RESEARCH ARTICLE

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Developmental evolution of the distal ankle in the dinosaur–bird transition

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Abstract

The adult ankle of early reptiles had five distal tarsal (dt) bones, but in Dinosauria, these were reduced to only two: dt3 and dt4, articulated to metatarsals (mt) mt3 and mt4. Birds have a single distal tarsal ossification center that fuses to the proximal metatarsals to form a new adult skeletal structure: the composite tarsometatarsus. This ossification center develops within a single large embryonic cartilage, but it is unclear if this cartilage results from fusion of earlier cartilages. We studied embryos in species from four different bird orders, an alligatorid, and an iguanid. In all embryos, cartilages dt2, dt3, and dt4 are formed. In the alligatorid and the iguanid, dt2 failed to ossify: only dt3 and dt4 develop into adult bones. In birds, dt2, dt3, and dt4 fuse to form the large distal tarsal cartilage; the ossification center then develops above mt3, in cartilage presumably derived from dt3. During the entire dinosaur–bird transition, a dt2 embryonic cartilage was always formed, as inferred from the embryology of extant birds and crocodilians. We propose that in the evolution of the avian ankle, fusion of cartilages dt3 and dt2 allowed ossification from dt3 to progress into dt2, which began to contribute bone medially, while fusion of dt3 to dt4 enabled the evolutionary loss of the dt4 ossification center. As a result, a single ossification center expands into a plate-like unit covering the proximal ends of the metatarsals, that is key to the development of an integrated tarsometatarsus.

KEYWORDS
ankle, birds, cartilage fusion, embryo, ossification, theropoda

1 | INTRODUCTION

The morphology and development of the ankle and foot of birds are highly specialized with regard to other tetrapods. Metatarsal 1 (mt1) is proximally reduced and does not articulate to the ankle; metatarsal 5 (mt5) is a small nonfunctional vestige, and mt2, mt3, and mt4 are fused to each other and to the distal ankle (Baumel & Witmer, 1993). The distal ankle of the most recent common ancestor of all living reptiles (turtles, lizards, crocodilians, and birds) had five independent (free) distal tarsal (dt) bones, articulating to the proximal end of each metatarsal (Figure 1a; Joyce et al., 2013; Peabody, 1951;

Schaeffer, 1941). In adult birds, there are no free elements of the distal ankle, which is entirely fused to the metatarsus, forming a single adult bone, the tarsometatarsus, that is a distinctive avian evolutionary innovation (Baumel & Witmer, 1993). During the embryonic development of birds, a large transversally (mediolaterally) expanded distal tarsal cartilage covers the proximal ends of mt2, mt3, and mt4, and then fuses to them, forming a cartilaginous tarsometatarsus (Hinchliffe, 1977, 1989; Maxwell & Larsson, 2009; Parker, 1891). Upon hatching, a single ossification center is formed in the region corresponding to the fused distal tarsal cartilage (Hogg, 1980; Maxwell & Larsson, 2009; McGowan, 1985). The
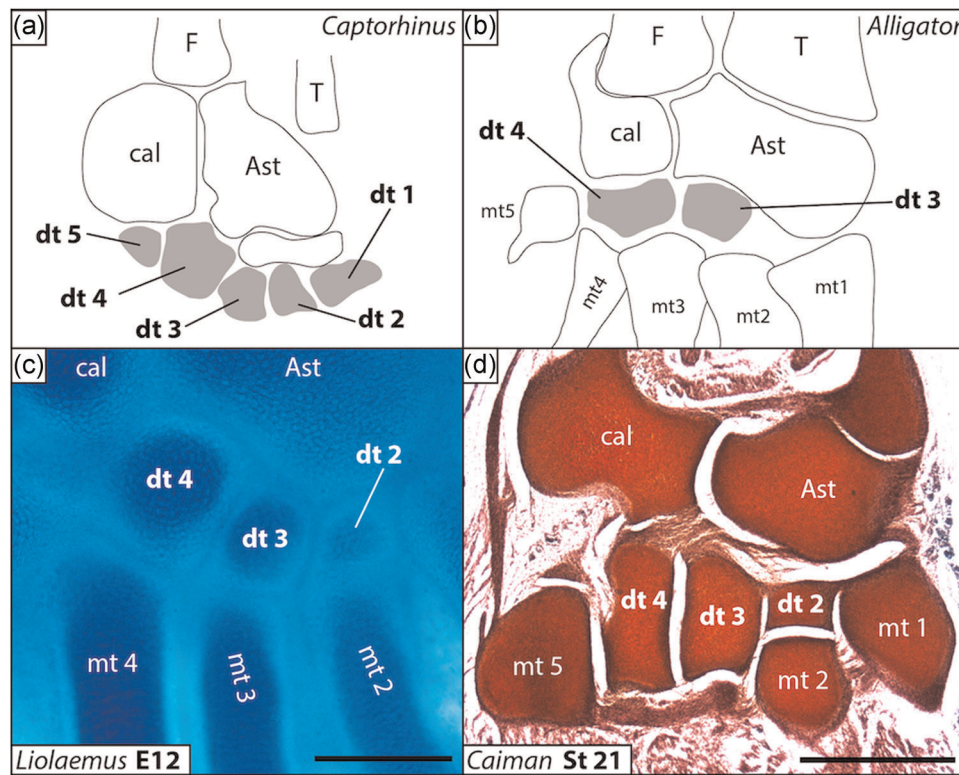
FIGURE 1 Distal tarsal elements in non-avian reptiles. (a) *Captorhinus* represents the ancestral condition present in early reptiles, with distal tarsal dt5 present as an independent bone (modified from Peabody, 1951). (b) Crocodilians like *Alligator* present only two distal tarsal bones; dt1, dt2, and dt5 are absent as independent bones (modified from Müller & Alberch, 1990). (c) and (d). Cartilage staining showing dt2, dt3, and dt4 in embryos of the lepidosaur *Liolaemus lemniscatus* and the crocodilian *Caiman yacare*. In both taxa, a dt2 cartilage is formed but fails to ossify; only dt3 and dt4 are present as ossified bones. ast, astragalus; cal, calcaneus; dt, distal tarsal; F, fibula; mt, metatarsal; T, tibia. Scale bars: c = 200 μm; d = 400 μm [Color figure can be viewed at wileyonlinelibrary.com]

