, D. A. in *Feathered Dragons: Studies on the Transition from Dinosaurs to Birds* (ed. Currie, P. J.) 67–111 (Indiana Univ. Press, Bloomington, 2004).
39. Ostrom, J. H. *Osteology of Deinonychus antirrhopus, an Unusual Theropod from the Lower Cretaceous of Montana* Vol. 30 (Peabody Museum of Natural History, Yale Univ. Press, New Haven, 1969).
40. Norell, M. A. et al. A new dromaeosaurid theropod from Ukhaa Tolgod (Ömnögov, Mongolia). *Am. Mus. Novit.* **3545**, 1–51 (2006).

41. Barsbold, R. & Osmólska, H. The skull of *Velociraptor* (Theropoda) from the Late Cretaceous of Mongolia. *Acta Palaeontol. Pol.* **44**, 189–219 (1999).
42. Currie, P. J. & Zhiming, D. New information on Cretaceous troodontids (Dinosauria, Theropoda) from the People's Republic of China. *Can. J. Earth Sci.* **38**, 1753–1766 (2001).
43. Maxwell, W. & Witmer, L. New material of *Deinonychus* (Dinosauria; Theropoda). *J. Vertebr. Paleontol.* **16**, 51A (1996).
44. Lü, J. et al. A new oviraptorid dinosaur (Dinosauria: Oviraptorosauria) from the Late Cretaceous of Southern China and its paleobiogeographical implications. *Sci. Rep.* **5**, 11490 (2015).
45. Lü, J., Tomida, Y., Azuma, Y., Dong, Z. & Lee, Y.-N. New oviraptorid dinosaur (Dinosauria: Oviraptorosauria) from the Nemegt Formation of southwestern Mongolia. *Bull. Nat. Sci. Mus. Tokyo Ser. C* **30**, 95–130 (2004).
46. Elzanowski, A. A novel reconstruction of the skull of *Archaeopteryx*. *Neth. J. Zool.* **51**, 207–215 (2001).
47. Rauhut, O. W. New observations on the skull of *Archaeopteryx*. *Paläont. Z.* **88**, 211–221 (2014).
48. Elzanowski, A. & Wellnhofer, P. Cranial morphology of *Archaeopteryx*: evidence from the seventh skeleton. *J. Vertebr. Paleontol.* **16**, 81–94 (1996).
49. Tischlinger, H. Neue informationen zum berliner exemplar von *Archaeopteryx lithographica* H. v. Meyer 1861. *Archaeopteryx* **23**, 33–50 (2005).
50. Wellnhofer, P. Das fünfte skelettexemplar von *Archaeopteryx*. *Palaeontogr. Abt. A* **A147**, 168–216 (1974).
51. Mayr, G., Pohl, B. & Peters, D. S. A well-preserved *Archaeopteryx* specimen with theropod features. *Science* **310**, 1483–1486 (2005).
52. Rauhut, O. W., Foth, C. & Tischlinger, H. The oldest *Archaeopteryx* (Theropoda: Avialiae): a new specimen from the Kimmeridgian/Tithonian boundary of Schamhaupten, Bavaria. *PeerJ* **6**, e4191 (2018).
53. Wang, M. & Hu, H. A comparative morphological study of the jugal and quadratojugal in early birds and their dinosaurian relatives. *Anat. Rec.* **300**, 62–75 (2017).
54. Bhullar, B. A. et al. Birds have paedomorphic dinosaur skulls. *Nature* **487**, 223–226 (2012).
55. Field, D. J. et al. Complete *Ichthyornis* skull illuminates mosaic assembly of the avian head. *Nature* **557**, 96–100 (2018).
56. O'Connor, J. K. & Chiappe, L. M. A revision of enantiornithine (Aves: Ornithothoraces) skull morphology. *J. Syst. Palaeontol.* **9**, 135–157 (2011).
57. Gould, S. J. *Hen's Teeth and Horse's Toes: Further Reflections in Natural History* (WW Norton & Company, New York, 2010).
58. Ossa-Fuentes, L., Mpodozis, J. & Vargas, A. O. Bird embryos uncover homology and evolution of the dinosaur ankle. *Nat. Commun.* **6**, 8902 (2015).
59. Diaz, R. E. & Trainor, P. A. Hand/foot splitting and the 're-evolution' of mesopodial skeletal elements during the evolution and radiation of chameleons. *BMC Evol. Biol.* **15**, 184 (2015).
60. Wake, D. B. Homoplasy: the result of natural selection, or evidence of design limitations? *Am. Nat.* **138**, 543–567 (1991).
61. Le Lièvre, C. S. & Le Douarin, N. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *Development* **34**, 125–154 (1975).
62. Le Lievre, C. S. Participation of neural crest-derived cells in the genesis of the skull in birds. *J. Embryol. Exp. Morphol.* **47**, 17–37 (1978).
63. Noden, D. M. The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev. Biol.* **96**, 144–165 (1983).
64. Evans, D. J. & Noden, D. M. Spatial relations between avian craniofacial neural crest and paraxial mesoderm cells. *Dev. Dyn.* **235**, 1310–1325 (2006).
65. Piekarski, N., Gross, J. B. & Hanken, J. Evolutionary innovation and conservation in the embryonic derivation of the vertebrate skull. *Nat. Commun.* **5**, 5661 (2014).
66. Maddin, H. C., Piekarski, N., Sefton, E. M. & Hanken, J. Homology of the cranial vault in birds: new insights based on embryonic fate-mapping and character analysis. *R. Soc. Open Sci.* **3**, 160356 (2016).
67. Noden, D. M. & Schneider, R. A. in *Neural Crest Induction and Differentiation* (ed. Saint-Jeannet, J.-P.) 1–23 (Landes Bioscience, Georgetown and Springer, New York, 2006).
68. Fabbri, M. et al. The skull roof tracks the brain during the evolution and development of reptiles including birds. *Nat. Ecol. Evol.* **1**, 1543 (2017).
69. Hamburger, V. & Hamilton, H. L. A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92 (1951).
70. Hays, H. & LeCroy, M. Field criteria for determining incubation stage in eggs of the common tern. *Wilson Bull.* **83**, 425–429 (1971).
71. Hall, B. & Miyake, T. The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. *Anat. Embryol.* **186**, 107–124 (1992).
72. Hall, B. K. & Miyake, T. Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int. J. Dev. Biol.* **39**, 881–893 (2004).
73. Yamazaki, Y., Yuguchi, M., Kubota, S. & Isokawa, K. Whole-mount bone and cartilage staining of chick embryos with minimal decalcification. *Biotech. Histochem.* **86**, 351–358 (2011).
74. Gingerich, P. Skull of *Hesperornis* and early evolution of birds. *Nature* **243**, 70–73 (1973).