ossification center of the distal ankle develops into a flat plate-like "bone" that covers the proximal ends of the now ossified metatarsals, before all elements co-ossify into the bony tarsometatarsus (Hogg, 1980; Maxwell & Larsson, 2009). Metatarsal bones also fuse to each other and undergo bone remodeling to produce a single periosteum and marrow cavity (Gutierrez et al., 2019).

It is tempting to speculate that the absence of dt1 and dt5 in birds is related to the reduction of the associated metatarsals mt1 and mt5. However, adult dt1 and dt5 bones were lost in evolution long before the reduction of their associated metatarsals. In adult crocodilians, there are no independent bones of dt1, dt2, and dt5; the only distal tarsal bones present are dt3 and dt4 (Figure 1b). Like birds, mt5 of crocodilians is reduced, but mt1 is well developed (Müller & Alberch, 1990). Birds and crocodilians are each other's closest living relatives, and are surviving representatives of the Archosauria, a vast clade of reptiles that includes a great diversity of fossil forms, including the dinosaurs (to readers unfamiliar with reptile evolution, we suggest consulting the phylogenetic tree provided in Figure S1). The earliest Archosauromorpha (the lineage leading to Archosauria) still had free dt1 and dt2 bones as adults; the loss of these bones occurred in the advanced Archosauriformes, shortly before the origin of the Archosauria (Ezcurra, 2016). The fossil record also shows that the adult dt5 was lost during the early

evolution of Archosauromorpha, when mt5 was still a well-developed bone (Joyce et al., 2013). Interestingly, the loss of an independent dt5 bone coincided with the evolution of a hook-like shape of the mt5, which suggests that the anlagen of dt5 could have fused to mt5 (Gauthier et al., 1988). This fusion actually occurs during the embryonic development of a hook-shaped mt5 ("ansulate bone") in turtles (Joyce et al., 2013), and has also been documented in the development of the hook-shaped mt5 of a lepidosaur, the bearded dragon *Pogona* (Diaz & Trainor, 2015). The earliest Archosauria still had a hook-like mt5, but mt5 became reduced shortly afterward in Avemetatarsalia (the archosaurian lineage leading to birds). In the Pseudosuchia (the archosaurian lineage leading to Crocodylia), basal forms retained a large and hook-shaped mt5, but it became reduced in Crocodyliformes, shortly before the origin of Crocodylia (Lecuona & Desojo, 2012). Therefore, although both birds and crocodilians currently have a reduced mt5, this reduction occurred independently in each lineage.

Within Avemetatarsalia, reduction of mt1 occurred towards the origin of the Neotheropod dinosaurs (Nesbitt et al., 2009; Sereno, 1999). Throughout most of the theropod-bird transition, only dt3 and dt4 were present as free bones in the distal ankle (See Supporting Information; Currie & Chen, 2001; Currie & Zhao, 1993; Dal Sasso et al., 2018; Funston et al., 2020; Osmólska et al., 1972;

Raath, 1990; Rowe, 1989). Close to the origin of birds, in the bird-like *Velociraptor*, and in a specimen of the basal Avialae *Archaeopteryx*, a large distal tarsal is present, but a midline indentation or notch has led to the suggestion that it is a composite, formed by late fusion at ossification stages of dt3 and dt4 (Norell & Makovicky, 1999; Rauhut et al., 2018). This has been recently confirmed by the evidence of late fusion of dt3 and dt4 in ontogenetic sequences of the oviraptorid *Oksoko*, in which these bones are separate as juveniles, but fused in adults (Funston et al., 2020). Thereafter, in the Pygostylia (birds that had already evolved reduced tails), a composite tarsometatarsus comparable to that of modern birds is present in adults, in which the distal tarsus has fused to the metatarsus (Chiappe et al., 1999). Closer to modern birds, in Ornithothoraces, a hatchling of the enantiornithine *Monoenantiornis* documents an ontogenetic stage with a single distal tarsal ossification center, that has not yet fused to the metatarsus (Hu & O'Connor, 2017). This suggests development like that of modern birds may have already been attained in these toothed birds of the Mesozoic. In summary, the greatest morphological differences between the distal ankle of birds and those of other archosaurs (such as crocodilians) occurred at the dinosaur–bird transition, towards the origin and early evolution of birds (for more detailed discussion of fossil taxa, see Supporting Information).

Several conceivable developmental mechanisms can explain the evolutionary reduction in the number of bones present in the adult distal ankle. Among early-acting mechanisms, cartilaginous precursors of distal tarsals may cease to form because a mesenchymal condensation fails to transition into cartilage formation, or because a mesenchymal condensation is no longer formed in the first place. A single cartilage may form at the position ancestrally occupied by two or more cartilages, by fusion of their prechondrogenic precursors, or formation of a larger mesenchymal condensation (Diaz & Trainor, 2015; Shubin & Alberch, 1986; Wake, 1991). Other mechanisms may act later, after cartilages have already formed (Cooper et al., 2014). The fusion of embryonic cartilages may result in a reduction in the number of distal tarsal elements, or an embryonic cartilage may fail to ossify. An embryonic cartilage may even "disappear" involving matrix degeneration, with cells trans-differentiating into another kind of connective tissue (Müller & Alberch, 1990), or becoming pycnotic and undergoing apoptosis (Hinchliffe & Hecht, 1984). The single ossification center in the distal tarsus of birds is known to develop from a large transversely expanded embryonic cartilage that covers the proximal ends of metatarsals mt2, mt3, and mt4 (Hinchliffe, 1977, 1989; Maxwell & Larsson, 2009; Mcgowan 1985; Parker, 1891). However, the earliest development of this cartilage is unclear. Previous authors had described it as the result of fusion of several earlier cartilages, with diverging accounts on the number of distal tarsal cartilages initially present (summarized in Romanoff, 1960). Zehntner reported four early distal tarsal elements in the swift (Zehntner, 1890, not figured), whereas Sieglbauer figured three

distal tarsals (dt2, dt3, and dt4) in the penguin *Pygoscelis*, and two distal tarsal cartilages in the mallard duck, suggesting the larger one was a composite dt3 + dt4 (Sieglbaur, 1911); Lutz in turn figured as many as five distal tarsalia in the mallard duck, and also five in the emu (Lutz, 1942). Broom reported three distal tarsalia in the ostrich (Broom, 1906, not figured) but Parker described and figured a single large, transversely elongated distal tarsal in the Kiwi (Parker, 1891). Unfortunately, these classic studies only used drawings; an analysis using photographs was made available by Hinchliffe, who documented a single large distal tarsal cartilage in the chicken, labeling it once as dt2 + dt3 (Hinchliffe, 1977) but maintaining that single cartilage is ever formed (Hinchliffe, 1989). A single large and transversely expanded distal tarsal cartilage has also been photographically documented in the quail (McGowan, 1985). The number of cartilaginous elements formed, and whether they fuse or not, is key to identify the mechanisms involved in the developmental evolution of the avian distal ankle. For instance, the evolution of large cartilage could have involved mechanisms that increased the growth of a single distal tarsal, with no fusion of earlier cartilages. An example of this is provided by the enlargement of dt4 in the chameleon's modified ball and socket zeugopodial-mesopodial articulation, which occurs without fusion of any neighboring elements, but rather, by greater growth of this element (Diaz & Trainor, 2015).

Here, we used different techniques to visualize chondrogenic patterns in the embryonic distal tarsus in a sample of diapsids, with a focus on birds. This includes the Galloanserae *Coturnix japonica* (Galliformes) and *Anas platyrhynchos* (Anseriformes), and the Neoaves *Vanellus chilensis* (Charadriiformes) and *Melopsittacus undulatus* (Psittaciformes). Non-avian reptiles are represented by the iguanid lizard *Lioalemus lemniscatus* (Lepidosauria, Squamata) and the alligatorid *Caiman yacare* (Crocodylia). The latter is especially relevant since non-avian dinosaurs also had only two distal tarsal bones (dt3 and dt4), and this condition has been conserved in *Caiman yacare* since their most recent common ancestor. Our study includes the first observations of avian distal tarsals through whole-mount immunofluorescence and confocal microscopy, using the quail *Coturnix japonica*. This allows optic sectioning to avoid the effects of element superposition, while at the same time fully appreciating the 3-D structure and relative position of other embryonic skeletal elements. While cartilage formation is sufficient to establish the presence of an embryonic skeletal element, lack of cartilage formation does not discard that a skeletal element may still be present as a mesenchymal condensation that fails to chondrify, or that fuses to another condensation before chondrification. The detection of mesenchymal condensations is an important following step to address matters left unsolved from observing chondrogenesis. However, as we will show below, our new data on early patterns of cartilage formation is sufficient to make significant inferences about the developmental evolution of the distal ankle of birds.