Acknowledgements

We wish to thank B.-A. Bhullar for kindly allowing us to examine and photograph embryos of *A. mississippiensis*. Special thanks go to M. Sallaberry and J. Mpodozis at Universidad de Chile. This work was funded by grants Anillo ACT172099 and Fondecyt 1150906 (Conicyt, Government of Chile) to A.O.V. This work is dedicated to the memory of Professor Juan Fernández Hidalgo.

Author contributions

D.S.-P. and A.O.V. conceived and planned the research. D.S.-P. and D.N.-L. collected, cleared and stained embryos and analysed embryological data. D.S.-P., S.S.-A. and J.O. analysed fossil specimens and palaeontological data. D.S.-P., D.N.-L., S.S.-A., J.O., J.F.B. and A.O.V contributed to the writing of the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41559-018-0713-1.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to D.S. or A.O.V.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

nature research

Corresponding author(s): Daniel Smith-Paredes, Alexander O. Vargas

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐	An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☒	☐	A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
☒	☐	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	NA
Data analysis	NA

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Provide your data availability statement here.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	We studied ontogenetic sequences in embryos of different species of archosaurs, including birds and alligator, focusing on the ossification sequence of the skull through the use of Alizarin staining, comparing our findings with the fossil record by reviewing the literature and direct osbervation of key fossil specimens..
Research sample	Eggs of different bird species were collected, either from the wild (Fulica armillata, Vanellus chilensis), from commercial farms (Gallus gallus, Anas platyrhynchos, Nothoprocta perdicaria, Alligator mississipiensis) or animals bred in our facilities (Melopsittacus undulatus). The number of embryos obtained varied and depended on the number of fertile eggs obtained.
Sampling strategy	We tried, when possible to obtain a series of embryos as closely spaced as we were able to collect.
Data collection	Embryos collected at the appropriate stages were stained and cleared with Alizarin red and ossification pattern was observed. Specimens reflecting important stages of ossification were photographed, documenting important events on the ossification sequence.
Timing and spatial scale	Depending on the availability of eggs, two or three embryos were collected twice a day or every couple of hours.
Data exclusions	Only embryos too young for presenting ossified elements in the skull were excluded from being stained and observed
Reproducibility	We tried to collect as many embryos as possible in close and sequential developmental stages.
Randomization	Not relevant. Of the whole sample of eggs we tried to just collect the most complete series of embryos as was possible
Blinding	No blinding was performed. All embryos were stained in the same fashion to observe the ossification pattern

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☐	☒ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials	Embryos represent the unique specimens used for the study. In order to study and photograph them sometimes embryos were subject to destructive sampling. Embryos are stored in Glicerol and could be still observed, although with time Aliarin tends to fade a little.

Palaeontology

Specimen provenance	The following specimens were directly analyzed: Deinonychus (MOR747) partial skull at the Museum of the Rockies, Bozeman;

Specimen provenance	Enantiornithe juvenile specimen (IVPPV15564A) at the Institute of Vertebrate Paleontology and Paleoanthropology, Beijing, China; Archaeopteryx (JM2257) at the Jura Museum in Eichstätt, Germany.
Specimen deposition	all specimens are deposited in the institutions mentioned above
Dating methods	NA

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Eggs from Melopsittacus undulatus were obtained from a breeding colony housed at the Universidad de Chile. Eggs were then incubated at 37,5 ºC and 70% humidity in an incubator with automatic rotation.
Wild animals	Eggs of different bird species were collected, either from the wild (Fulica armillata, Vanellus chilensis), from commercial farms (Gallus gallus, Anas platyrhynchos, Nothoprocta perdicaria, Alligator mississipiensis). Avian eggs were then incubated at 37,5 ºC and 70% humidity in an incubator with automatic rotation.
Field-collected samples	Eggs of different bird species were collected, either from the wild (Fulica armillata, Vanellus chilensis). Eggs were then incubated at 37,5 ºC and 70% humidity in an incubator with automatic rotation.