2 | METHODS

2.1 | Animal embryos

All procedures were formally approved by the Comité de Etica de la Facultad de Ciencias, Universidad de Chile, which certifies compliance with all aspects required for government funding (http://www.conicyt.cl/fondecyt/2012/10/31/bioetica/). None of the wild species used is in a conservation category of concern (http://www.iucnredlist.org). Eggs from *Liolaemus lemniscatus* (wreath lizard) were laid by gravid females captured with field permits of the Servicio Agrícola y Ganadero (SAG) and were incubated following published procedures for *Liolaemus tenuis* (Lemus et al., 1981). *Caiman yacare* embryos (Yacare caiman, Alligatoridae) belong to Paula Bona (Museo de La Plata). Fertilized eggs of *Gallus gallus* (Chicken, Galliformes), *Anas platyrhynchos* (Mallard duck, Anseriformes) were purchased from local farms: Chorombo S.A, Avícola Metrenco. Fertilized eggs of *Melopsittacus undulatus* (Budgerigar, Psittaciformes) were obtained from birds kept at facilities of the Faculty of Science, University of Chile. Fertilized eggs of *Vanellus chilensis* (Chilean lapwing, Charadriiformes) were collected with permission from SAG (MS).

2.2 | Cartilage and bone staining

Embryos were fixed in 100% methanol for 2–3 days at room temperature (RT). Methanol was replaced by 5:1 ethanol/acetic acid solution with 0.03% 8G alcian blue for 2 days at RT in an orbital shaker. Late embryos for bone staining were submerged in 0.03% alizarin red: 0.5% KOH solution for 1–4 h at RT and washed in distilled water. Then, embryos were cleared in a sequence of 1:3, 1:1, and 3:1 glycerol/water, and photographed in a stereoscopic microscope.

2.3 | Whole-mount immunofluorescence

Coturnix japonica (Quail, Galliformes) embryos were immuno-stained following the methods of Botelho et al. (2014). Embryos were fixed in Dent's Fix (4:1 methanol/DMSO) for 2 h at RT, dehydrated in 100% methanol, and left at −20°C overnight. Before immunostaining, they were bleached in Dent's bleaching (4:1:1 methanol/DMSO/H_2O_2) for 24 h at RT. For anti-collagen immunostaining, embryos were fixed and bleached as above. Then, hindlimbs were dissected and digested with 2 mg/ml of hyaluronidase (Sigma) in PBS for 2 h at 37°C. Embryos were rehydrated in PBS 1% triton (PBST) and incubated in primary antibodies for 2 days at 4°C in an orbital shaker. Primary antibodies were diluted in 2% horse serum, 5% DMSO in PBST at the following concentrations: 1:40 collagen Type-II (II-II6B3, DSHB); and 1:40 collagen Type-IX (2C2-II, DSHB) and washed in PBST (3 × 10 min and 3 × 1 h in an orbital shaker). Secondary antibodies anti-mouse (Alexa-488 Jackson ImmunoResearch) diluted in 5% goat serum, 5% DMSO in PBST, and incubated for 24 h at 4°C in. After

that, they were washed, cleared with Urea 4 M, and photographed in a fluorescent stereoscopic microscope (Nikon). For 3D reconstructions, 10 μm stacks were obtained in a spinning disk confocal microscope (Olympus) and projected in cellSens software (analysis for Z stacks obtained for 3D reconstruction, 2D deconvolution/Nearest Neighbor analysis for removal of background fluorescence).

3 | RESULTS

3.1 | Cartilage formation in the distal tarsal region of non-avian reptiles

In embryos of *Liolaemus lemniscatus* (whole-mount alcian blue at 12 days of incubation, Figure 1c) and *Caiman yacare* (histological sections at stage 21, Figure 1d), we observed well-developed embryonic cartilages of dt2, dt3, and dt4. The presence of a dt2 cartilage is relevant because in both of these species, dt2 is not present as an adult bone. A dt2 cartilage in *Caiman yacare* was also photographically documented and labeled in Fernández Blanco et al. (2020), and previously also in other crocodilians, *Alligator mississippiensis* and *Melanosuchus niger*, where it is reported to become a fibrous tendon (Müller & Alberch, 1990; Vieira et al., 2016). However, in other species of *Liolaemus*, an embryonic dt2 cartilage is not observable in published photographs, or mentioned in any descriptions (Fabrezi et al., 2007), which suggests variable chondrification of this element within this genus. Only dt3 and dt4 are present as adult bones in *Liolaemus*, and no variation has been described for the number of adult distal tarsals within this genus (Lobo & Abdala, 2001). In both *Liolaemus lemniscatus* and *Caiman yacare*, we did not detect any cartilage formation for dt1 and dt5, although these elements may still be formed as mesenchymal condensations.

3.2 | Cartilage and bone formation in avian embryos and juveniles

In embryos at stage HH30 of *Gallus gallus* (chicken) and *Anas platyrhynchos* (mallard duck), conventional alcian blue whole-mounts were sufficient to detect the formation of three distinct domains of cartilage formation in the distal ankle, otherwise connected by only tenuous staining. Each domain was spatially associated with the proximal ends of mt2, mt3, and mt4, respectively (Figure 2a, b). We therefore identify each of these domains as cartilages of distal tarsals dt2, dt3, and dt4. In *Melopsittacus undulatus* (budgerigar) and *Vanellus chilensis* (Chilean lapwing), distinct cartilage domains corresponding to dt2, dt3, and dt4 are observable at a later stage HH35 (Figure 2c,d). We observed the expression of collagen Type II (Col2) which marks earliest cartilage formation (Eames et al., 2003; Miller & Matukas, 1969; Zhang et al., 2009) in embryos of *Coturnix japonica* at stage HH29, as well as collagen Type IX (Col9), which is indicative of early cartilage differentiation (Eames et al., 2003; Ninomiya et al., 1984; Zhang et al., 2009). Both Col2 and Col9 show three
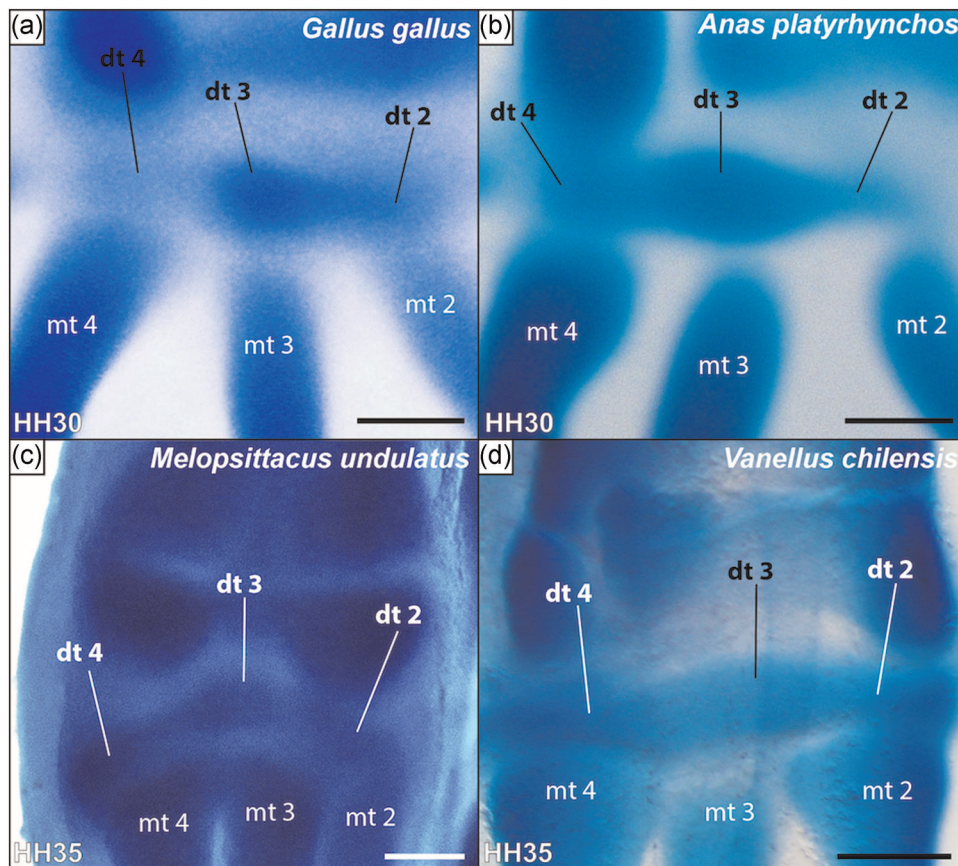
FIGURE 2 Alcian blue whole-mount staining for cartilage showing three domains of cartilage formation in the embryonic distal ankle of *Gallus gallus* (a), *Anas platyrhynchos* (b), *Melopsittacus undulatus* (c), and *Vanellus chilensis* (d). dt, distal tarsal; mt, metatarsal. Scale bars: a, b = 100 μm; c, d = 200 μm [Color figure can be viewed at wileyonlinelibrary.com]

distinct domains of expression (Figure 3, Supporting Movie) that correspond to dt2, dt3, and dt4. These thereafter fuse to form the single large and transversally expanded distal tarsal cartilage that has been previously described by several authors (Hinchliffe, 1977; Maxwell & Larsson, 2009; McGowan, 1985; Parker, 1891). In all birds here analyzed, we did not detect any cartilage formation for dt1 and dt5, although these elements may still be formed as

mesenchymal condensations that fail to enter chondrogenesis. Alizarin red staining in a hatchling specimen of *Coturnix japonica* (quail) and a juvenile of *Vanellus chilensis* (Chilean lapwing) match with the data provided by Hogg (1980) and McGowan (1984, 1985) that describe a single ossification center is formed in the distal ankle of birds. No additional ossification centers were found (Figure 4). Further studies on paleognathous birds are especially important for a
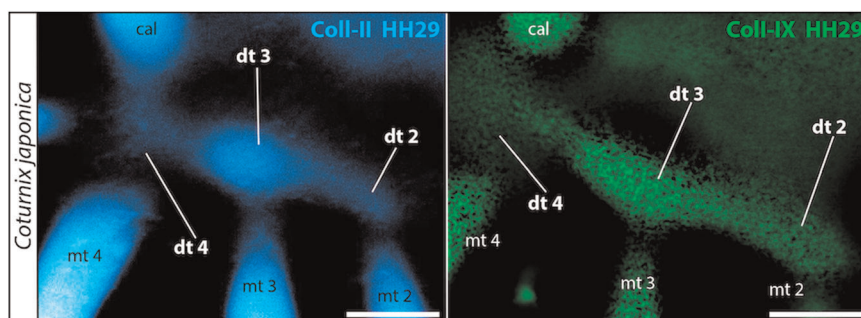

FIGURE 3 Whole-mount immunofluorescence against collagen Type-II (a) and Type-IX (b) in early embryos *Coturnix japonica* (HH29) reveals the presence of three distinct domains of cartilage formation corresponding to distal tarsals 2, 3, and 4. cal, calcaneus; dt, distal tarsal; mt, metatarsal. Scale bar = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]
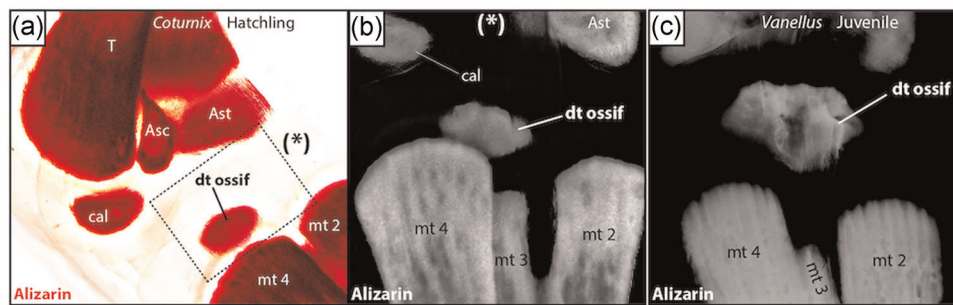
FIGURE 4 Whole-mount alizarin red staining for ossification centers and bones shows a single ossification center in the distal tarsal region of a *Coturnix japonica* hatchling (a,b) and in a *Vanellus chilensis* juvenile (c). The distal tarsal ossification center develops above mt3, as best appreciated in (b); mt3 is in a more plantar position, behind mt2 and mt4. Asc, ascending process ossification center; ast, astragalus ossification center; cal, calcaneus ossification center; dt osiff, distal tarsal ossification center; mt, metatarsal bones; T = tibia [Color figure can be viewed at wileyonlinelibrary.com]

more general assessment of tarsal development in birds. However, we emphasize that Neognathae are not nested within Paleognathae, and as sister clades, both are informative about the most recent common ancestor of the crown birds (Neornithes).

4 | DISCUSSION

In all reptiles analyzed here, including birds, a dt2 cartilage was always formed, implying that post-chondrogenic mechanisms were involved in its failure to become an independent bone. Among the lepidosaurian relatives of *Liolaemus lemniscatus*, an adult bone of dt2 is present in basal rhynchocephalians (Hughes, 1968), and arguably also in some squamates (Fabrezi et al., 2007 and references therein). As in basal Lepidosauria, an adult bone of dt2 was also present in basal Archosauromorpha (Supporting Information), which further confirms that its failure to develop into an adult bone evolved independently in Lepidosauria and Archosauria. It is worth noting that in many squamates, a dt2 is not only absent as an adult bone, but an embryonic dt2 cartilage is no longer formed (Fabrezi et al., 2007), indicating the additional involvement of earlier mechanisms that impede chondrogenesis.

The absence of embryonic cartilages dt1 and dt5 in all diapsids studied here implies that early prechondrogenic mechanisms became involved. If mesenchymal condensations are still formed, they either fail to chondrify, or alternatively, they fuse to another condensation before chondrogenesis. The latter may be the case for dt5 in *Liolaemus lemniscatus*, which may fuse to mt5 to produce the hook-like mt5 (Diaz & Trainor, 2015; Joyce et al., 2013). The possibility that a prechondrogenic dt5 may fuse to mt5 in birds or in *Caiman yacare* seems less likely, since mt5 is not hook-shaped, and is very reduced. However, this issue may deserve further exploration, given that early archosaurs did have a large, hook-shaped mt5, and even the small-sized mt5 of Crocodyliformes can be hook-shaped as in *Protosuchus*, or wide and rounded as in the modern crocodilian *Melanosuchus* (Colbert & Mook, 1951; Vieira et al., 2016).

As discussed above, the early skeletogenic pattern for dt2, dt3, and dt4 is conserved in birds. Three distinct domains of cartilage formation are formed, but then fuse to form a large transversally expanded cartilage (Hinchliffe, 1977, 1989; Maxwell & Larsson, 2009; Mcgowan 1985). Conservation of the dt2 embryonic cartilage in both birds and crocodilians implies this cartilage has likely remained present since early archosaurs, including the entire dinosaur–bird transition, despite the fact it did not ossify: only dt3 and dt4 were present as adult bones (Figure 5a–c). A small putative dt2 bone has been reported in some coelurosaurian dinosaurs (Xing et al., 2020; Supporting Information). This would be consistent with the inferred presence of a dt2 cartilage, that could ossify occasionally. However, these rare cases are isolated, with no evidence of an ossified dt2 in their closest relatives. To our knowledge, no specimen with a putative dt2 bone has ever been reported in the extensive fossil record of the bird-like Maniraptora and basal Avialae, which comprises countless articulated specimens. Therefore, there is no evidence to suggest dt2 could have ossified immediately before the evolution of the avian tarsometatarsus.

While the single large cartilage in the embryonic distal ankle of modern birds is clearly a composite of dt2 + dt3 + dt4, the same cannot be said for the single ossification center that forms within this cartilage. Because dt2 had ceased to ossify in Archosauria, this excludes its contribution to the ossification center of the avian distal ankle (Figure 5). Further, the ossification center in the transversally expanded cartilage of birds develops at a central position aligned with mt3 (Figure 4b), which suggests it develops within tissue-derived only from the dt3 cartilage. We propose that fusion among the distal tarsal cartilages allowed for bone replacement starting at the dt3 region to extend medially into dt2, and laterally into dt3 (Figure 5d). Even before fusion, direct contact between cartilages is probably enough to disrupt the feedback loop of paracrine signaling between PTHrP and Indian Hedgehog, which normally inhibits the progress of ossification near articular surfaces (Botelho et al., 2016). Upon cartilage fusion, there would be no PTHrP signaling (which normally is maintained only near articular surfaces) or structural impediment such as a gap or perichondrium that could arguably stop
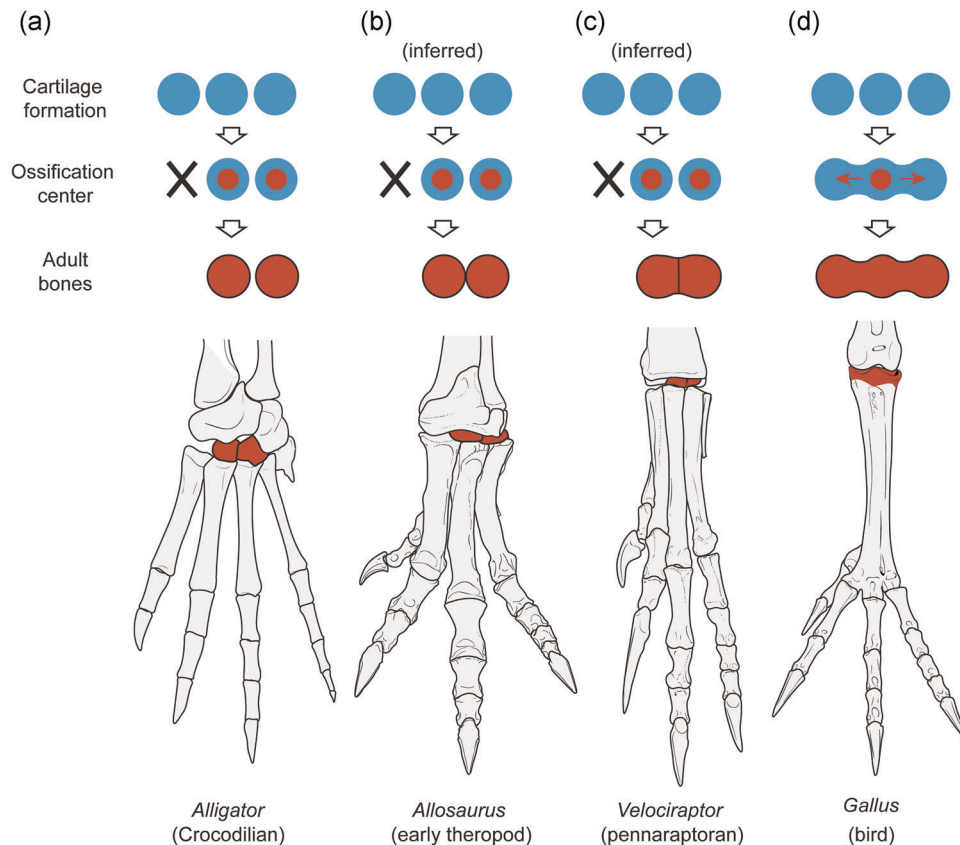
FIGURE 5 Developmental evolution of the avian distal ankle. Successive taxa to the right share a more recent common ancestor with modern birds. Blue circles represent cartilage. Red dots show the presence and position of early ossification centers, and red represents bone. As in crocodilians (a), non-avian dinosaurs like *Allosaurus* (b) present only dt3 and dt4 as adult bones, but likely also developed a dt2 cartilage that failed to ossify. In bird-like dinosaurs like *Velociraptor* (c), dt3 and dt4 fused at late ossification stages. In modern birds (d), cartilages dt2, dt3, and dt4 fuse, and ossification beginning in cartilage derived from dt3 progresses into the dt2 cartilage (left red arrow), which now contributes bone medially; ossification also progresses laterally into dt4 (right red arrow), which likely enabled the evolutionary loss of the dt4 ossification center. As a result, a single plate-like ossified unit covers the proximal metatarsals and fuses to them, forming the proximal region of the avian tarsometatarsus (highlighted in red; adult fusion is seamless) [Color figure can be viewed at wileyonlinelibrary.com]

ossification from dt3 from progressing into dt2. Importantly, the non-ossifying dt2 cartilage could now begin to contribute bone in the medial region of the distal ankle above mt2, expanding the distal tarsal "bone" that fuses to the metatarsus. The evolutionary history of dt2 is akin to that of the intermedium and prefrontal of birds, in that these had also ceased to develop into independent skeletal elements of the adult; however, their continued formation as independent embryonic elements allowed them to participate in the evolution of new composite structures (Ossa-Fuentes et al., 2015; Smith-Paredes et al., 2018).

Towards the origin of birds, in paravians like *Velociraptor* and *Archaeopteryx*, dt3 and dt4 began fusing at late ossification stages (Figure 5c), to form a single large bone with a mid-indentation or notch (Norell & Makovicky, 1997, 1999; Rauhut et al., 2018). In the evolution of other composite basipodial elements, such as the astragalus in the ankle of amniotes, or the semilunate carpal in the wrist of Pennaraptora, skeletal elements initially fuse at late ossification stages. At a later step in evolution, the fusion event is displaced earlier in the developmental sequence, to fusion of the

embryonic cartilages (Botelho et al., 2014; Meyer and Anderson, 2013). Additionally, the number of ossification centers decreases, and the cartilage resulting from fusion develops a single ossification center: a simplification that is likely enabled by the fact that ossification can now progress into all fused embryonic cartilages. Taking these evolutionary trends into account, we hypothesize that Paraves like *Archaeopteryx* represents the first step of bony fusion between dt3 and dt4, and that fusion of their embryonic cartilages occurred thereafter. Cartilage fusion could have enabled the loss of an independent ossification in dt4, since ossification starting in dt3 could now progress laterally into dt4. This may have already occurred in the Ornithothoraces, where a single ossification center is documented in the ontogeny of an enantiornithine (Hu & O'Connor, 2017).

It is interesting to speculate whether the fusion of the embryonic cartilages dt2 and dt3 could have occurred first in evolution, before the fusion of the dt4 cartilage. Several bird-like dinosaurs show a dt3 that articulates to the proximal ends of both mt2 and mt3 (see Supporting Information and Figure S2). This could arguably reflect

cartilage fusion of dt2 and dt3, with ossification from dt3 progressing medially, and coming in contact with mt2. If so, the dt4 cartilage would have fused later, in Pygostylia or Ornithothoraces. Regardless of how many evolutionary steps were involved, the fusion of these three cartilaginous distal tarsals allowed the development of a single ossified unit that expands over the proximal ends of the metatarsals, arguably a key step for the development and evolution of an integrated tarsometatarsus (Figure 5d).

From a more general standpoint, it is worth noting that most of the inferences and hypotheses above can only be arrived at through a combination of embryological and paleontological evidence, and are difficult to achieve through any of these data sources on its own. The development of the distal ankle of birds is also an example of how an ancestral developmental pattern is conserved (the formation of three distal tarsal cartilages, as in crocodilians) but then modified through a post-patterning mechanism (cartilage fusion), producing a "recapitulatory" developmental pattern. Conceivably, the phenotype of the distal ankle of birds could have been achieved through the formation of single cartilage, as in the proposal by Hinchliffe (1977, 1989), which remained unquestioned for a long time. It is therefore interesting that this is not the case. In a modern interpretative framework, such conservation of an early ancestral pattern may reflect how other relevant developmental mechanisms have been built around it ("entrenchment," as in Wimsatt, 2003). Therefore, the presence of this ancestral pattern of three cartilages may be coupled to developmental mechanisms that have remained key to producing a functional phenotype.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ANATOMICAL NOMENCLATURE

ast, astragalus; cal, calcaneum; dt1, distal tarsal 1; dt2, distal tarsal 2; dt3, distal tarsal 3; dt4, distal tarsal 4; dt5, distal tarsal 5; F, fibula; fib, fibulare; mt, metatarsus; tib, tibiale; T, tibia.

PEER REVIEW

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